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DEVELOPMENT OF DELIVERY SYSTEMS FOR MELANOTROPIC PEPTIDES

by

Sydney Onye Ugwu

A Dissertation submitted to the Faculty of the
DEPARTMENT OF PHARMACEUTICAL SCIENCES
In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1 9 9 4
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Sydney O. Ugwu entitled Delivery Systems for Melanotropic Peptides and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy/Pharmaceutical Sciences.

James Blanchard, Ph.D.  
Date 5/27/94

Michael Mayersohn, Ph.D.  
Date 5/27/94

Arnold Martin, Ph.D.  
Date 5/27/94

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

James Blanchard  
Dissertation Director  
Date July 1, 1994
STATEMENT BY AUTHOR

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Finally, my special thanks go to my parents, family and friends for their support, encouragement and patience throughout my long academic training.
DEDICATION

Dedicated to my family and especially to my father.
FOREWORD

The findings of this dissertation are organized as follows:

There are six chapters of this dissertation. The first chapter gives the overall background of the research. The second chapter is about the development and validation of a HPLC assay for α-Melanotropic peptide analog (MT-II). The comparison of the HPLC and frog-skin bioassay methods is described in chapter three. The fourth chapter is about the study of stability of MT-II in aqueous buffered solutions. The fifth chapter deals with the development of a novel method for the determination of acid dissociation constants of a model peptide. The final chapter is about the enteral absorption of the two melanotropic peptides from the rat intestine.

Relevant figures and tables are placed at the end of each chapter. Legends to figures are placed on its own numbered page immediately preceding the page it describes. References of all chapters are placed at the end of the last chapter.

Each chapter is treated as a separate entity in the dissertation. Therefore, the figures, tables and references of each chapter are numbered starting from one onwards.
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ABSTRACT

The overall objective of this dissertation was to develop formulations suitable for the delivery of α-melanotropic peptides.

A HPLC assay with UV detection was developed to evaluate the performance of melanotan-II (MT-II) in vitro and in vivo. The method was validated to establish linearity, precision and accuracy and also was successfully applied to a pharmacokinetic study in rats. Correlation of the plasma MT-II concentrations determined using HPLC and frog skin bioassay methods indicated the existence of a significant linear correlation.

Next, the stability of MT-II in aqueous buffered solution was studied in order to facilitate the formulation of stable dosage forms. Results indicated that the degradation of MT-II followed apparent first-order kinetics. MT-II was most stable at approximately pH 5.0. Shelf-life at 25°C and activation energy were 27 hr and 7.5 kcal/mole, respectively. The degradation rate was directly proportional to phosphate concentration and temperature, but independent of the ionic strength.

In another phase of the study, a novel method was developed for the determination of acid dissociation
constants of peptides. The method involved potentiometric titrations, following which data were analyzed using the Gran plot and the Best program. The method was also capable of accurately determining the contamination of the peptide sample with weak acids, strong acids, or water. The method was validated using boric acid and a peptide molecule.

Finally, bioavailability (F) of Melanotan-I (MT-I) and Melanotan-II (MT-II) solutions, administered intra-jejunally to rats were 1.6 and 5.0%, respectively. The administration of MT-I with dimethyl-β-cyclodextrin (DMCD), a permeation enhancer, did not significantly increase the absorption of MT-I. However, after intrajejunal administration of MT-I in the presence of DMCD and Aprotinin (APRO), a protease inhibitor, the F was significantly increased by 4-fold. This result showed that enzymatic degradation appeared to be a significant barrier to the intestinal absorption of MT-I.
CHAPTER 1

BACKGROUND

Skin cancer is the most common type of cancer in the United States--more than twice as common as all other types of cancer combined. Recent figures indicate that there are over 600,000 cases of skin cancer per year in the United States (1). The most serious form of skin cancer is malignant melanoma, which was diagnosed in about 32,000 persons in 1991. It was estimated that skin cancers resulted in about 8,500 deaths in 1991, 6500 from malignant melanoma and 2,000 due to other skin cancers (1). The overall incidence is increasing at the rate of 4% per year. This is probably due, at least in part, to the continued depletion of the ozone layer by chlorofluorocarbon (CFC) propellants. Arizona's incidence of skin cancer is the highest in the nation (2). According to the Arizona Cancer Center Skin Registry, Arizona residents are developing basal cell cancer at a rate that is 1.5 to 3.9 times the national average and squamous cell cancer at a rate 2.5 to 7.6 times the national average. The incidence of malignant melanoma, a life-threatening form of cancer is twice as prevalent in Arizona as in other regions of the United States.

Several measures have been attempted to decrease the
incidence of skin cancers. These measures include public information programs to alert the public to the dangers of excessive exposure to sunlight and to promote the use of sun-blocking creams and ointments. However, these measures have met with only limited success. Despite the recent trend toward the increased use of sunscreens and those with higher sun-protection factor (SPF) values, the incidence of skin cancers continues to rise. Thus there is clearly a need for better methods of protecting the skin from the harmful ultraviolet rays of the sun if the incidence of skin cancer is to be reduced.

Recent work with two melanotropic peptides synthesized by Dr. Victor Hruby and co-workers in the Department of Chemistry, University of Arizona indicates that both of these agents, referred to as Melanotan I and II (MT-I and MT-II), are capable of tanning the skin (3,4). However, a considerable number of additional clinical trials are necessary before the therapeutic potential of these agents can be realized. The performance of these studies, in turn, requires the development of suitable formulations for administering the Melanotans to human volunteers. The overall objective of this dissertation was to develop formulations suitable for delivering these melanotropic peptides.
In order to evaluate the performance of the peptide formulations in vivo and in vitro, a sensitive and specific assay is needed to measure concentrations of the melanotropic peptides in the blood. A radioimmunoassay has been developed for MT-I. Chapter 2 of this dissertation presents the development and validation of a high-performance liquid chromatographic assay (HPLC) for MT-II in Rat Plasma. Chapter 3 compares the HPLC assay to the frog-skin bioassay (FSB) for measuring the biological activity of the melanotropic peptides. The goal of this study was to validate the HPLC assay and to establish a relationship between plasma MT-II concentrations measured by both methods.

In chapter 4, the stability and degradation of MT-II in aqueous solutions was investigated. Specifically, the influence of pH, temperature, ionic strength and phosphate buffer concentration on the degradation of MT-II was investigated. The development and testing of a novel method for the determination of acid ionization constants (pKa) of peptides is introduced in chapter 5. This method is unique because it allows the determination of pKa values for overlapping ionization processes for both salt and non-salt forms of peptides. It also overcomes most of the traditional problems associated with the potentiometric
determination of pKa of peptides.

Chapter 6 presents the study of the enteral absorption of α-MSH analogs from the rat intestine. In this study an in situ rat model was used to compare the absorption efficiencies of various formulations of MT-I and also to compare the enteral absorption efficiencies of MT-I and MT-II.
CHAPTER 2

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE α-MELANOTROPIN [4,10] FRAGMENT ANALOGUE (MELANOTAN-II) IN RAT PLASMA

SUMMARY

A high-performance liquid chromatographic (HPLC) procedure has been developed for the quantification of Melanotan-II (MT-II), a cyclic heptapeptide which promotes rapid tanning of the skin, in rat plasma. The method involves precipitation of plasma proteins followed by direct-injection HPLC with ultraviolet detection. Calibration curves were linear over the range 100-1000 ng/ml for rat plasma. The method is reproducible and reliable with a detection limit of 50 ng/ml in plasma. Within- and between-day precision and accuracy reported as coefficient of variation (% CV) and relative error (% RE), respectively were < 7%. The application of the assay was successfully demonstrated by quantifying the concentration of MT-II in rat plasma samples following an intravenous dose of 0.3 mg/kg.

KEY WORDS: high-performance liquid chromatography (HPLC), assay, Melanotan-II, α-melanotropin, peptide.
INTRODUCTION

Melanotan-II (MT-II) is a synthetic cyclic α-melanotropin analog (1). This cyclic heptapeptide (Figure 1) is capable of stimulating melanin synthesis and promoting rapid tanning of the skin (2). This peptide is currently in phase-I clinical evaluation for use in the prevention of sunlight-induced skin cancers.

We report here a sensitive, specific, reproducible, and stability-indicating assay of MT-II in plasma using high-performance liquid chromatography (HPLC). To our knowledge, this assay is the first quantitative method for measuring Melanotan-II in a biological fluid. Previously, the presence of this peptide in biological fluids (e.g., urine) was monitored using frog skin and lizard skin bioassays (3). However, these bioassays are only semi-quantitative and can be nonspecific, variable and expensive.

HPLC procedures have been reported for the separation of mixtures of other cyclic α-melanotropic peptides (4,5). However, these procedures were primarily developed for preparative purification and were aimed at examining the chromatographic behavior and mechanism of separation of mixtures of these analogs (4,5).

The present method uses a reversed-phase column at
35°C. Sample preparation involves precipitation of plasma proteins followed by direct injection of the supernatant. The mobile phase uses triethylamine phosphate (TEAP) buffer as the ion-pairing reagent and acetonitrile as the organic modifier. The elution is isocratic and the analysis time per sample is about 13-15 min.

**MATERIALS AND METHODS**

**Apparatus**

The HPLC system consisted of a Spectra-Physics (Fremont, CA, USA) Isochrom pump, a Rheodyne (Cotati, CA, USA) model 7125 injector valve with a 50 μl loop and a Spectra-Physics model 100 variable-wavelength UV detector set at 214 nm. The analytical column was a Vydac (Hesperia, CA, USA) C$_{18}$ (5 μm) microbore cartridge (150 x 2.1 mm i.d.), fitted with a Whatman (Clifton, NJ, USA) C$_{18}$ (10 μm) guard column (10 x 4.6 mm i.d.). The guard column was routinely changed after about 100 injections of plasma supernatant as a precautionary measure against pressure build-up in the HPLC system and break-through contamination of the analytical column. Peak recording and area integrations were made with a Spectra-Physics model 4400 integrator. All injections were made with a Hamilton (Reno, NV, USA) model 702-SNR 100 μl syringe.
Column temperature as maintained at 35 ± 0.1°C with a Timberline (Boulder, CO, USA) model H-500 heater to maintain retention time reproducibility to within 0.5 mins and to improve detector baseline stability.

Experimental

Drug Standard

Purified Melanotan-II was obtained from Dr. Victor Hruby of the Department of Chemistry, University of Arizona. MT-II was synthesized by a conventional solid-phase method of peptide synthesis (1). Following synthesis, the crude peptide powder was purified by cation-exchange chromatography on a carboxy methyl cellulose column (1). The final purification was effected by preparative reversed-phase HPLC on a C18 bonded silica column, with separations monitored at 280 nm. The purity of the finished peptide was verified by thin-layer liquid chromatography (TLC) in at least three solvent systems and analytical reversed-phase HPLC at 280 nm and 220 nm. The structure of the purified peptide was confirmed by fast-atom bombardment (FAB) mass spectrometry, amino acid analysis and two-dimensional nuclear magnetic resonance (NMR) spectroscopy.

Preparation of Standard (Calibration) Solutions

A stock solution of MT-II (1 mg/ml) was prepared in rat plasma and stored at -20°C. The stability of the stock
solution was periodically checked by assaying aliquots of the stored solution at various time intervals. It was found that the MT-II stock solution was stable at the storage temperature for at least four months. Serial dilutions of the stock solution with drug-free rat plasma were used to obtain the desired concentrations of the calibration standards. The standard solutions were then stored at 4°C and analyzed over a period of 8 h. No change in the peak area of repeated injections of the calibration standards was observed over the 8 h period during storage at 4°C.

