Journal of Chromatography, 584 (1992) 175–180
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6604

High-performance liquid chromatographic assay for the α-melanotropin[4,10] fragment analogue (Melanotan-II) in rat plasma

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(First received June 15th, 1992; revised manuscript received September 17th, 1992)

ABSTRACT

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 and relative error, 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.

INTRODUCTION

Melanotan-II (MT-II) is a synthetic cyclic α-melanotropin analogue [1]. This cyclic heptapeptide (Fig. 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, selective 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 (*i.e.*, urine) was monitored using frog skin and lizard skin bioassays [3]. However, these bioassays are only semi-quantitative and can be non-specific, 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 analogues [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.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Spectra-Phys-

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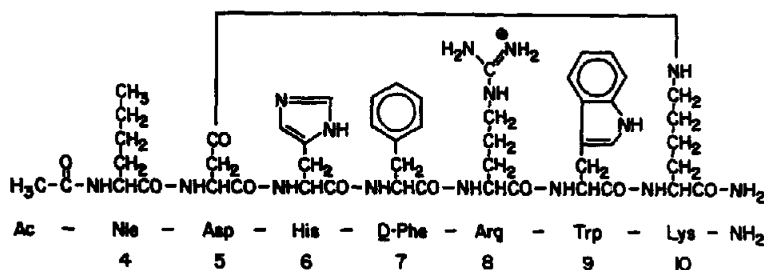

Fig. 1. Structure of α-melanotropin analogue (Melanotan-II).

ics (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 mm × 2.1 mm I.D.), fitted with a Whatman (Clifton, NJ, USA) C_{18} (10 μm) guard column (10 mm × 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 was 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 min and to improve detector baseline stability.

Drug standard

Purified MT-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 carboxymethyl cellulose column [1]. The final purification was effected by preparative reversed-phase HPLC on a C_{18} bonded silica column, with separations monitored at 280 nm. The purity of the finished peptide was verified by thin-layer chromatography in at least three solvent systems and analytical reversed-phase HPLC at 280 and 220 nm. The structure of the purified peptide was confirmed by fast-atom bombardment 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 were 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 s and centrifuged at 10 800 *g* 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 MT-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 (R.E.) of the mean of five determinations of each concentration relative to the known concentration, as shown in Table II. The coefficient of variation (C.V.) served as a measure of precision.

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.

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 one 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 mg/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 of EDTA solution (0.7 mg Na_2EDTA in 15 μl saline) and 12 μl of 8 *M* acetic acid. Following centrifugation at 4°C (10 min, 2000 *g*), the plasma was collected and stored at −20°C until the day of analysis.

RESULTS

Fig. 2 shows representative chromatograms for unsupplemented (blank) plasma (A) and for plasma supplemented with MT-II (B). As can be seen from the chromatograms, the peptide peak was well resolved from endogenous plasma com-

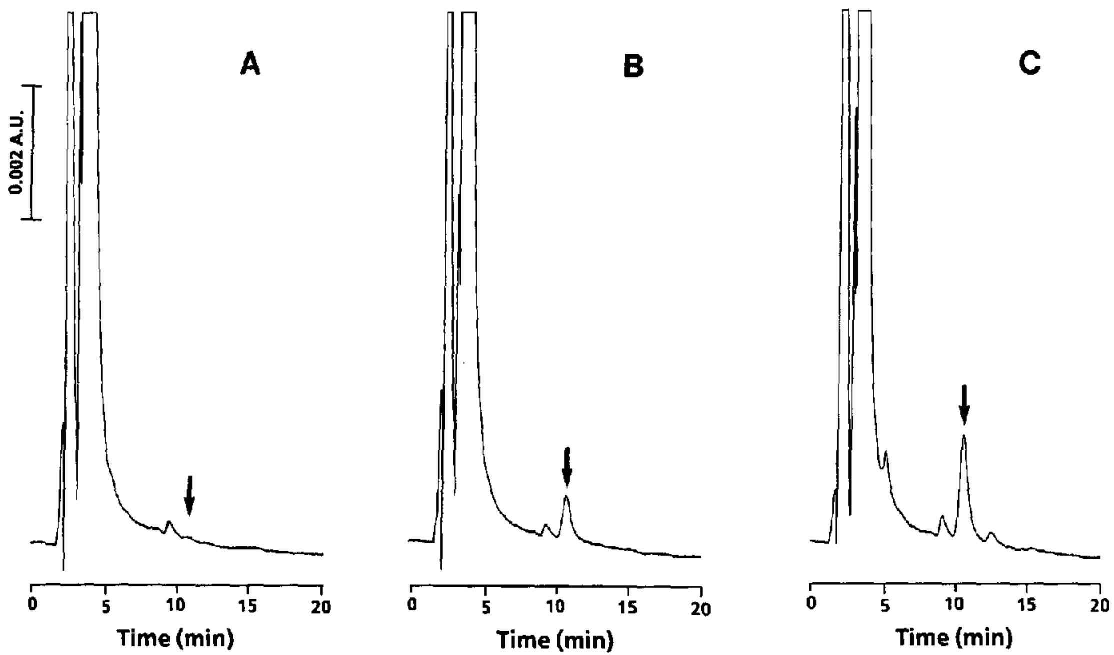

Fig. 2. Chromatograms of (A) drug-free rat plasma, (B) rat plasma spiked with 500 ng/ml Melanotan-II and (C) plasma of rat given MT-II intravenously. The rat plasmas illustrated in A and B were collected from the same rat dosed intravenously with MT-II immediately before dosing. The arrows on the chromatograms refer to the elution position of MT-II.

