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Determination of melanotan II in rabbit urine using solid-phase extraction sample preparation followed by reversed-phase high-performance liquid chromatography

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Abstract

A solid-phase extraction (SPE) method has been developed for the isolation of melanotan II from rabbit urine. The proposed extraction method makes it possible to selectively isolate melanotan II without significant loss of the peptide. Standard curves obtained from high-performance liquid chromatographic (HPLC) analysis of spiked urine extracts are linear from 0.1 to 4.0 μg/ml. The analytical method is shown to be highly reproducible, giving a relative standard deviation of less than 5% for both between-day and same-day analyses. The accuracy of the method obtained from standard plots ranges from −3.3 to 3.1%.

Keywords: Melanotan II

1. Introduction

Melanotan II is a synthetic, cyclic derivative of α-melanocyte stimulating hormone (α-MSH) (Fig. 1). Like its endogenous analog, melanotan II produces a darkening of the skin upon contact with skin cells. Because of the growing incidence of skin cancer in the USA, many researchers are investigating the use of this compound as an alternative to tanning by ultraviolet radiation. The "tan" produced by this peptide not only reduces the need for traditional tanning, but also provides protective pigmentation in cases when exposure to ultraviolet radiation is inevitable.

At present, there are only a few standard assays for the analysis of melanotropins in biological samples. One such bioassay, described by Castrucci et al. [1], is based on the activities of α-MSH and its analogs towards dermal melanocytes of the frog. Samples containing melanotropins are placed in contact with these cells, which respond by producing a natural pigment that brings about a rapid darkening of the frog's skin. The intensity of this color change is then measured with a reflectometer.

Melanotropins can also be quantitated by radioimmunoassay [2]. In these types of methods, a radiolabeled melanotropin is reacted in vitro with an antibody that shows specificity for the peptide. A

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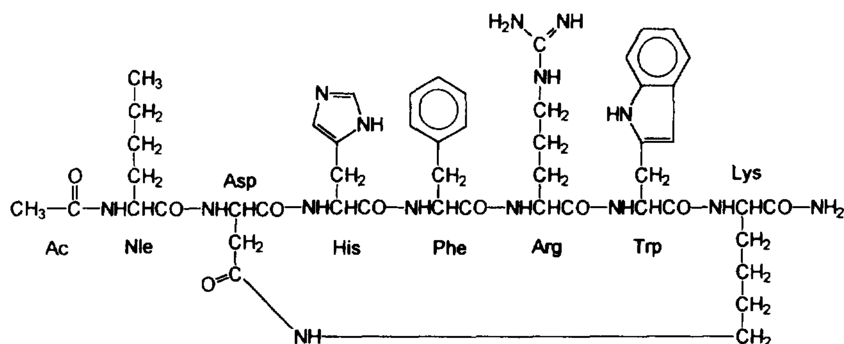

Fig. 1. Structure of melanotan II.

scintillation counter is then used to quantify the amount of radioactive material bound to the antibody.

The extraction of peptides from biological samples is often a difficult task because of their highly polar nature and their limited extractability into organic solvents. For this reason, biological samples are often analyzed directly following a sample clean-up procedure. These procedures typically remove endogenous interferences from the supernatant, either by precipitation or extraction [3–7]. In a recent publication by Ugwu et al. [8], a HPLC method was developed for melanotan II. Solid-phase extraction (SPE) was used to extract melanotan II from rat plasma. Although this extraction effectively isolates the peptide from compounds found in plasma, it was found to be inefficient at removing many urinary impurities. Hence, in order to make it applicable to urine, the SPE method proposed by Ugwu et al. [8] must be modified.

The goal of this work is to develop an analytical method that uses SPE to selectively isolate melanotan II from rabbit urine. The addition of this assay to the currently existing collection of analytical methods for melanotan II will allow for more comprehensive clinical studies of this peptide.

2. Experimental

2.1. Apparatus

Samples were analyzed by reversed-phase HPLC using a Beckman 126 solvent delivery system equipped with a Beckman 168 diode array detector (Beckman Instruments, Fullerton, CA, USA) and a Rheodyne 710 manual injector (Rheodyne, Cotati, CA, USA). Data analysis was performed using System Gold chromatographic acquisition software (Beckman Instruments). Melanotan II was extracted from rabbit urine using Bakerbond C_8 SPE cartridges (VWR Scientific, Ceritos, CA, USA). The samples were drawn through the extraction cartridges under negative pressure using a Vac-Elut vacuum manifold (Varian Sample Preparation Products, Harbor City, CA, USA). Separations were carried out on a 150× 4.6 mm, Adsorbosphere column packed with 5 μm C_8 bonded silica (Alltech Associates, Deerfield, IL, USA).