**Mobile Phase**

Acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). Phosphate buffer (100 mmol/l) was prepared by adding 17.42 g of dibasic potassium phosphate (J.T. Baker, Phillipsburg, NJ, USA) to 1 l of distilled, deionized water (Millipore water purification system). The pH of the solution was adjusted to 2.2 with phosphoric acid (Fisher Scientific, Fairlawn, NJ, USA). The mobile phase was prepared by mixing 21% (v/v) acetonitrile and 79% (v/v) phosphate buffer. Next, 18 μl of 99% (v/v) triethylamine was added to reduce adsorption, improve peak shape and hence, improve sensitivity (6,7) and the mixture was continuously stirred for 10 min. The pH was then adjusted to 2.5 with phosphoric acid, if necessary. The mobile phase
was filtered through a nylon-66 membrane filter, 0.45 μm (Rainin, Woburn, MA, USA), and degassed by sonication for at least 15 min. The mobile phase was continuously purged with helium gas (99.995% pure; U.S. Airweld, Tucson, AZ, USA) prior to equilibration of the column and during each sample run. A mobile phase flow rate of 0.25 ml/min was utilized.

**Sample Preparation**

A 30 μl volume of 6% (v/v) perchloric acid (Eastman Kodak, Rochester, NY, USA) was added to 100 μl of rat plasma containing MT-II. The mixture was stirred on a vortex mixer for 10 sec and centrifuged at 10,800 Xg for 10 min at 25°C. A 100 μl volume of the supernatant was injected onto the chromatograph.

**Standard (Calibration) Curves**

Plasma samples containing 100, 250, 500, 750, and 1000 ng/ml of Melanotan-II were prepared. The plasma standards were prepared and chromatographed using the same procedures employed for unknown (e.g., rat plasma) samples. Standard curves were constructed using peak areas of MT-II. The linearity of the curves was verified by means of correlation and regression analysis, as shown in Table I.

**Validation: Accuracy and Precision**

The validation of the HPLC procedure was designed to test the accuracy and precision of the method. Validation
was accomplished by assaying five different concentrations of MT-II in rat plasma each day for five consecutive days. The accuracy was assessed by calculating the relative error of the mean (\%RE) of five determinations of each concentration relative to the known concentration, as shown in Table II. The coefficient of variation (\% CV) served as a measure of precision.

**Validation: Stability-Indicating Procedure**

An aqueous solution of perchloric acid (3 M) was used to accelerate the degradation of MT-II. MT-II (500 ng/ml) was left for 75 h at room temperature in the perchloric acid solution. Then, aliquots (50 µl) were collected at various time intervals (i.e., 0, 3, 24, and 75 h) and assayed using the identical conditions described above.

**Validation: Mass spectrometric analysis**

A plasma sample of MT-II collected from rats given MT-II intravenously was assayed using the same conditions described above. The peak corresponding to the retention time of MT-II (12.3 min) was fraction collected. The fraction was then evaporated under nitrogen to remove the organic modifier (i.e., acetonitrile) of the mobile phase. The aqueous layer was then freeze-dried and the dried sample was subjected to fast-atom bombardment mass spectrometric analysis.
Column Care

For overnight storage, the column was washed with distilled water at 0.4 ml/min for 10 min, followed by a wash with 50:50 (v/v) acetonitrile:water at the same flow-rate for 10 min. At the end of the week, in addition to the routine outlined above, the column was washed with 95:5 (v/v) acetonitrile:water at 0.4 ml/min for 30 min to remove any adsorbed materials.

Preliminary Pharmacokinetic Study

A male Sprague-Dawley rat, weighing 424 g was used in this study. The animal was acclimatized to the laboratory environment in the animal care room for 1 week before study. Under mild ether anesthesia (by inhalation), a cannula for blood sampling was implanted in the right external jugular vein 24 h prior to drug administration. The surgical procedure was identical to that reported by Chow et al. (8). The animal received 0.3 μg/kg MT-II by slow intravenous infusion over 0.7 min, and blood samples were drawn via the cannula at specific time intervals over a 24 h period. Blood samples were collected in chilled polypropylene tubes containing 15 μl EDTA-solution (0.7 mg Na₂EDTA in 15 μl saline) and 12 μl of 8 M acetic acid. Following centrifugation at 4°C (10 min, 2,000 Xg), the plasma was collected and stored at -20°C until the day of analysis.
RESULTS

Figure 2 shows representative chromatograms for unsupplemented (blank) plasma (2A) and for plasma supplemented with Melanotan-II (2B). As can be seen from the chromatograms, the peptide peak was well resolved from endogenous plasma components. The retention time for the peptide was 11.0 min. Figure 2 (C) illustrates plasma of a rat given a 0.3 $\mu$g/kg dose of MT-II at 0.5 h post injection.

The specificity of the analytical method for measuring unchanged MT-II was determined by analysis of samples of MT-II in distilled water that were intentionally degraded with 3M aqueous perchloric acid at 25°C. A control solution, consisting of MT-II in aqueous perchloric acid at time zero, exhibited a well-resolved peptide peak at 11 min (Figure 3A). Figure 3B shows a chromatogram of this sample after 24 h. MT-II degraded approximately 60% in 24 h in 3M aqueous perchloric acid solution and the only apparent degradation product did not co-elute with the unchanged peptide (Figure 3B). The peak for the degradation product had a retention time of 12.3 min and was completely resolved from MT-II.

The MT-II peak areas were plotted vs MT-II concentrations and a least-squares regression was
performed. The slopes were significantly different from zero at $p<0.0005$. Plots were linear over the concentration range tested with correlation coefficients ($r$) $> 0.999$ (Table I). Statistical analysis also showed that the intercepts were not significantly different ($p<0.01$) from zero.

The between-day and within-day assay precision and accuracy, as measured by %CV, and %RE, respectively are summarized in Tables II and III. The %CV and %RE, for the lowest concentration assayed were less than 7%. The results indicate that the assay was both accurate and reproducible. The quantification limit for this assay was 50 ng/ml using the criterion that the signal from the minimum quantifiable peak should be $\geq$ three times the baseline noise level.

As shown in Figure 4, the mass spectrum of the peak fraction corresponding to the retention time of MT-II shows the presence of a molecular ion (MW) of 1024. This molecular ion was indeed the molecular weight of MT-II, thus confirming the identity of the MT-II peak in the chromatogram.

The plasma concentration versus time profile for MT-II in the rat following intravenous dosing is shown in Figure 5. The data could be described by a two-compartment model with an elimination ($\beta$-phase) half-life of 61.2 hours.
DISCUSSION

The method reported here represents the first assay that is suitable for determining the pharmacokinetic profile of MT-II in rat plasma. This peptide was previously shown to have excellent stability in trypsin, pepsin, and chymotrypsin (2,3). The degradation in strongly acidic conditions, observed here, indicates that it may be possible to deliver this potentially important therapeutic agent via the oral route because the degradation half-life is relatively long compared to typical gastric emptying rates (9). It is recommended that polypropylene tubes be used for sample preparation instead of glass tubes because peptides (including MT-II) are known to bind avidly to glassware.

The plasma concentration versus time profile of MT-II obtained in rat plasma illustrates the applicability of the assay to an actual pharmacokinetic study in rats. Further recent work in our laboratory indicates that the assay can be applied without modification to the analysis of dog and human plasma samples.
Figure 1. Structure of α-melanotropin analog (Melanotan-II).
STRUCTURE OF MELANOTAN-II (MT-II)

Ac-[Nle₄,Asp⁵,D-Phe⁷,Lys¹⁰] α-MSH₄₁₀-NH₂
Figure 2. HPLC chromatograms of (a) Drug-free rat plasma; (b), rat plasma spiked with 500 ng/mL Melanotan-II; (c) plasma of rat given MT-II intravenously. The rat plasmas illustrated in figures A and B were collected from the same rat dose iv with MT-II immediately before dosing. The arrows on the chromatograms refer to the elution time of MT-II.
Figure 3. Sample chromatograms for (a) control solution (MT-II at time 0) and (b) solution of MT-II degraded with 3M aqueous perchloric acid at 25°C for 24 h. The arrows on the chromatograms refer to the elution position of MT-II.
Figure 4. Fast-atom bombardment mass spectrum of MT-II peak fraction collected following injection of plasma of rat given MT-II intravenously into the HPLC.
FAB MASS SPECTRUM

PERCENT INTENSITY

120
110
100
90
80
70
60
50
40
30
20
10
0

M/Z

100 200 300 400 500 600 700 800 900 1000 1100 1200

102
194
131
283
378
651
1024
Figure 5. Plasma concentration versus time profile of MT-II in rat following an iv of 0.3 mg/kg.
PLASMA CONCENTRATION VS. TIME PROFILE OF MT-II

CONCENTRATION (ng/mL)

TIME (hr)
Table I. Summary of HPLC Calibration Curves for Melanotan-II (MT-II)

<table>
<thead>
<tr>
<th>Determination</th>
<th>Slope*</th>
<th>95% Confidence Limit</th>
<th>r</th>
<th>Interceptb</th>
</tr>
</thead>
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<td>± 0.0005</td>
<td>0.9999</td>
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<td>4</td>
<td>0.0324</td>
<td>± 0.0022</td>
<td>0.9993</td>
<td>-1.0200</td>
</tr>
<tr>
<td>5</td>
<td>0.0322</td>
<td>± 0.0019</td>
<td>0.9995</td>
<td>-0.7985</td>
</tr>
<tr>
<td>Mean</td>
<td>0.0319</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Stand. Dev. 0.006
% CV 1.81

* Slopes were significantly different from zero at p < 0.0005.

b Intercepts were not significantly different from zero at p < 0.01.
Table II. Between-Day Variability and Accuracy in the Analytical Method for Melanotan-II (MT-II) in Plasma

<table>
<thead>
<tr>
<th>MT-II Concentration (ng/mL)</th>
<th>Measured MT-II Concentration (ng/mL)</th>
<th>%CV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>%RE&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>106.7 ± 5.3</td>
<td>4.98</td>
<td>-6.70</td>
</tr>
<tr>
<td>250</td>
<td>246.9 ± 8.2</td>
<td>3.31</td>
<td>1.22</td>
</tr>
<tr>
<td>500</td>
<td>494.7 ± 11.5</td>
<td>2.32</td>
<td>1.06</td>
</tr>
<tr>
<td>750</td>
<td>745.9 ± 11.5</td>
<td>1.53</td>
<td>0.55</td>
</tr>
<tr>
<td>1000</td>
<td>1005.9 ± 11.0</td>
<td>1.09</td>
<td>-0.59</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD, n = 5.  
<sup>b</sup> Percent coefficient of variation.  
<sup>c</sup> Percent relative error of the mean.  

%RE = True Conc. - Mean Conc.  
\[ \text{X 100} \]
Table III. Within-Day Variability and Accuracy in the Analytical Method for Melanotan-II (MT-II) in Plasma

<table>
<thead>
<tr>
<th>MT-II Concentration (ng/mL)</th>
<th>Measured MT-II Concentration (ng/mL)</th>
<th>%CV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>%RE&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>516.0 ± 13.9</td>
<td>4.12</td>
<td>-3.21</td>
</tr>
<tr>
<td>1000</td>
<td>984.1 ± 40.6</td>
<td>2.68</td>
<td>1.59</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD, n = 5 injections.  
<sup>b</sup> Percent coefficient of variation.  
<sup>c</sup> Percent relative error of the mean.
CHAPTER 3

COMPARISON OF HPLC AND BIOASSAY METHODS FOR MELANOTAN-II (MT-II) DETERMINATION: APPLICATION TO PHARMACOKINETIC STUDY IN RATS

SUMMARY

The pharmacokinetic profile of the melanotropic peptide Melanotan-II (MT-II) was determined in rats following a 0.3 mg/kg intravenous dose. Regression analysis of the plasma MT-II concentrations determined using HPLC and bioassay methods indicated the existence of a significant linear correlation ($r = 0.90, p < 0.001$). The plasma concentration vs. time plots determined using the two assay methods yielded biphasic disposition profiles that were essentially superimposable. The following pharmacokinetic parameters were assessed from plasma concentration vs. time data using both methods: $C_{\text{max}}$, AUC, $CL_s$, $t_{1/2\beta}$, MRT, $V_d\beta$ and $V_{ss}$. Statistical comparison of the parameters measured by each method were not significantly different (at the 0.05 level) except for $t_{1/2\beta}$, MRT and $V_{ss}$. The presence of even one aberrant data point in the $\beta$-phase can significantly influence $t_{1/2\beta}$ when only a few data points are available in the $\beta$-phase. Since MRT and $V_{ss}$ were calculated from $t_{1/2\beta}$ it is not surprising that these two parameters also differed between methods.
INTRODUCTION

The α-melanocyte stimulating hormone (α-melanotropin or α-MSH) is a linear tridecapeptide, which is synthesized and secreted by the pars intermedia of vertebrate pituitary glands (1). α-MSH plays a role in integumental color changes by its action on melanocytes within the skin (2). Several analogs and fragments of α-MSH have been synthesized (3), many of which have been shown to have superpotent and prolonged melanotropic activity in the in vitro frog and lizard skin bioassays (4).