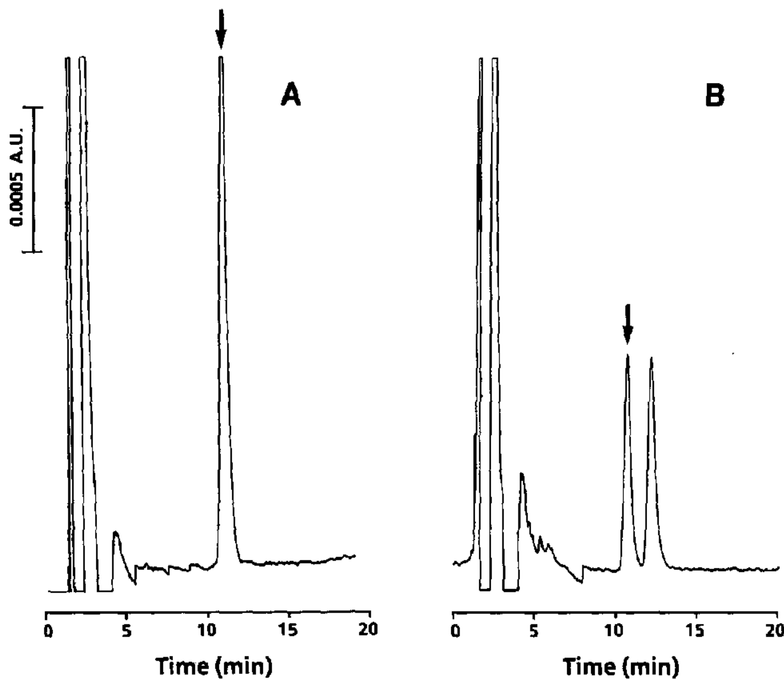

Fig. 3. Sample chromatograms for (A) control solution (MT-II at time 0) and (B) solution of MT-II degraded with 3 M aqueous perchloric acid at 25°C for 24 h. The arrows on the chromatograms refer to the elution position of MT-II.

TABLE I

SUMMARY OF HPLC CALIBRATION CURVES FOR MELANOTAN-II

Determination	Slope[a]	95% Confidence limit	r	Intercept[b]
1	0.0314	±0.0005	0.9999	−0.0649
2	0.0312	±0.0020	0.9994	0.0227
3	0.0324	±0.0021	0.9994	−0.6793
4	0.0324	±0.0022	0.9993	−1.0200
5	0.0322	±0.0019	0.9995	−0.7985
Mean	0.0319			
S.D.	0.0006			
C.V. (%)	1.81			

[a] Slopes were significantly different from zero at $p < 0.0005$.
[b] Intercepts were not significantly different from zero at $p < 0.01$.

ponents. The retention time for the peptide was 11.0 min. Fig. 2C illustrates plasma of a rat given a 0.3 mg/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 3 *M* 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 (Fig. 3A). Fig. 3B shows a chromatogram of this sample after 24 h. MT-II degraded approximately 60% in 24 h in 3 *M* aqueous perchloric acid solution, and the only apparent degradation product did not co-elute with the unchanged peptide (Fig. 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 *versus* 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 C.V. and R.E., respectively, are summarized in Tables II and III. The C.V. and R.E. 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 ≥ three times the baseline noise level.

The plasma concentration *versus* time profile for MT-II in the rat following intravenous dosing

TABLE II

BETWEEN-DAY VARIABILITY AND ACCURACY IN THE ANALYTICAL METHOD FOR MELANOTAN-II IN PLASMA

Concentration added (ng/ml)	Measured concentration[a] (ng/ml)	C.V.[b] (%)	R.E.[c] (%)
100	106.7 ± 5.3	4.98	−6.70
250	246.9 ± 8.2	3.31	1.22
500	494.7 ± 11.5	2.32	1.06
750	745.9 ± 11.5	1.53	0.55
1000	1005.9 ± 11.0	1.09	−0.59

[a] Mean ± S.D., $n = 5$.
[b] Coefficient of variation.
[c] Relative error of the mean (%) =

$$\frac{\text{true concentration} - \text{mean measured concentration}}{\text{true concentration}} \times 100.$$

TABLE III

WITHIN-DAY VARIABILITY AND ACCURACY IN THE ANALYTICAL METHOD FOR MELANOTAN-II IN PLASMA

Concentration added (ng/ml)	Measured concentration[a] (ng/ml)	C.V.[b] (%)	R.E.[c] (%)
500	516.0 ± 13.9	4.12	−3.21
1000	984.1 ± 40.6	2.68	1.59

[a] Mean ± S.D., $n = 5$.
[b] Coefficient of variation.
[c] Relative error of the mean.

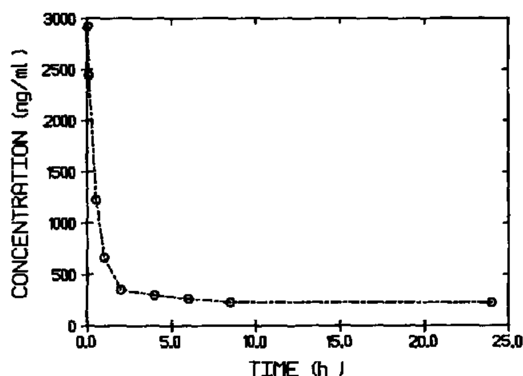

Fig. 4. Plasma concentration *versus* time profile of MT-II in rat following an intravenous dose of 0.3 mg/kg.

is shown in Fig. 4. The data could be described by a two-compartment model with an elimination (β-phase) half-life of 61.2 h.

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 concentrations *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.

ACKNOWLEDGEMENTS

The authors thank Dr. Victor J. Hruby of the Department of Chemistry, University of Arizone, for his generous gift of the Melanotan-II samples. The kind assistance of the Dr. Shubh Sharma and Mr. Gifford Hoyer is gratefully acknowledged.

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