2.2. Reagents

Melanotan II was supplied by the laboratory of Dr. Victor Hruby at The University of Arizona, Department of Chemistry (Tucson, AZ, USA). Acetonitrile, methanol and ethanol were of the JT Baker brand, HPLC-grade solvents (VWR Scientific). Triethylamine (HPLC-grade) was purchased from Aldrich (Milwaukee, WI, USA). All other reagents (phosphoric acid, sodium hydroxide, monobasic potassium phosphate and dibasic sodium phosphate) were of analytical grade and were obtained form the University of Arizona Stores (Tucson, AZ, USA).

2.3. Sample preparation

Standard urine solutions containing 4.0, 2.0, 1.0, 0.5, 0.25 and 0.10 μg/ml of melanotan II were

prepared by serial dilution of a 50 μg/ml stock solution (in ethanol) with blank rabbit urine. A 3-ml volume of each urine standard was placed into a 10-ml polypropylene culture tube. The pH of each sample was adjusted to 2.2±0.1 with 20 μl of concentrated phosphoric acid. The resulting solutions were extracted according to the procedure described in Section 2.4.

2.4. Solid-phase extraction

The extraction cartridges and stopcocks were placed into the solvent delivery ports of the Vac-Elut 10 vacuum manifold. Each cartridge was activated with a 3-ml methanol wash, which was immediately followed by a 3-ml rinse with 0.1 *M* phosphate buffer, pH 2.2.

In order to prevent drying of the solid support, stopcocks were needed to stop the flow as soon as the fluid reached the top of the packed bed. The pretreated urine samples were then quantitatively transferred to the extraction cartridge reservoir. The samples were drawn through the cartridges under a vacuum pressure of 2–3 in. Hg. After the samples had completely passed through the cartridges, the following rinse procedure was used to eliminate various interferences: (1) with 3 ml of 0.1 *M* phosphate buffer, pH 12.0; (2) with 3 ml of 0.1 *M* phosphate pH 12.0–acetonitrile (80:20, v/v) and (3) with 3 ml of deionized water. Each rinse step was performed under a vacuum pressure of 5–10 in. Hg. Melanotan II was then eluted from the cartridges using two 0.49-ml volumes of a mixture containing 0.1 *M* phosphate buffer (containing 0.1% triethylamine, pH 2.2) and acetonitrile (70:30, v/v). Elution was carried out without the aid of a vacuum. The extracts were collected into 1-ml volumetric flasks and the final volumes were adjusted appropriately with the elution mixture.

2.5. Chromatographic conditions

The extracted samples were loaded onto a 100-μl loop and passed through the analytical column described above. The analyses were performed at ambient temperature using a mobile phase consisting of 28% acetonitrile and 72% 0.04 *M* phosphate buffer (containing 0.1% triethylamine, pH 3.6). The column effluent was monitored at 217 nm.

2.6. Standard curves

Standard curves for melanotan II were obtained over a period of three days using the above standard concentrations.

2.7. Accuracy and precision

The accuracy and precision of the analytical method were determined by performing six, individual concentration, measurements on a test sample containing 0.75 μg/ml melanotan II. This test sample was prepared and separated into six 3-ml aliquots, all of which were extracted along with the standards. The concentration of each test sample was then predicted from the resulting standard curve. In order to obtain meaningful statistics, this procedure was repeated over three separate days. The accuracy of the method was determined for each day of the validation by averaging the six predicted concentrations and then comparing the mean to the true value. The precision of the analytical method was computed for both same-day (intra-day) and between-day (inter-day) measurements. The variability in each case is reported as the percentage relative standard deviation [R.S.D. (%)], which relates the mean estimated concentration for the test standards to their standard deviation.