More recently, Melanotan-II (MT-II), a cyclic lactam analog of α-MSH (Figure 1) was synthesized (5). This peptide also stimulates melanin synthesis and thereby darkens the skin rapidly in the in vitro frog and lizard skin bioassays (6). Furthermore, recent preliminary clinical trials have demonstrated the capability of this peptide to provide a rapid and prolonged tanning of human skin (7).

The bioassay method has predominantly been used to measure the potency of melanotrophic peptides. One major advantage of this method is its extremely high sensitivity, with minimum detectable concentration in the low pg/mL range. However, there are several disadvantages of this method, i.e., it is laborious, can be highly variable, and
involves the use of animals. Thus, there is a need for an alternative method for measuring the concentration of melanotropic peptides. We recently reported a HPLC assay for the quantitation of MT-II in rat plasma (8). The first objective of the present study was to determine the relationship between the plasma concentration of MT-II measured by the bioassay and HPLC methods. Many peptides, including Melanotan-II, are very potent (i.e., "superpotent"), thus it often becomes necessary to have a highly sensitive assay available to adequately characterize the pharmacokinetic profile of these peptides, due to the very low doses required to elicit a therapeutic response. Our second objective was therefore to evaluate the ability of the two methods to characterize the pharmacokinetic profile of MT-II in the rat.

MATERIALS AND METHODS

Materials

Purified MT-II was obtained from Dr. Victor Hruby of the Department of Chemistry, University of Arizona. The purity of the peptide was greater than 99% as determined by reversed-phase HPLC on a C<sub>18</sub> bonded silica column, with UV detection at 280 nm and 220 nm. The MT-II sample was a powdered lyophilized diacetate salt form. HPLC-grade
acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). Potassium phosphate, monobasic and dibasic (analytical grade), was purchased from J.T. Baker Chemical Company, Phillipsburg, NJ, USA. The water used in the experiments was deionized and distilled using a Millipore filter system (Millipore Corp., Bedford, MA, USA). Bond Elut C₈ solid phase extraction columns were obtained from Varian sample preparation products, Harbor City, CA, USA. Sodium Chloride Irrigation Solution, 0.9%, was obtained from Baxter Healthcare Corp., Deerfield, IL, USA. Aprotinin, Biotechnology grade, was obtained from Miles, Kankakee, IL, USA. Ethylenediaminetetraacetic acid dihydrate, tripotassium salt (EDTA) was obtained from Aldrich Chemical Co., Milwaukee, WI. Ketamine HCl was obtained from Aveco Co., Fort Dodge, IA. Xylazine was obtained from Lloyd Labs., Shenandoah, IA. Frogs (Rana Pipiens) were obtained from Kons Scientific Co., Oshkosh, WI.

Animal studies

Male Sprague-Dawley rats weighing 250-300 g were used for this study. The animals were acclimatized to the laboratory environment for one week prior to the study. The rats were fasted for 16-20 hr prior to each experiment. Water was allowed ad libitum. The animals were anesthetized
with an intraperitoneal injection of a mixture of 80 mg/kg Ketamine and 10 mg/kg Xylazine. Thereafter, one third of the anesthetic dose was given every 60 minutes to maintain anesthesia. The core body temperature was maintained at 37°C by placing the animal on a heating pad and monitored using a rectal probe (Model 51, Fluke Instruments, Rolling Meadows, IL). A length of PE-50 polyethylene tubing (Becton Dickinson, Parsippany, NJ) was inserted into the right external jugular vein for periodic blood sampling. The surgical procedure for cannulation of the jugular vein was similar to that reported elsewhere (9). A 23-guage needle blunted at one end was inserted into the cannula and was used with a 1-ml polypropylene syringe for removal of blood samples. The animals received 0.3 mg/kg of MT-II in normal saline by intravenous infusion over 0.5 min. After dosing, the cannula was washed with sterile normal saline solution prior to blood sampling. Blood samples (300-350 μL) were drawn at specific time intervals over a 2 hr period. Blood samples were collected in chilled polypropylene tubes containing 10 μL of EDTA solution (0.7 mg K₂EDTA in 10 μL saline) and 10 μL Aprotinin (18,000 Kallekrein Inhibitory Units/mL saline). Following centrifugation at 4°C (10 min, 2000 Xg), the plasma was collected and stored at -20°C until the day of analysis. All samples were analyzed within 4
days of collection.

**HPLC Analysis**

Prior to HPLC analysis, plasma samples were prepared using a solid-phase extraction method. The extraction column (Bond-Elut, C₈) was first conditioned with 2 mL of acetonitrile, followed by 2 mL of 0.1 M phosphate buffer (pH 2.5). During the conditioning step, care was taken to avoid letting the column dry out. An aliquot of each plasma sample (50 μL) was added to 250 μL of 0.1 M phosphate buffer (pH 2.5). This mixture was then applied to the column and allowed to run through it at a vacuum pressure of approximately 3 mm Hg. Then, 2 mL of a mixture of 10% acetonitrile and 90% phosphate buffer was applied to the column to wash out impurities and unwanted plasma components. After the column was allowed to dry at 3 mm Hg vacuum pressure, MT-II was eluted with 300 μL of a mixture of 33% acetonitrile:67% phosphate buffer (pH 2.5). 50 μL of the eluant was injected directly onto the HPLC column. The HPLC method for measuring plasma MT-II concentrations was described in detail elsewhere (8).

**Frog-Skin Bioassay**

The *in vitro* biological activity (i.e., frog-skin darkening) of MT-II in plasma was determined using a frog-skin bioassay method as described elsewhere (10,11). Prior
to the bioassay, plasma samples were first prepared using the solid-phase extraction method described above.

**Data Analysis**

Plasma concentration vs. time data for MT-II were analyzed by conventional noncompartmental methods using RSTRIP, a nonlinear least-squares optimization program (12). The estimates of elimination half-life ($t_{1/2p}$), area under the curve (AUC) and mean residence time (MRT) were obtained from the RSTRIP analyses. MRT was calculated by dividing the area under the first moment curve (AUMC) by AUC. Total body clearance ($CL_s$) and volume of distribution at steady state ($V_{ss}$) were calculated as follows:

$$CL_s = \frac{\text{Dose}}{\text{AUC}}$$

$$V_{ss} = CL_s \times MRT$$

$Vd_\beta$ was calculated by dividing $CL_s$ by $K_\beta$ (the $\beta$-phase elimination rate constant). $C_{max}$ was estimated visually from the plasma concentration-time plot. The regression analysis of MT-II plasma concentrations measured using the HPLC and bioassay methods was performed using a least-squares linear regression program (13).

**RESULTS AND DISCUSSION**

**Correlation between HPLC assay and Bioassay**

Plasma samples obtained from the pharmacokinetic
experiments were analyzed by both analytical methods. A good linear correlation was obtained for plasma concentrations measured by both assay methods (Fig. 2). The equation of the regression line was

\[ y = 0.78x - 44 \quad (n = 17, \ r = 0.90, \ p < 0.001). \]

In general, the plasma concentrations measured by the bioassay method were slightly higher than those measured by the HPLC method. This may be due to the presence of trace amounts of MT-II metabolites in the plasma. Although MT-II was extracted from plasma prior to analysis, small amount of metabolites may have been present in the extracted samples. For the HPLC assay, these metabolites could be easily resolved, which is not the case for the bioassay. In fact, the bioassay may measure metabolites as well as MT-II, which may explain the slightly higher plasma MT-II concentrations observed when using the bioassay method.

One major distinction between the two assay methods is the precision. As previously reported, the within- and between-day assay precision, as measured by the coefficient of variation (% CV), for the HPLC assay was less than 7%. In contrast, the % CV for the bioassay was approximately 25%, which is within the normal variation for this method.

**Disposition Kinetics of MT-II**

Figure 3 shows typical concentration vs. time plots of
MT-II in a rat following an i.v. bolus dose of 0.3 mg/kg, along with the fitted curves. Pharmacokinetic analyses of the plasma concentration vs. time curves showed that the disposition profile was best described by a biexponential equation. Pharmacokinetic parameters calculated in four rats using the two assay methods are presented in Table 1. Statistical comparison of the parameters measured by each method were not significantly different (at the 0.05 level) except for $t_{1/2\beta}$, MRT and $V_{ss}$. As can be seen in Fig. 2 the presence of even one aberrant data point in the $\alpha$-phase can significantly influence $t_{1/2\beta}$ when only a few data points are available in the $\beta$-phase. Since MRT and $V_{ss}$ were calculated from $t_{1/2\beta}$ it is not surprising that these two parameters also differed between methods.

The mean $CL_s$ values calculated for both methods were approximately 1.5 mL min$^{-1}$. This value was $\approx 1/7$ of either the renal or portal blood flows ($\approx 9.0$ mL min$^{-1}$) in rats (14). This indicates that MT-II has a low extraction ratio and is restrictively cleared. Most peptides and proteins are rapidly cleared from the blood due to rapid receptor uptake in the liver and peripheral tissues and/or degradation by plasma proteases (15). The low clearance of MT-II observed in this study can be largely attributed to the chemical modifications of the peptide structure which
enhance its stability to proteolytic enzymes (5). $V_{ss}$ determined using HPLC and bioassay methods was $0.5 \pm 0.1 \text{ L.Kg}^{-1}$ and $0.2 \pm 0.02 \text{ L.Kg}^{-1}$, respectively; values much greater than the whole blood volume of the rat ($\approx 60 \text{ mL.Kg}^{-1}$) (13). This may be due to extensive distribution of MT-II to tissues or other extravascular sites. The short alpha phase half life (15 min) also suggests rapid distribution of MT-II from blood to tissues. The relatively long elimination ($\beta$-phase) half life of the MT-II molecule can be attributed to the low clearance which, in turn, can be attributed to its enhanced chemical stability.

CONCLUSIONS

In conclusion, this study shows that both assay methods are linearly correlated. However, because of major differences in the selectivity, sensitivity and precision of the methods, the HPLC assay should be employed when a precise, selective determination of the plasma MT-II concentration is desired. The bioassay should generally be performed to ensure that biological activity is retained in the plasma samples and to provide an estimate of the plasma concentration of MT-II when high assay sensitivity is required. The pharmacokinetic study indicated a biphasic disposition for MT-II. As can be seen in Fig. 2, the
presence of even one aberrant data point in the $\beta$-phase can significantly influence $t_{1/2\beta}$ when only a few data points are available in this portion of the concentration-time profile. Due to the higher precision of the HPLC assay we believe the $t_{1/2\beta}$ of $1.5 \pm 0.5$ hr is the more accurate of the two values. This value is fairly long in comparison to typical values for other peptides.
Figure 1. Structure of Melanotan-II
STRUCTURE OF MELANOTAN-II (MT-II)

\[ \text{Ac-}[\text{Nle}^4,\text{Asp}^5,\text{D-Phe}^7,\text{Lys}^{10}] \alpha\text{-MSH}_{4-10}\text{-NH}_2 \]
Figure 2. Correlation of the plasma concentration of MT-II obtained by HPLC and bioassay methods. Open circles represent the actual data points. The solid line represent the regression line \((n = 17, r = 0.90, p < 0.001)\).
CORRELATION OF PLASMA CONCENTRATION DETERMINED BY HPLC AND BIOASSAY METHODS

$r = 0.90$
$p < 0.001$
Figure 3. Plasma concentrations of MT-II in rats after an iv dose of 0.3 mg/kg determined by HPLC and the bioassay method. Each point represents the mean of four experiments.
PLASMA CONCENTRATION VS. TIME PROFILE OF MT-II

Concentration (ng/mL)

Time (hr)

- HPLC
- BIOASSAY
Table 1. Comparison of Pharmacokinetic Parameters for Melanotan-II (MT-II) After the Bolus Intravenous Administration of 0.3 mg/kg MT-II to Rats (n = 4) determined using HPLC vs Bioassay.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HPLC (Mean ± SD)</th>
<th>Bioassay (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (ng.mL$^{-1}$)</td>
<td>2278.2 ± 374.0</td>
<td>1911.2 ± 249.3</td>
</tr>
<tr>
<td>AUC (ng.mL$^{-1}$.hr)</td>
<td>1188.0 ± 205.2</td>
<td>1102.1 ± 202.3</td>
</tr>
<tr>
<td>$\text{CL}_S$ (L.Kg$^{-1}$.hr$^{-1}$)</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>$t_{\alpha\beta}$ (hr)</td>
<td>1.5 ± 0.5</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>1.9 ± 0.6</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>$V_d$ (L.Kg$^{-1}$)</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>$V_{ss}$ (L.Kg$^{-1}$)</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.02*</td>
</tr>
</tbody>
</table>

* Significantly different at the $p<0.05$ level
CHAPTER 4

KINETICS OF DEGRADATION OF A CYCLIC LACTAM ANALOG OF α-MELANOTROPIN (MT-II) IN AQUEOUS SOLUTION

SUMMARY

The kinetics of degradation of MT-II in aqueous buffered solution was studied in order to facilitate the formulation of a stable oral dosage form. A stability-indicating high-performance liquid chromatographic (HPLC) assay was used to measure the concentrations of MT-II remaining at various time periods. The rate of degradation of MT-II was studied as a function of pH, phosphate buffer concentration, temperature and ionic strength. Results indicated that the degradation of MT-II followed apparent first-order kinetics. The pH-rate profile showed that MT-II was most stable at approximately pH 5.0. Data obtained from this study also indicated that the degradation rate of this peptide was directly proportional to phosphate buffer concentration and temperature. The shelf-life of MT-II in aqueous buffer solutions at 25°C was 27 hr. The activation energy was 7.5 kcal/mole. The degradation rate of MT-II appeared to be independent of the ionic strength of the aqueous buffered solution.

Key words: Degradation; Peptide; α-Melanotropin; Analog; Kinetics; Stability.
INTRODUCTION

MT-II is a cyclic heptapeptide derivative of α-melanocyte stimulating hormone (Figure 1) (Al-Obeidi et al., 1989). MT-II stimulates melanin synthesis and thereby tans the skin rapidly (Hadley et al., 1989). This peptide is currently in phase I clinical trials for use in the prevention of sunlight-induced skin cancers (Griego, R.D. and Levine, N., 1992).

The potential therapeutic importance of this peptide prompted us to undertake a study of its chemical stability and degradation kinetics in aqueous buffered solutions, as no previous data were available. Furthermore, stability data obtained from this study will provide fundamental information needed to develop stable delivery systems of MT-II for both animal and human studies.

The purpose of this investigation was to determine the influence of pH, phosphate buffer concentration, temperature and ionic strength on the rate of degradation of MT-II.

MATERIALS AND METHODS

Materials

Purified MT-II was obtained from Dr. Victor Hruby of the Department of Chemistry, University of Arizona. The
purity of the peptide was greater than 99% as determined by reversed-phase HPLC on a C18 bonded silica column, with UV detection at 280 and 220 nm. The MT-II sample was a powdered lyophilized diacetate salt form. HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Potassium phosphate, monobasic and dibasic (analytical grade), was purchased from J.T. Baker Chemical Company, Phillipsburg, NJ, U.S.A. The water used in the experiments was deionized and distilled using a Millipore filter system (Millipore Corp., Bedford, MA, U.S.A.).

**HPLC analysis**

The HPLC system consisted of a Spectra-Physics (Fremont, CA, U.S.A.) Isochrom pump, a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector with a 50-μL loop and a Hitachi/Spectra-Physics (Fremont, CA, U.S.A.) Model 100-30 variable-wavelength UV detector set at 214 nm. The analytical column was a Zorbax (Dupont Instruments, Wilmington, DE, U.S.A.) C8 column (10-μm, 150 mm x 4.6 mm I.D.), fitted with a Whatman (Clifton, NJ, U.S.A.) C18 (30-μm) guard column (10 mm x 4.6 mm I.D.). Peak recording and area integrations were made with a Spectra-Physics model 4400 integrator. The mobile phase consisted of 0.1 M aqueous K2HPO4:acetonitrile (73:27 % v/v) containing 18 μl
99% v/v triethylamine per liter of mobile phase (pH 2.5). A flow-rate of 1.0 ml/min was utilized. Duplicate 100 μL injections were made for each sample assayed using a Hamilton (Reno, NV, U.S.A.) Model 702-SNR 100-μL syringe.

**Kinetic Studies**

A stock solution of MT-II (1000 μg/ml) was prepared in water and stored at 4°C until use. This solution was prepared fresh weekly. The sample solution (10 μg/ml) was prepared by mixing appropriate volumes of MT-II stock solution, phosphate buffer (0.5 M) and potassium chloride (1.5 M). Potassium chloride (1.5 M) and KOH or HCl (1.0 M) were used to adjust ionic strength and pH of the solution, respectively. In some experiments, it was necessary to use a low phosphate buffer concentration (0.02 M), to minimize buffer catalysis and a low ionic strength (μ) to reduce any possible salt effect. The temperature dependence of MT-II degradation was studied at 50, 60, and 70°C in 0.02 M phosphate buffer (pH 7.0, μ = 0.15). The influence of different μ (0.15, 0.25, 0.5, 0.75, 1.25, 1.5) was studied at 60°C (0.02 M phosphate, pH 9.11). The degradation of MT-II in the pH range 2 to 9 was studied at 60°C, with μ and phosphate buffer concentration held constant at 0.15 and 0.02 M, respectively. The effect of different phosphate buffer concentrations (0.02, 0.10, 0.5M) was investigated at
60°C ($\mu = 1.5$, pH = 9.11). The pH of all sample solutions was measured at the various study temperatures. All sample solutions were placed in 5-mL screw-capped polypropylene tubes and stored in an oven set at the desired temperature. At appropriate time intervals, aliquots of samples were withdrawn and analyzed by HPLC.

RESULTS AND DISCUSSION

HPLC Analysis

The assay was found to be linear between 1 µg/ml and 10 µg/ml MT-II ($r = 0.9997$). The assay precision of a 2 µg/ml MT-II sample was less than 1 % RSD (n=5). The homogeneity and identity of the MT-II peak were determined as reported elsewhere (Ugwu, S.O. and Blanchard, J., 1992). The stability-indicating nature of this assay is shown by the chromatograms (Figure 2) of samples of MT-II (10 µg/ml) in pH 2.4 and 7.0 buffer solutions that were stored at 4°C for 300 days. The amount of the intact MT-II remaining was 33%. The degradation peaks eluted separately without any interference from the peak representing the intact peptide. The degradation mechanism and the identification of the major degradation products have not yet been determined.

MT-II Degradation Kinetics

A typical logarithmic plot of the percentage of MT-II
remaining versus time for the degradation kinetics in pH 7.0, 0.02 M phosphate buffer is shown in Figure 3. The plot indicates that the degradation of MT-II in the aqueous buffer solution followed apparent first-order kinetics under conditions of constant pH, temperature and μ. The apparent first-order rate constant (K\text{obs}) was obtained from the slope of the following equation:

\[
\ln \% A_t = -K_{\text{obs}} \cdot t + \ln \% A_0
\]

where \% A_0 and \% A_t denote the percentage of MT-II remaining at time zero and time t, respectively and K_{\text{obs}} represents the degradation rate constant.

The Effect of Temperature on Degradation Rate

The effect of temperature on the degradation rate of MT-II was investigated at 50, 60 and 70°C in 0.02 M phosphate buffer at pH 7.0 and μ = 0.15. At all three temperatures, decomposition of MT-II followed an apparent first-order process. The values of K_{\text{obs}} calculated at the different temperatures are summarized in Table 1. The rate constant (K_{\text{obs}}) of many chemical reactions can be empirically related to the absolute temperature (T) by the Arrhenius equation:

\[
\log K_{\text{obs}} = \log A - \frac{E_a}{2.303} / RT
\]

where A, E_a and R are the pre-exponential constant, experimental activation energy and gas constant,
respectively. In this study, the dependence of $K_{obs}$ on temperature followed a linear Arrhenius plot (Figure 4). The linear Arrhenius plot obtained here indicates that the degradation mechanism did not change over the temperature range studied. On the basis of these data, the $E_a$ calculated from the slope of the Arrhenius plot was 7.53 kcal/mole. The pre-exponential constant was calculated from the intercept to be 1301.4 hr$^{-1}$. The enthalpy ($\Delta H^\dagger$) at the different temperatures (Table 1) was calculated as follows:

$$\Delta H^\dagger = E_a - RT$$

and the shelf-life at 25°C was calculated to be 26.92 hr from the equation:

$$t_{90} = 0.105/K_{obs}$$

Where $t_{90}$ represent the time at which 10% degradation has occurred. The above shelf-life calculation was made on the assumption that the $E_a$ remained constant over the temperature range studied.

**pH-Rate Profile**

The rate of degradation of MT-II at various pH values followed apparent first-order kinetics over the time course of the studies. From the pH-rate profile, shown in Figure 5, the pH of optimum stability was estimated to be approximately 5.0. A general hypothetical rate equation for this process can be written as follows:
\[ K_{\text{obs}} = K_{H^+} [H^+]^n + K_o + K_{OH^-} [OH^-]^m \]

where \( K_{H^+}, K_{OH^-}, K_o \) are the rate constants for the specific acid-, base- and water-catalyzed reactions, respectively. The decreasing (n) and increasing (m) slopes of the pH-rate profile were estimated to be -0.102 and 0.127, respectively. Therefore, the orders with respect to hydrogen and hydroxide ions were -0.102 and 0.127, respectively. The other three parameters (\( K_{H^+}, K_{OH^-} \) and \( K_o \)) were obtained by fitting the pH-rate data to above equation using a nonlinear least-square regression program. The estimated n and m values were fixed during the regression analysis. The best fit of the data provided values of \( K_{H^+} = 0.015 \) (S.E. = 0.004), \( K_o = -0.003 \) (S.E. = 0.002), and \( K_{OH^-} = 0.047 \) (S.E. = 0.006). Since \( K_o \) is not significantly different from zero the rate equation can be written as follows:

\[ K_{\text{obs}} = 0.015[H^+]^{-0.102} + 0.047[OH^-]^{0.127} \]

From the above analysis, the following conclusions can be made:

1. The degradation reaction is not specific acid- or base-catalyzed by hydrogen or hydroxide ion, respectively, since the slopes in the acid or base regions of the pH-rate profile were not +1 nor -1, respectively. This finding may indicate that MT-II degrades by several different pathways, each with its own pH dependence and true catalytic
2. The uncatalytic term (water) does not play a role in the
degradation of MT-II.
3. The hydroxyl ion catalyzes MT-II degradation to a greater
degree than the hydrogen ion.

**Effect of Ionic Strength**

The following modified form of the Debye-Huckel
equation was used to examine the effect of ionic strength on
the degradation rate of MT-II:

\[
\log K_{\text{obs}} = \log K_0 + 2Q.Z_A Z_B [\sqrt{\mu}/(1+\sqrt{\mu})] 
\]

where \( K_0 \) is the rate constant at \( \mu = 0 \), \( Q \) is a constant for
a given solvent and temperature, and \( Z_A \) and \( Z_B \) are the
charges on species A and B, respectively. In this study,
the plot of \( \log K_{\text{obs}} \) against \( \sqrt{\mu}/(1+\sqrt{\mu}) \) gave a slope that was
not significantly different from zero (\( p = 0.983 \)) (Figure 6). Thus, the kinetic salt effect on the degradation
kinetics of MT-II was interpreted to be negligible.