3. Results

3.1. Chromatography

Fig. 2 shows the chromatogram obtained from an aqueous standard solution containing 0.5 μg/ml melanotan II. Fig. 3 corresponds to the chromatograms obtained from extracted blank rabbit urine (A) and from extracted rabbit urine spiked with 0.5 μg/ml of the peptide (B). As shown in Figs. 2 and 3B, melanotan II has a retention time of 13.8 min. A comparison of Fig. 3A and B shows that the peak corresponding to the peptide is free of interferences. The chromatogram was reproducible in each rabbit. There was always baseline separation between

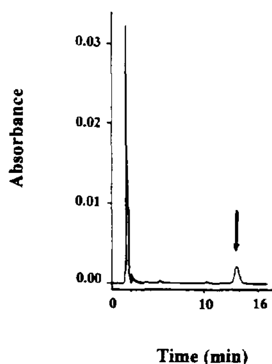

Fig. 2. Chromatogram obtained from an aqueous standard solution containing 0.5 μg/ml melanotan II (50 ng on-column).

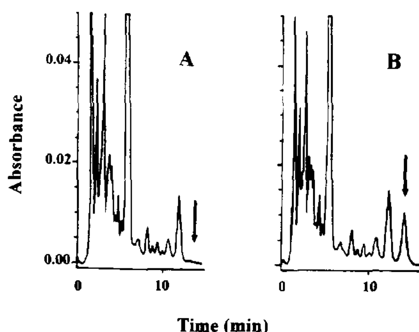

Fig. 3. Chromatogram obtained from (A) extracted blank rabbit urine and (B) extracted rabbit urine spiked with 0.5 μg/ml melanotan II (150 ng on-column).

melanotan II and the most closely eluting component (between 12 and 14 min), as shown in the chromatogram, which allowed for identification of melanotan II. The minimum resolution between melanotan II and the most closely eluting component is greater than 1.2.

3.2. Standard curves

Each standard plot was found to be linear from 0.1 to 4.0 μg/ml, giving a correlation coefficient (r^2) of greater than 0.997.

3.3. Accuracy and precision

Table 1 shows the inter-day and intra-day reproducibility of the melanotan II assay. The accuracy of the average predicted concentration for each day is reported in terms of percentage error. It is clear that the average estimated concentration compares quite favorably with the true value, indicating a high extraction efficiency for melanotan II from rabbit urine. It is apparent from this table that the analytical method gives highly reproducible results for both same-day and between-day determinations. The inter-day and intra-day variation of retention time of melanotan II is also illustrated in Table 1.

4. Discussion

As suggested earlier, the extraction of melanotan II from rabbit urine requires some modification of the plasma assay developed by Ugwu et al. [8]. These modifications are necessary because many endogenous substances are present in much higher concentrations in urine than in plasma. For this reason, most urine analyses call for more selective sample purification techniques than do plasma assays.

The three-step wash procedure enables the separation of melanotan II from the endogenous compounds found in urine. The first wash step uses an

Table 1
Inter-day and intra-day reproducibility of the assay for a 0.750 μg/ml sample of melanotan II in rabbit urine

	Day 1	Day 2	Day 3	Three-day average
Retention time (t_R)	13.726	13.795	13.884	13.802
Standard deviation of t_R	0.12	0.07	0.09	0.11
Average measured concentration (μg/ml)	0.727	0.775	0.748	0.750
Error (%)	3.07	−3.33	0.27	0.00
Standard deviation	0.01	0.03	0.01	0.02
Relative standard deviation (%)	1.63	4.36	1.26	3.17

n=6 for each day.

alkaline buffer to ensure that most of the urinary amines are unionized. In the next wash step, a mixture of 20% acetonitrile and 80% alkaline buffer is used to elute the uncharged amines from the extraction cartridge. It is believed that peptide adsorption is maintained by the interaction between the partially cationic guanidino moiety of melanotan II and the negatively charged silanol groups on the solid support. The third wash step, with deionized water, eliminates various water-soluble impurities but does not overcome the Van der Waals interactions between the peptide and the C_8 portion of the bonded phase. The result of this wash process is that urine samples are purified without significant loss of the peptide.

The proposed extraction method is highly reproducible. As shown in Table 1, the relative standard deviation for both inter-day and intra-day determinations is far below the acceptable limit for a biological assay. These statistics are still more impressive considering the nature of the analyte and the fact that no internal standard was used in the analysis. Although this method cannot identify the metabolites in rabbit urine, it can resolve the parent drug from potential metabolites and degradation products that may be present in rabbit urine, giving a more accurate determination of the parent compound.

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