**Effect of Phosphate Buffer Concentration**

An increase in phosphate buffer concentration increases
the rate of degradation of MT-II, as shown in Figure 7.
MT-II diacetate may exist as the neutral, monoacidic or
diacidic cationic species depending on the pH of the
reaction medium. Therefore, in aqueous media, the following
expressions may be written for MT-II:
\[ \text{BH}_2^{2+} \rightarrow \text{BH}^+ + \text{H}^+ \quad (1) \]
\[ \text{BH}^+ \rightarrow \text{B} + \text{H}^+ \quad (2) \]

where B, BH\(^+\) and BH\(_2^{2+}\) denote the neutral, monoacidic and diacidic cationic species, respectively. The effect of buffer concentration on the rate of degradation was studied at a constant pH of 9.11. At this pH, the equilibrium represented by equation 2 predominates, since pH \(\gg\) pK\(_1\). Therefore, it can be assumed that either BH\(^+\) or B can undergo degradation. The following kinetic schemes may then be written:

\[ \text{BH}^+ \rightarrow \text{Products} \quad (3), \text{ or} \]
\[ \text{B} \rightarrow \text{Products} \quad (4) \]

In the above reactions, the two forms of MT-II can react with hydroxide ion, hydrogen ion, or the phosphate buffer species to form products. In a previous experiment, we had shown that the ionic strength had no effect on the rate of degradation of MT-II. Therefore, the possibility of two charged species reacting can be eliminated from consideration since one of the reactants must be the neutral species (Carstensen, 1970). Since hydroxide ion, hydrogen ion, and the phosphate buffer species are all charged, it follows that the uncharged reactant has to be the neutral form (B) of MT-II. Therefore, the reaction represented by equation 3 can be assumed not to occur. From equation 4,
the following generalized rate equation may be written as follows:

\[ \frac{d(\text{Products})}{dt} = K_{\text{obs}} \, [B] \]  

(5)

\( K_{\text{obs}} \) can be decomposed into individual equations for specific acid/base and general acid/base catalysis. Hence, the generalized rate equation may be written as:

\[ K_{\text{obs}} = K_0 + K_1 [H^+] + K_2 [OH^-] + K_3 [H_2PO_4^-] + K_4 [HPO_4^{2-}] \]  

(6)

Since the rate of degradation was observed to increase with an increase in phosphate buffer concentration, the contribution of general acid/base catalysis to the overall rate of degradation is highly significant. At the experimental pH (9.11), the predominant phosphate buffer species is HPO_4^{2-} and the hydrogen ion concentration is very low. Therefore, the overall rate equation can be simplified to:

\[ K_{\text{obs}} = K_2 [OH^-] + K_0 + K_4 [HPO_4^{2-}] \]  

(7)

**CONCLUSIONS**

From the above results, it may be concluded that MT-II is relatively stable and has the potential to be successfully formulated into an oral solid (tablet or capsule) dosage form, or a liquid dosage form to be taken soon after preparation. However, the pH of all formulations should be maintained as close to 5.0 at as low a phosphate
buffer concentration as possible. Finally, such aqueous formulations should be stored at refrigerator temperature (4°C) unless used within 24 hours of preparation.

The effects of temperature, ionic strength and phosphate concentration on MT-II degradation were not studied at the pH of optimum stability in order to accelerate the degradation process and hence obtain data more quickly. Nevertheless, these results should closely mimic the pattern observed at pH 5.0.
Figure 1. Structure of α-melanotropin analog (MT-II).
STRUCTURE OF MELANOTAN-II (MT-II)

Ac-[Nle^4, Asp^5, D-Phe^7, Lys^{10}] α-MSH_{4-10}-NH_2
Figure 2. Chromatograms of MT-II (10 µg/ml) in (A) pH 2.4 and (B) pH 7.0, 0.02 M phosphate buffers when stored at 4°C for 300 days. Key (1) MT-II, (2-4) degradation products. The number assigned to the degradation products in A and B are arbitrary and are not meant to imply that the peak so numbered represents identical molecules.
CHROMATOGRAMS OF MT-II (10μg/mL)
(0.02M PO₄, 4°C, 300 DAYS)
Figure 3. Typical apparent first-order plot for the degradation of MT-II in pH 7.0, 0.02 M phosphate buffer ($\mu = 0.15$) at $60 \pm 0.2^\circ$C. The solid line represent the fitted line.
TYPICAL APPARENT FIRST-ORDER PLOT FOR DEGRADATION OF MT-II (pH 7.0, 0.02M PO₄)
Figure 4. Arrhenius plot of the degradation of MT-II in pH 7.0, 0.02 M phosphate buffer and constant $\mu$ of 0.15 ($r^2 = 0.98$). The solid line represents the fitted line.
ARRHENIUS PLOT FOR THE DEGRADATION OF MT-II
(pH 7.0, 0.02M PO₄, μ = 0.15)
Figure 5. pH-rate profile for the degradation of MT-II in 0.02 M phosphate buffer, pH 2.0-9.5, $\mu = 0.15$, at $60 \pm 0.2^\circ C$. The solid line represents the fitted line.
pH-RATE PROFILE FOR THE DEGRADATION OF MT-II
(pH 2.0-9.5, 0.02M PO₄, μ = 0.15)
Figure 6. Effect of ionic strength on the degradation of MT-II in 0.02 M phosphate buffer, pH 9.11 at 60 ± 0.2°C. The solid line represent the fitted line.
EFFECT OF IONIC STRENGTH ON THE DEGRADATION OF MT-II (pH 9.11, 0.02M PO₄)
Figure 7. Effect of phosphate buffer concentration on the degradation of MT-II, pH 9.11 and $\mu = 1.5$, at $60 \pm 0.2^\circ C$. The solid line represent the fitted line.
EFFECT OF PHOSPHATE BUFFER CONCENTRATION ON THE DEGRADATION OF MT-II (pH 9.11, 0.02M PO₄)

Log Percent Remaining

0  2  4  6  8  10
Time (hr)

〇: 0.5M
●: 0.1M
△: 0.02M

0  0.02M  0.05M
2.05  2.00  1.95  1.90  1.85  1.80
Table 1. Observed Rate Constants, Arrhenius Parameters and Shelf-Lives for MT-II Degradation.  

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$K_{obs}$ (hr$^{-1}$)</th>
<th>$t_{90}$ (hr)</th>
<th>$\Delta H^\ddagger$ (Kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ± 0.2</td>
<td>0.0102</td>
<td>10.29</td>
<td>6.89</td>
</tr>
<tr>
<td>60 ± 0.2</td>
<td>0.0155</td>
<td>6.77</td>
<td>6.87</td>
</tr>
<tr>
<td>70 ± 0.2</td>
<td>0.0202</td>
<td>5.87</td>
<td>6.85</td>
</tr>
</tbody>
</table>

* Conditions: pH 7.0, $\mu = 0.15$ and $[\text{PO}_4] = 0.02$ M.

$^b$ Activation energy (Ea) = 7.5 Kcal/mole

$^c$ Log $K_{obs} = -Ea/2.303RT + \log A$
CHAPTER 5
A METHOD FOR THE DETERMINATION OF ACID DISSOCIATION CONSTANTS OF MODEL PEPTIDES

SUMMARY

A novel method is reported for determining the acid dissociation constants of peptides. The method involves the potentiometric titration of the peptide, following which the data are analyzed utilizing the Gran plot and the Best program. In addition to the dissociation constants of the peptide, the method is capable of accurately determining the contamination of the peptide sample with weak acids, strong acids, or water. The method is validated using boric acid and a peptide molecule.

INTRODUCTION

Recent advances in recombinant DNA and hybridoma techniques coupled with progress in large-scale purification and fermentation processes have resulted in the production of an increasing number of peptide and protein drugs (1,2). At present most of these agents must be given by injection since their large size, instability, and high polarity severely limit their formulation into dosage forms intended for other routes of administration. Thus, for these types
of compounds, formulation becomes a particularly challenging obstacle to overcome before their clinical utility can be realized. Knowledge of the physical properties becomes especially important in helping to guide the formulator to prepare efficacious dosage formulations. One of the important properties of peptides and protein molecules is the degree of dissociation of their constituent amino acids. In order to assess this important parameter it is necessary to know their dissociation constants, i.e., their pKa values.

One of the most widely used methods for the determination of acid dissociation constants is potentiometry. Other methods, such as spectrophotometry, are generally less accurate, more time consuming, or difficult to use in complicated systems. These methods are generally employed in cases where potentiometric titrations are inappropriate, e.g., in determination of pKa's above 12 or below 2, or for a compound of low aqueous solubility (3,4).

Despite many advantages of the potentiometric method for pKa determinations, there are a few common problems encountered in the pKa determination of commercially available peptides. Many commonly available peptides are purified by gradient HPLC using trifluoroacetic acid (TFA)-acetonitrile mobile phase. The peptide-containing fraction
is collected and freeze-dried (5). The purified peptide may be converted to its acetate or chloride salt by redissoving it in either glacial acetic acid or HCl solution, respectively, followed by freeze-drying. It is therefore extremely difficult to obtain a peptide of high purity free of low levels of acidic and basic contaminants for the following reasons:

1. Salts of peptides are often contaminated by small amounts of acetic acid or HCl left after the freeze-drying process. The amount of residual acids depends on the type of vacuum employed during the freeze-drying process and on the nature of the peptide, and therefore varies with each batch synthesized.

2. Most chloride or acetate salts of peptides are hygroscopic, and therefore the determination of the amount of peptide by weighing is subject to errors. This error can be significant when titration experiments are performed with small quantities of peptide.

3. If the peptide is supplied in the form of a chloride salt, it will likely contain a small amount of unreacted HCl. Any weak acid or strong acid contaminant in a peptide sample introduces uncertainties in the potentiometric determination of the pKa values because these contaminants consume the strong base added during the
potentiometric titration.

The purpose of this paper is to establish a general method which provides answers to the following questions:

1. Is the peptide acetate salt sample contaminated by acetic acid or acetate ions? If so, how many millimoles of the contaminant are present?

2. Is the peptide salt contaminated by strong acids? If so, how many millimoles of the strong acid contaminants are present?

3. What are the pKa values of the ionizable groups on the peptide?

**THEORETICAL CONSIDERATIONS**

Peptides are usually polyprotic weak acids/bases in nature and their successive dissociation constants can be determined with the aid of the BEST program (6), which is a multiparametric non-linear weighted least-squares fitting program for all points on the potentiometric titration curve. The BEST program calculates the \(-\log[H^+]\) on the basis of input parameters and compares these values with experimental values. The pKa's and other parameters are then refined to their "best" values until the standard deviation (\(\sigma\)) of the differences between the calculated and experimental values is a minimum. This program also has
the capability of determining the number of millimoles of all ionizable species initially present in the system (7).

In any non-linear optimization procedure, the number of parameters should be the smallest possible estimated simultaneously should be the smallest possible. Therefore, in this study, we employed a linear least-squares regression method to determine the total number of millimoles of titratable protons in the system, following which we will use the BEST program to determine the number of millimoles of the peptide, the pKa values of the peptide, and any ions present, e.g., acetate, by estimating these values simultaneously.

A fully protonated peptide with \( m \) protonation sites, after acidification with excess strong acid, e.g., HCl, can be represented as \( \text{HA}^n^- \). It is necessary to acidify the peptide solution before performing the potentiometric titration in order to measure the first pKa of the peptide. The number of moles of strong acid added is \( (N_{\text{SA}})_{\text{add}} \). After acidification, a solution of the peptide will generally also contain acetic acid, HAc, and Cl\(^{-}\) as a counter ion. The total number of titratable protons in solution, which include the free hydrogen ions, the hydrogen ions on the peptide and acetic acid, is represented as \( (N_{\text{H}^+})_{\text{tot}} \) after acidification. The total number of millimoles of the
peptide and acetic acid are represented by \((N_{A1})_{tot}\) and 
\((N_{Ac})_{tot}\), respectively. The number of millimoles of free \(H^+\) and 
the volume of the peptide solution after acidification are represented by \((N_{SA})_{free}\) and \(V_a\) (mL), respectively. During 
the titration of the above acidified peptide solution, a volume \(V_b\) (mL), of a strong base titrant, e.g., \(NaOH\), of 
concentration \(C_b\) (moles/L), is added to the solution.

In the high pH region of the titration curve, where all 
titratable hydrogen ions are neutralized by the \(NaOH\) added and 
the pH value is approximately 2 units higher than the 
highest pKa estimated for the peptide, the charge balance 
equation for this system is approximated as:

\[
[Na^+] + (n-m) A_1^{(n-m)+} = [OH^-] + [Cl^-] + [Ac^-] \quad (1)
\]

The mass balance equation for \(Cl^-\) and \(Na^+\) at high pH is:

\[
[Cl^-] = \left\{ (N_{SA})_{free} + n A_1^{(n-m)+} \right\} \frac{(V_a + V_b)}{(V_a + V_b)} \quad (2)
\]

\[
[Na^+] = \frac{C_b V_b}{(V_a + V_b)} \quad (3)
\]

and the mass balance for \(Ac^-\) is given by

\[
[Ac^-] = \frac{(N_{Ac})_{tot}}{(V_a + V_b)} \quad (4)
\]

By substitution of Equations 2, 3 and 4 into equation 1, 
multiplying the resulting equation by \((V_a + V_b)\) and 
rearranging, equation 5 is obtained:

\[
[OH^-] (V_a + V_b) = C_b V_b - \left\{ (N_{SA})_{free} + (m A_1^{(n-m)+}) + [Ac^-] \right\} (V_a + V_b) \quad (5)
\]

The mass balance for the total number of millimoles of
$\text{H}^+$, which includes the free hydrogen ions as well as the hydrogen ions bound to the peptide and acetic acid, after acidification with strong acid can be represented as:

$$\left( N_{\text{H}^+} \right) = \left( N_{\text{SA}} \right)_{\text{free}} + \left( m[A_1^{(n-m)^+}] + [\text{Ac}^-] \right) (V_a + V_b) \quad (6)$$

at pH 2 units higher than the highest pKa of the peptide. Substitution of equation 6 into equation 5 gives:

$$[\text{OH}^-] (V_a + V_b) = C_b V_b - \left( N_{\text{H}^+} \right)_{\text{tot}} \quad (7)$$

A plot of $[\text{OH}^-] (V_a + V_b)$ versus $V_b$ yields a straight line. The slope of the straight line is $C_b$ and the Y-intercept is $\left( N_{\text{H}^+} \right)_{\text{tot}}$. The pKa values of the peptide, as well as the number of millimoles of acetate ions, $\left( N_{\text{Ac}^-} \right)_{\text{tot}}$, and the number of millimoles of the peptide, $\left( N_{A_1} \right)_{\text{tot}}$, are determined by the BEST program. The total number of millimoles of the titratable protons in solution, which includes the free hydrogen ions, and the hydrogen ions in the peptide as well as the acetic acid, is represented as $\left( N_{\text{H}^+} \right)_{\text{init}}$ before acidification with strong acid, and is calculated as follows:

$$\left( N_{\text{H}^+} \right)_{\text{init}} = \left( N_{\text{H}^+} \right)_{\text{tot}} - \left( N_{\text{SA}} \right)_{\text{add}} \quad (8)$$

The number of millimoles of strong acid contaminants such as hydrochloric acid, designated as $\left( N_{\text{SA}} \right)_{\text{cont}}$, in the initial peptide sample are determined as follows:

$$\left( N_{\text{SA}} \right)_{\text{cont.}} = \left( N_{\text{H}^+} \right)_{\text{init}} - \left( m \left( N_{A_1} \right)_{\text{tot}} + \left( N_{\text{Ac}^-} \right)_{\text{tot}} \right) \quad (9)$$

A comparison of $\left( N_{\text{H}^+} \right)_{\text{init}}$, $\left( N_{A_1} \right)_{\text{tot}}$ and $\left( N_{\text{Ac}^-} \right)_{\text{tot}}$ provide some
information about the purity of the peptide sample.

MATERIALS AND METHODS

MATERIALS:

A 50% (w/w) NaOH solution was purchased from Fisher Scientific (Tustin, CA), potassium acid phthalate (99.95-100.05%) was purchased from Aldrich Chemical Co. (Milwaukee, WI), Concentrated nitric acid (HNO₃) was purchased from EM Science (Gibbtown, NJ), Schizophrenia related peptide (TVL), a tripeptide, was purchased from Peptide International (Louisville, KY), and was used as received. All other analytical grade chemicals used in the study were purchased from Mallinckrodt, Inc. (Paris, KY).

METHODS:

Potentiometry:

Potentiometric measurements of hydrogen ion concentration were performed in a 150 mL glass jacketed vessel provided with a magnetic stirrer and a tightly fitting rubber stopper. The latter was equipped with inlet and outlet tubes for nitrogen, a buret for delivery of NaOH, a glass pH electrode, a salt-bridge, made of 2% agarose gel containing saturated KCl, which was connected to a saturated calomel reference electrode, a thermometer, and a glass rod bent into a right angle at its end for removal of NaOH drops
suspended on the tip of the buret. During the titration, the tip of the buret was positioned above the surface of the solution to minimize the possible replacement of the NaOH on the tip of the buret with titration solution. The temperature in the titration vessel was maintained at 25 ± 0.1°C by circulating thermostated water through the jacket using a VWR 1160 refrigerated circulator. To minimize the effect of nitrogen flow on the evaporation of the titration solution, the nitrogen gas was saturated with water vapor by passing it through a solution of 0.1 M NaNO₃.

A standard NaOH solution was prepared from 50% (w/w) NaOH solution and CO₂-free deionized H₂O and the prepared NaOH solution was stored in a KIMAX precision bore three-way stopcock buret maintained under a nitrogen atmosphere to prevent the absorption of CO₂. The carbonate content of the standard NaOH solution (0.5%) was determined by the method of Martell and Montekaitis (6). In the above calculation (6), -log[H⁺] values were used instead of the pH meter readings. The molarity of the NaOH solution was determined with potassium acid phthalate using phenolphthalein as indicator. A stock HNO₃ solution, prepared from concentrated HNO₃ and CO₂-free deionized water, was standardized with the standard NaOH. A stock NaNO₃ solution (1.000 M) was prepared by dissolving 42.50 g NaNO₃ in 500.0
mL CO₂-free deionized water.

**pH Meter Calibration**

A BECKMAN model IP-72 pH Meter, equipped with an electrode pair, was calibrated with standard buffers (pH = 4.00 and 7.00). Titration of 50.00 mL of 2.06x10⁻³ M nitric acid (HNO₃) (0.1028 millimoles) solution (in 0.100 M sodium nitrate; NaNO₃) with standard 0.1207 M NaOH was performed to obtain an experimental curve for the conversion of measured pH to -log[H⁺]. The conversion curve was prepared by using the titration data obtained from the acidic region only and then extrapolated to the basic region based on the assumption that the glass electrode respond linearly up to pH 11. This assumption was later verified by our experimental results. The ion-product of water (Kw) was calculated using the Debye-Hückel equation (8) at 25°C and an ionic strength of 0.10.

**Determination of Acid Dissociation Constants of Boric Acid**

A stock solution of boric acid (H₃BO₃), 40.00mM, was prepared by dissolving a weighed amount of dried H₃BO₃ in CO₂-free deionized water. A stock solution of boric acid (5.000 mL) was transferred into a 50 mL volumetric flask followed by addition of 1.000 mL (0.1028 millimoles) of HNO₃ and 5.000 mL of 1.000 M sodium nitrate (NaNO₃). The resulting solution was diluted to 50.000 mL with CO₂-free
water, then 40 mL of the diluted solution was transferred into a titration vessel and potentiometric titration was performed at 25.0°C under a nitrogen atmosphere.

**Determination of Acid Dissociation Constants of Peptides**

A weighed amount of the peptide (Fig. 1) was placed in the titration vessel, followed by addition of 1.000 mL (0.1028 millimoles) of HNO₃, 4.000 mL of 1.000 M NaNO₃ and 35.00 mL of CO₂-free deionized water. The potentiometric titration was performed at 25°C under nitrogen atmosphere.

### RESULTS AND DISCUSSION

#### pH Calibration

The pH calibration curve determined for the pH measuring system is shown in Fig. 2a, and the resulting linear least squares regression is shown below (equation 10). The correlation coefficient was 0.9999.

\[-\log[H^+] = 1.000(4) \times \text{pH}_{\text{measured}} - 0.038(2) \quad (10)\]

The numbers in parentheses denote the uncertainties in the last digits of the slope and y-intercept, respectively. The uncertainty in the values of pH$_{\text{measured}}$ was 0.001. Based on the calculated propagation of uncertainties (9), the uncertainty in the values of -log[H⁺] was about 0.015 at pH$_{\text{measured}}$ < 3.5 and 0.014 at pH$_{\text{measured}}$ of approximately 11, which ensured the reliability of the experimentally
determined pKa's of the peptide. Theoretically, the slope of the straight line (equation 10) should be 1 with Y-intercept of -0.084, as calculated from the Debye-Hückel equation (8) under the experimental conditions. In practice, however, the value of the Y-intercept is rarely the same as the value calculated from the Debye-Hückel equation because of the uncertainties introduced by the variation in the experimental conditions under which the parameters in the Debye-Hückel equation were calculated, including:

1. the variation in the background electrolyte
2. the variation in the manner in which the junction potential at various interfaces are maintained.
3. the uncertainties involved in the determination of the parameters used by the Debye-Hückel equation, which is not reported in the literature.

Therefore, in our laboratory, we prefer to employ an experimental calibration curve to obtain the values of hydrogen ion concentration rather than to calculate the hydrogen ion concentration from activity coefficients of the hydrogen ion obtained from the Debye-Hückel equation (8). The value of the slope may deviate from 1. This deviation mainly originated from the errors introduced during the calibration of the pH meter with the two standard pH
buffers. The uncertainty associated with the experimentally determined slope is the major source of the uncertainty in the determination of \(-\log[H^+]\) especially in the high pH region. The validity of the experimental curve for the conversion of measured pH to \(-\log[H^+]\) was examined using the Gran plot method (11):

\[
[H^+] (V_a + V_b) = -C_b V_b + C_a V_a \tag{11}
\]

\[
[OH^-] (V_a + V_b) = C_b V_b - C_a V_a \tag{12}
\]

where \(C_a\) represents the initial concentration of HNO₃. After an experimental curve (Fig. 2a) was obtained from the titration of HNO₃ with NaOH, \(pH_{\text{measured}}\) at each titration point was converted to \(-\log[H^+]\) using the least-squares linear regression equation (equation 10). A plot of the left side of equation 11 versus \(V_b\), using titration data obtained in the acidic region gave a straight line, with a slope of \(C_b\) and a y-intercept of \(-C_a V_a\). A plot of the left side of equation 12 versus \(V_b\), using titration data obtained in the basic region gave a straight line with slope of \(-C_b\) and y-intercept of \(C_a V_a\). If the experimental conversion curve is valid, the two y-intercepts and slopes calculated from equations 11 and 12 should be identical within the experimental uncertainties. In reality, due to the contamination of NaOH with CO₂, the slope calculated from equation 12 should be smaller than the slope calculated from
equation 11 by a percentage equal to the percentage of \( \text{CO}_2 \) present in the \( \text{NaOH} \) solution. The titration curve of \( \text{HNO}_3 \) with \( \text{NaOH} \) and the Gran plots are shown in Fig. 2(b). The summary of the parameters obtained from the experimental conversion curve are shown in Table I. As can be seen from this table, the slopes and intercepts were essentially equal.

**Verification of the Method with Boric Acid**

Since it was difficult to obtain a peptide of high purity with known pKa values to serve as a standard, boric acid was used to verify the proposed method. Boric acid is a good candidate to serve as a standard acid because it is not hygroscopic and thus the amount of boric acid used can be weighed accurately. The first acid dissociation constant of boric acid has been reported (9). However, the dissociation equilibria of boric acid are complicated, therefore, it was not feasible to calculate the total number of millimoles of \( H^+ \), \( (N_{H^+})_{\text{tot}} \) in the very high pH region. During the titration of boric acid, the addition of \( \text{NaOH} \) was stopped after the first equivalence point was reached. The \( (N_{H^+})_{\text{tot}} \) was determined on the basis of stoichiometry while the pKa and the amount of boric acid used in the titration were determined using the BEST program. Acetic acid was assumed to be initially present in the titration solution.
and its amount was determined by the BEST program as well. The pKa of acetic acid at 25.0°C and 0.1 M ionic strength calculated using the Debye-Hückel equation (10) was 4.564 (8,9). The experimental and fitted titration curves are shown in Fig. 3 and the refined parameters obtained from the BEST program are listed in Table II. The pKa value determined was in good agreement with the value previously reported (9).

The use of boric acid as a standard has established the validity of the proposed method. The uncertainty in the amount of the major weak acid component was about 1%. As expected, the number of millimoles of acetate present in the titration solution was zero and the uncertainty in the amount of acetic acid was 1.8%.

**Determination of Acid Dissociation Constants of the Peptide**

The experimental titration curve as well as the fitted titration curve are shown in Fig. 4. The results of the linear least-squares method and the refined parameters are listed in Table III. Since the \( (N_{SA})_{cont} \) determined was slightly less than zero, the initial peptide was not contaminated by strong acid. The \( (N_{AC})_{tot} \) was found to be 0 within experimental error, therefore, neither acetic acid nor acetate ions was initially present in the peptide sample. The \( (N_{H^+})_{init} \) determined from the linear least-
squares method was 0.0267 millimoles which was slightly less than the number of millimoles of peptide (0.03288 millimoles). We therefore conclude that the peptide supplied was a zwitterion and not the acetate salt as claimed. The two pKa's determined were close to values expected for α-amino and α-carboxylic acid groups of peptides.

The method developed above is also suitable for determination of acid dissociation constants of polypeptides with several pKa values. However, the reliability of the pKa values and the amount of acetate determined by potentiometry are dependent upon the reliability of the potentiometric determination of hydrogen ion concentration. The reliability of the parameters obtained using this method is also limited by the number of data points obtained in the buffer region of the titration curve. In general, the uncertainty of the estimated parameters increases when fewer data points are used in the non-linear fitting program. It is therefore necessary to collect a large number of data points in the buffer region. During the titration, the smallest increments of NaOH volume should be added to the titration vessel in order to increase the total number of data points. This is the fundamental limitation of the accuracy of this method since the amount of peptide
available for potentiometric titration is usually very small. Alternatively, more data points can be collected by adding more dilute NaOH to the titration vessel. However, it is very difficult to maintain the ionic strength during the titration because of the dilution effect following NaOH addition. Furthermore, it may be necessary to neglect some data points in the high and low pH region to increase the weight of data points in the buffer region.
Figure 1. Structure of a fully protonated peptide, TVL.
STRUCTURE OF TVL PEPTIDE

Thr — Val — Leu
Figure 2a. pH Calibration Curve at 25.0°C and at an ionic strength of 0.10. Solid line: \(-\log[H^+]\) vs pH$_{\text{measured}}$; ■: experimental data points calculated from the concentration of the standard solutions; □: experimental data points calculated from the concentration of the standard solution and pKw of 13.80, using Debye-Hückel equation.
CONVERSION CURVE FOR THE pH METER
Figure 2b. Potentiometric titration curve of HNO$_3$;

■: experimental data points; +: data points for the Gran plot in the acidic region; *: data points for Gran plot in the basic region; solid lines are fitted lines by least-squares linear regression.
POTENTIOMETRIC CURVE OF STANDARD HNO,

Volume of NaOH added (mL)

[+H]601-
Figure 3. Potentiometric titration curve of a mixture of boric acid and HNO₃. ■: experimental data points; solid line represents the titration curve determined by the BEST program.
POTENTIOMETRIC CURVE OF BORIC ACID

-\log[H^+]

Volume of NaOH (mL)
Figure 4. Potentiometric titration curve of TVL peptide acidified with HNO₃. •: experimental data along with titration curve fitted by BEST program. ■: data points for Gran plot in the basic region along with fitted line by linear least-squares regression.
POTENTIOMETRIC TITRATION CURVE OF TVL PEPTIDE

\[ \text{Vb (mL)} \]

\[ \text{[H}^+\text{]} \]

\[ [\text{OH}^-=V_a+V_b] \]
Table I. Analysis of the Experimental Conversion Curve by Gran Plot$^{a,b,c}$

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S2</th>
<th>I1</th>
<th>I2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.1208 (6)</td>
<td>0.1201 (1)</td>
<td>0.1028 (3)</td>
<td>-0.1031 (2)</td>
</tr>
</tbody>
</table>

$^a$S1 and S2 represent the slopes from Equations 11 and 12 respectively.

$^b$I1 and I2 represent y-intercepts calculated from Equations 11 and 12, respectively.

$^c$The numbers in parenthesis are uncertainties in last digits of values of slopes and intercepts obtained by linear least-squares regression.
Table II. Summary of the Titration Data for Boric acid at 25°C

<table>
<thead>
<tr>
<th>( {b(N_A)}_{\text{tot}} )</th>
<th>( {b(N_A)}_{\text{tot}} )</th>
<th>( {a(N_A)}_{\text{tot}} )</th>
<th>( {b(N_{A1})}_{\text{tot}} )</th>
<th>pKa*</th>
<th>pKa®</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0028</td>
<td>0</td>
<td>0.1582</td>
<td>0.1604</td>
<td>9.062</td>
<td>9.061</td>
</tr>
</tbody>
</table>

*Values determined by the BEST program, \( \sigma_{fit} = 0.0009 \). The uncertainty in the number of millimoles of boric acid calculated is not obtainable from the BEST program.

bValues calculated from the concentration of the standard solution.

A1 represents conjugate base of boric acid.

* Value determined by the BEST program; the uncertainty in the pKa is less than 0.020.

® pka value obtained from ref. 9.
Table III. Summary of the Titration Data for TVL at 25°C

<table>
<thead>
<tr>
<th>$(N_{H^+})_{tot}$</th>
<th>$(N_{SA})_{add}$</th>
<th>$(N_{H^+})_{init}$</th>
<th>$(N_{SA})_{cont}$</th>
<th>$(N_{AC})_{tot}$</th>
<th>$(N_{TVL})_{tot}$</th>
<th>$pK_1$</th>
<th>$pK_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1295$^a$</td>
<td>0.1028</td>
<td>0.0267</td>
<td>-0.0391</td>
<td>0.00003$^b$</td>
<td>0.03288$^b$</td>
<td>3.64$^b$</td>
<td>7.11$^b$</td>
</tr>
</tbody>
</table>

$^a$Values determined by the linear least-squares method.
$^b$Values refined by the BEST program, the $\sigma_{fit} = 0.0157$. 
$^*$Values in the first six columns are in millimoles.
CHAPTER 6

ENTERAL ABSORPTION OF \( \alpha \)-MELANOTROPIN ANALOGS (MT-I AND MT-II) FROM THE RAT INTESTINE

SUMMARY

The extent of absorption of MT-I (Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Gly-Pro-Lys-Val-NH\(_2\)) and MT-II (cyclic Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH\(_2\)) from the rat intestine was studied using an in situ closed-loop experimental method similar to one described by Schilling and Mitra (1). The plasma MT-I and MT-II concentrations was quantitated using RIA and HPLC, respectively. The absolute bioavailability (F) of MT-I solution, administered intra-jejunally to rats at a dose of 620 \( \mu \)g/kg, was 1.6 ± 0.1%. The administration of MT-I with dimethyl-\( \beta \)-cyclodextrin (DMCD), a permeation enhancer, did not significantly increase the absorption of MT-I. However, after intrajejunal administration of MT-I in the presence of DMCD and Aprotinin (APRO), a protease inhibitor, the bioavailability was significantly increased to 6.6 ± 1.4% (\( p < 0.05 \)). This result showed that enzymatic degradation was a significant barrier to the intestinal absorption of MT-I. The F of MT-II solution administered to a rat at a dose of 6.76 mg/kg, was 5.0%. This value was three times greater than that reported above for MT-I.
INTRODUCTION

The α-Melanocyte stimulating hormone (α-MSH or α-Melanotropin) is a tridecapeptide which regulates skin color in vertebrates (2). Melanotan-I (MT-I; Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Gly-Pro-Lys-Val-NH₂) is an analog of α-Melanotropin (3). MT-II is a cyclic lactam heptapeptide analog of α-Melanotropin (4). Both peptides have been shown to induce rapid skin darkening in in vitro frog- and lizard-skin bioassays (5) and also in humans (6,7).

Like most other peptides, MT-I and MT-II are routinely administered subcutaneously. Oral administration is the best route since it is preferred by most patients. However, the oral absorption of peptide and protein-based drugs is very low due to low gastric pH, proteolytic enzymes in the intestine and their low permeability across the gastrointestinal mucosa (8,9). Due to the aforementioned barriers, absorption promoters and protease inhibitors have been used in an attempt to enhance the gastrointestinal absorption of peptides (10).

In comparison to the natural hormone (α-MSH), MT-I and MT-II were reported to be fairly stable to purified proteolytic enzymes (i.e., pepsin, trypsin and chymotrypsin) in vitro (5). Although the results of the above study
demonstrated the metabolic stability of this peptide in vitro, its in vivo stability and bioavailability still need to be determined.

The first objective of this study was to determine the bioavailability of MT-I following its intra-jejunal administration using an in situ rat model. Our second objective was to evaluate the effect of Dimethyl-β-cyclodextrin (DMCD) and Aprotinin (APRO) on the absorption of MT-I across the rat jejunum. APRO has inhibitory effects on various proteolytic enzymes (11) and therefore inhibits the degradation of peptides and proteins in the gastrointestinal tract (12) and subcutaneous tissues (13). Cyclodextrins are cyclic oligomers of glucose with α-1,4 glycosidic bonds. DMCD is a methylated derivative of cyclodextrin which has been reported to enhance the absorption of insulin across the nasal mucosa of rats (14). Our third objective was to compare the absorption efficiencies of the two α-MSH analogs.

MATERIAL AND METHODS

Materials:

Purified MT-I Acetate (≥ 99% pure) was obtained from Bachem California, Torrance, CA. Purified MT-II was obtained from Dr. Victor Hruby of the Department of
Chemistry at the University of Arizona. Aprotinin (APRO), Biotechnology grade, was obtained from Miles, Kankakee, IL. Dimethyl-β-cyclodextrin (DMCD) was obtained from Pharmatec, Alachua, FL. Sodium Chloride Irrigation solution, 0.9% was obtained from Baxter Healthcare Corp., Deerfield, IL. Ethylenediaminetetraacetic acid dihydrate, tripotassium was obtained from Aldrich Chemical Co., Milwaukee, WI. Ketamine HCl was obtained from Aveco Co., Fort Dodge, IA. Xylazine was obtained from Lloyd Labs., Shenandoah, IA. Acetonitrile was obtained from Burdick & Jackson (Muskegon, MI). Potassium phosphates (monobasic and dibasic) was obtained from JT Baker (Phillipsburg, NJ). The RIA kits for MT-I analysis were obtained from Incstar Corp., Stillwater, MN. The water used in the experiments was deionized and distilled using a Millipore filter system (Millipore Corp., Bedford, MA.)

**Closed Loop-Intestinal Absorption Technique:**

Male Sprague-Dawley rats weighing 200-300 g were used for this study. The animals were acclimatized to the laboratory environment for one week prior to the study. The rats were fasted for 16-20 hr prior to each experiment; water was allowed ad libitum. The jejunal absorption of MT-I in the presence and absence of absorption promoters was studied using the closed-loop technique described by
Schilling and Mitra (1). This procedure essentially involves cannulation of the right external jugular vein of the anesthetized rat for periodic blood sampling. This was followed by a midline abdominal incision to expose a segment of the small intestine. A 15 cm segment of proximal jejunum (beginning 10 cm beyond the ligament of Trietz) was cannulated, washed with Normal Saline and ligated at the distal end. The appropriate test solution (~400 μL) was then instilled into the segment and the proximal end was ligated to form a closed loop. Blood samples (250-350 μL) were then collected from the jugular vein cannula into 1 mL plastic syringes at various time intervals following the start of each absorption experiment. An equal volume of Normal Saline was injected into the jugular vein to replace the volume of blood sampled. In order to determine the absolute bioavailability of each formulation of MT-I and MT-II administered enterally, intravenous doses of the two peptides were injected into the jugular vein. Blood samples were then collected at various times after dosing. All blood samples were collected in polypropylene tubes containing 10 μL of tripotassium EDTA (0.7 mg in 10 μL) and Aprotinin (18,000 Kallekrein inhibitor units in 10 μL). The blood samples were centrifuged at 10,000 Xg for 10 minutes at 4°C and the plasma samples harvested and stored at -70°C.
until assayed. The study design for MT-I is summarized below:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (N=3)</td>
<td>intravenous (186 µg/kg MT-I)</td>
</tr>
<tr>
<td>II (N=3)</td>
<td>jejunal (620 µg/kg MT-I)</td>
</tr>
<tr>
<td>III (N=3)</td>
<td>jejunal (620 µg/kg MT-I + DMCD (10µmole/kg))</td>
</tr>
<tr>
<td>IV (N=3)</td>
<td>jejunal (620 µg/kg MT-I + DMCD (10 µmole/kg) + APRO (1000 KIU/kg))</td>
</tr>
</tbody>
</table>

MT-II was administered intravenously and intra-jejunally at doses of 0.3 mg/kg and 6.76 mg/kg, respectively.

**Validation of the Intestinal Absorption Model:**

In order to assess the biochemical viability of the intestinal preparation during the time frame of the study, plasma samples obtained at various time intervals during one of the in situ experiments (Group IV) were analyzed for albumin, total protein, alkaline phosphatase. As a control, serum indices for serum samples collected from rats prior to their use in the intestinal absorption experiments were determined. All plasma samples were assayed by standard methods at the Animal Diagnostic Laboratory (Tucson, AZ).

**Radioimmunoassay (RIA) of Plasma MT-I:**

The MT-I plasma concentrations were determined using a RIA method reported previously (15).
High-Performance liquid chromatographic (HPLC) analysis of Plasma MT-II:

The MT-II plasma concentrations were analyzed using a HPLC method previously reported (16).

Data Analysis:

Plasma concentration vs. time data for MT-I and MT-II were analyzed by conventional noncompartmental methods using RSTRIP, a non-linear least-squares optimization program (17). The estimates of area under the curve (AUC) and mean residence time (MRT) were obtained from the RSTRIP analyses. MRT was calculated by dividing the area under the first moment curve (AUMC) by AUC. Total body clearance (CLs) and volume of distribution at steady state (Vss) were calculated as follows:

\[
CLs = \frac{\text{Dose}}{\text{AUC}}
\]

\[
Vss = CLs \times MRT
\]

Maximum concentration values (Cmax) were estimated visually from the plasma concentration-time plots. The bioavailability, F was calculated by comparing the AUC for each oral treatment to that of the intravenous dose as shown below:

\[
F = \frac{(AUC)_{po}}{(AUC)_{iv}} \times \frac{(Dose)_{iv}}{(Dose)_{po}}
\]
The data were expressed as mean ± SEM. Group differences were determined using Student’s t-test. A significance level of p < 0.05 was used in all treatment comparisons.

RESULTS AND DISCUSSION

To verify the validity of the closed-loop in situ technique, various serum indices were measured in order to assess the biochemical viability of the intestinal preparation (Table 1). Alkaline phosphatase is primarily localized in the intestinal mucosa (18,19), hence its release into the blood is an indication of intestinal damage. As shown in Table 1, the level of this enzyme remains essentially unchanged and within the normal range for rats during the 1.5 hour experimental period, indicating that the intestinal preparation was indeed viable during the period of the in situ experiments. There was a 20% decrease in total protein and albumin concentrations during the time course of the study which is indicative of blood loss as a result of multiple sampling. In the first one hour of experimental period, the total protein and albumin decreased by 6%. Based on these results, plasma MT-I and MT-II concentrations determined during the first 60 minutes of the iv and in situ experiments were used for the bioavailability
calculations.

Figure 1a shows a plasma concentration vs. time plot of MT-I in rats following an iv bolus dose of 186 μg/kg. Pharmacokinetic analysis of the plasma concentration vs. time data showed that the disposition profile was best described by a biexponential equation. The pharmacokinetic parameters calculated from the profiles are summarized in Table 2. The total clearance (CLs) and volume of distribution at steady state (Vss) were 0.8 ± 0.1 L.Kg\(^{-1}\)hr\(^{-1}\) and 0.1 ± 0.02 L.Kg\(^{-1}\), respectively. The CLs of the peptide in rats was about one-half of the average body clearance reported for rats (20), indicating that the peptide is restrictively cleared. Vss was greater than the total blood volume of a rat which may suggest that MT-I was distributed extravascularly.

Figure 1b shows the plasma MT-I concentraton vs. time plots following administration of MT-I alone (control), MT-I plus DMCD and MT-I plus DMCD/APRO to the jejunal segments of rat intestine. The absorption parameters calculated from the plasma concentration vs. time profiles are summarized in Table 3. The absolute bioavailability (%F) of MT-I (control) was determined to be 1.6 ± 0.1 %. The observed bioavailability of MT-I is comparable to values reported for other peptides of similar molecular weight and number of
127

amino acid residues (21,22). In general, most peptides and proteins are highly potent, hence their biological activity is observed at relatively low plasma concentrations (23). Although the absorption efficiency of MT-I reported here is low, the plasma concentrations achieved in this study were more than adequate to induce skin darkening in frogs.

The absorption efficiency of MT-I in the presence of DMCD was 1.8 ± 0.6 %. This value was not significantly different from the control value (p > 0.05) (Table 3). A previous study has shown DMCD to be a potent permeation enhancer of the nasal absorption of insulin (14) in rats. It has also been shown that DMCD can extract lipids from the nasal mucosal membrane, thereby increasing the nasal absorption efficiency of insulin (24). We anticipated that DMCD would enhance the oral absorption of MT-I by a similar mechanism, however, our results indicate that MT-I absorption efficiency was not significantly increased by DMCD. A recent study by Shao and Mitra (25) showed that the intestinal mucosa of rats was more resistant to the absorption-enhancing effects of DMCD than the nasal mucosa and alveolar sacs. This suggests that the intestinal mucosal membrane may be structurally different from nasal and alveolar membranes. Furthermore, cyclodextrins are degraded by amylases present in the gastrointestinal tract.
and, as a result, their absorption-enhancing effects may be reduced in the intestinal mucosa.

The %F of MT-I in the presence of DMCD and APRO was 6.6 ± 1.4 %, which was significantly different from control values (Table 3). This is most likely due to the inhibition of intestinal proteolytic enzyme activity by APRO.

The plots in Figure 2 represent the plasma pharmacokinetic profile of intravenously and intra-jejenally administered MT-II. Based on these plots, the F of MT-II was calculated to be approximately 5% (Table 4). Comparatively, the F for MT-II was three times greater than the value for MT-I. The difference in F of the two peptides can be attributed to differences in their molecular weights and in vivo stability. The molecular weight of MT-I is about two-fold greater than that for MT-II and in a previous report (5), MT-II was shown to be more resistant to proteolytic enzymatic degradation in vitro than MT-I, which further substantiates its better bioavailability.

The results of this study indicate that the development of oral delivery systems for these peptides is feasible. Thus, the optimization of the oral formulation of these α-MSH analogs appear warranted.
Figure 1: MT-I plasma level profile in rats following bolus intravenous administrations of 0.186 mg/kg dose (A) and in situ jejunal administration of various formulations of 620 μg/kg MT-I in absence or presence of absorption promoters (DMCD or DMCD/APRO). Each data point represent mean values ± SEM (n = 3).
PLASMA CONCENTRATION VS. TIME PROFILES FOLLOWING IV AND ENTERAL ADMINISTRATIONS OF MT-II

CONCENTRATION (ng/mL)

TIME (hr)

--- iv

--- in-situ
Figure 2: MT-II plasma level profile in rats following bolus intravenous administration of 0.3 mg/kg dose (●) and in-situ jejunal administration of 6.76 mg/kg dose (○).
PLASMA CONCENTRATION VS. TIME PROFILE FOLLOWING IV ADMINISTRATION OF MT-I

A

PLASMA CONCENTRATION VS. TIME PROFILES FOLLOWING ENTERAL ADMINISTRATION OF MT-I

B

TIME (min)
Table 1. Effect of Study Time on the values of Serum Indices of Biochemical Viability of the Rat Intestinal Preparation

<table>
<thead>
<tr>
<th>Time, Min</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase (I.U./L)</td>
<td>347</td>
<td>343</td>
<td>359</td>
<td>372</td>
<td>305</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td>5.2</td>
<td>4.9</td>
<td>4.9</td>
<td>4.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.4</td>
<td>3.3</td>
<td>-</td>
<td>-</td>
<td>2.4</td>
</tr>
<tr>
<td>Parameter</td>
<td>Value</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>--------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng.mL(^{-1}))</td>
<td>1960.0 ± 145.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (ng.mL(^{-1}).hr)</td>
<td>264.9 ± 45.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLs (L.Kg(^{-1}).hr(^{-1}))</td>
<td>0.8 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vss (L.Kg(^{-1}))</td>
<td>0.1 ± 0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \text{n} = 4 \text{ rats} \\
stext{bMean ± SEM}
Table 3. Comparison of Pharmacokinetic Parameters observed in In-Situ Intestinal Absorption Studies of MT-I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC$_{0-60\text{min}}$ (ng.mL$^{-1}$·hr)</th>
<th>Absorption Efficiency (%)</th>
<th>Cmax (ng.mL$^{-1}$)</th>
<th>t$_{max}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>285 ± 94.0</td>
<td>100</td>
<td>1960.0 ± 145.4</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>CONTROL</td>
<td>15.8 ± 0.8</td>
<td>1.6 ± 0.1</td>
<td>33.0 ± 1.8</td>
<td>14.5 ± 0.5</td>
</tr>
<tr>
<td>DMCD</td>
<td>17.5 ± 5.1</td>
<td>1.8 ± 0.6</td>
<td>24.1 ± 10.0</td>
<td>14.9 ± 0.5</td>
</tr>
<tr>
<td>DMCD/APRO</td>
<td>62.9 ± 13.5*</td>
<td>6.6 ± 1.4*</td>
<td>86.2 ± 16.5*</td>
<td>15.1 ± 0.9</td>
</tr>
</tbody>
</table>

*significant increase compared to control at p < 0.05.
Table 4. Pharmacokinetic Parameters following IV and In-Situ Intestinal Absorption Studies of MT-II in a Rat*  

<table>
<thead>
<tr>
<th></th>
<th>IV</th>
<th>IN-SITU</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AUC_{0-60\text{min}}$ (ng.mL$^{-1}$.hr)</td>
<td>804.1</td>
<td>732.5</td>
</tr>
<tr>
<td>F (%)</td>
<td>100</td>
<td>4.6</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng.mL$^{-1}$)</td>
<td>2300</td>
<td>860.0</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>1.20</td>
<td>12.0</td>
</tr>
</tbody>
</table>

*Anesthetized rat was administered 6.76 mg/kg MT-II in 0.4 mL Normal Saline intra-jejunally, reference animals received an iv dose of 0.3 mg/kg MT-II.
References


CHAPTER 2
HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR
THE α-MELANOTROPIN [4,10] FRAGMENT ANALOGUE
(MELANOTAN-II) IN RAT PLASMA

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CHAPTER 3

COMPARISON OF HPLC AND BIOASSAY METHODS FOR MELANOTAN-II (MT-II) DETERMINATION: APPLICATION TO PHARMACOKINETIC STUDY IN RATS

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CHAPTER 4

KINETICS OF DEGRADATION OF A CYCLIC LACTAM ANALOG OF α-MELANOTROPIN (MT-II) IN AQUEOUS SOLUTION

References


CHAPTER 5
A METHOD FOR THE DETERMINATION OF ACID DISSOCIATION CONSTANTS OF MODEL PEPTIDES

References


CHAPTER 6

ENTERAL ABSORPTION OF $\alpha$-MELANOTROPIN ANALOGS
(MT-I AND MT-II) FROM THE RAT INTESTINE

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