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Journal of Chromatography B, 670 (1995) 235–242

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic assay for Melanotan-1 ([Nle^4-DPhe^7]α-melanocyte-stimulating hormone) in biological matrices

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First received 16 January 1995; revised manuscript received 11 April 1995; accepted 11 April 1995

Abstract

The overall objective of this research was to develop a sensitive, specific, and stability-indicating HPLC assay for the determination of the [Nle^4-DPhe^7]α-melanocyte-stimulating hormone analog known as Melanotan-1 (MT-1) in biological matrices, i.e., cell culture transport media and human plasma. Separation was accomplished isocratically within 8.0 min using a C_8 reversed-phase column. The mobile phase consisted of 0.1 *M* phosphate buffer–acetonitrile (80:20, v/v) with 18 μl/l triethylamine at pH 2.50. The flow-rate was 1 ml/min with detection at 214 nm. Standard curves ($n = 5$) were linear over the concentration range 100–1000 ng/ml. The precision, accuracy, intra- and inter-day variations were good with C.V.s typically within 8.7% for concentrations greater than 100 ng/ml. This method was applied to a study of the transport of MT-1 in the Caco-2 cell monolayer model.

1. Introduction

Melanotan-1 (Fig. 1) is a tridecapeptide (M_r 1647), an analog of α-melanocyte-stimulating hormone [1]. It is currently in phase I trials at the Arizona Cancer Center (University of Arizona, Tucson, USA) to evaluate its potential as a chemopreventive agent for sunlight-induced skin cancers. At present there are two methods available for quantitating MT-1 in biological samples—radio-immunoassay (RIA) and frog skin bioassay.

Although much of the information obtained to date (in vitro transport, bioavailability, pharmacokinetics, etc.) has used quantitation of MT-1 by RIA [2], this method has several drawbacks (e.g., cross reactivity, availability and purity of RIA kits, reproducibility and reliability). These factors limit its use for routine analyses. In addition, RIA kits have short shelf lives (3–4 weeks) and are costly ($ 1.25/sample). Frog skin bioassay has been used in the past to determine the concentrations of MT-1 in blood and urine samples [3]. Briefly, this method consists of monitoring the changes in the darkening of a piece of skin excised from frogs (*Rana pipiens*) on exposure to different known concentrations of MT-1. This is achieved using reflectance measurements. The changes in skin darkening are then correlated with known concentrations to construct standard curves. The lack of specificity and relatively low precision greatly limit the use of this procedure for routine quantitative analyses of MT-1 in biological samples. The objectives of this re-

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0378-4347/95/$09.50
SSDI 0378-4347(95)00177-8

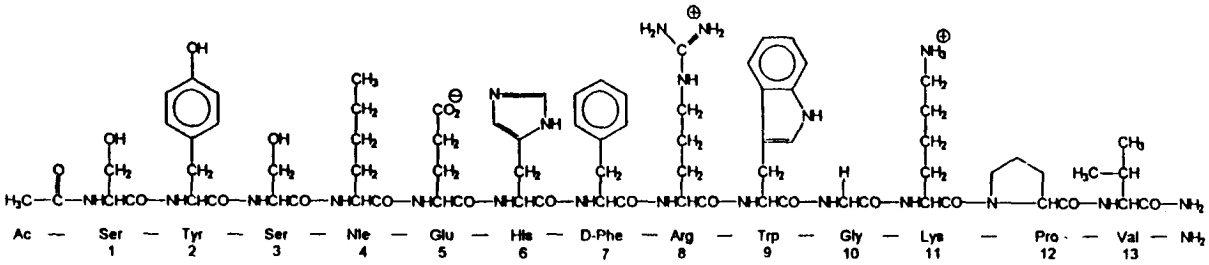

Fig. 1. Structure of [Nle^4-DPhe^7]α-melanocyte-stimulating hormone (Melanotan-1).

search were two-fold. First, to develop a sensitive, specific, and stability-indicating assay for the determination of MT-1 in cell culture transport media. Second, to extend this procedure to the quantitation of MT-1 in human plasma. Analysis of MT-1 in the cell culture transport buffer was achieved without any prior sample clean-up. For plasma, a solid-phase extraction was performed before injection onto the column. The elution was isocratic and the analysis time per sample was less than 20 min.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Spectra-Physics (Fremont, CA, USA) Isochrom pump, a Rheodyne (Cotati, CA, USA) Model 9125 injector valve with 40-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_8 (5 μm) microbore cartridge (150 mm $\times$ 2.1 mm I.D.) fitted with a Whatman (Clifton, NJ, USA) C_{18} (10 μm) guard column (10 mm $\times$ 4.6 mm I.D.). The guard column was routinely changed after about 100 injections as a precautionary measure to avoid pressure build-up in the HPLC system. Peak recording and integration were accomplished with a Spectra-Physics Model 4290 integrator. All injections were made at room temperature with a Hamilton (Reno, NV, USA) Model 725-SNR 100 μl syringe previously coated with Sigmacote (Sigma, St. Louis, MO, USA) to minimize binding of MT-1 to syringe components.

2.2. Preparation of standard solutions

Purified MT-1 (>99%) was obtained from Bachem (Torrance, CA, USA). A stock solution of MT-1 (1 mg/ml) was prepared in water and stored at −20°C. The stability of the stock solution was checked periodically by assaying aliquots of the stored solution at various time intervals. MT-1 was found to be stable at this temperature for at least 6 months. Serial dilutions of the stock solution with cell culture transport buffer were used to obtain the desired concentrations of the calibration standards. The cell culture transport buffer consisted of Hank's Balanced Salt Solution (HBSS, Gibco, Long Island, NY, USA, with 5.6 m*M* glucose) and 25 m*M* HEPES (Sigma, St. Louis, MO, USA), pH 7.4.

2.3. 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 (purified using a Millipore water purification system). The pH of the solution was adjusted to 2.20 with phosphoric acid (Fisher Scientific, Fairlawn, NJ, USA). The mobile phase was prepared by mixing 800 ml of 0.1 *M* phosphate buffer and 18 μl/l of 99% (v/v) triethylamine with stirring for 15 min. To this, 200 ml acetonitrile was added and the mixture stirred for 10 min. The pH was adjusted to 2.50 with phosphoric acid when 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 flow-rate used was 1 ml/min. All tubing in the chromatograph assembly was made of Polyetherether ketone (PEEK) in order to minimize any non-specific adsorption of the peptide. The injector was flushed with 2 ml of water between successive injections to eliminate carryover of MT-1 from one injection to the next.

2.4. Sample preparation

Sample preparation using the cell culture transport media was achieved by serial dilutions of the stock solution with the transport media. Samples were prepared fresh daily. For plasma samples, solid-phase extraction (SPE) of the drug was performed to eliminate plasma components. This was accomplished by loading 250 μl of plasma onto C_{18} SPE Bond-Elut cartridges (Varian, Harbor City, CA, USA) previously conditioned with 100% acetonitrile followed by 0.1 *M* phosphate buffer (pH 2.5). The sample was then washed successively with 1 ml each of 10% and 15% acetonitrile in 0.1 *M* phosphate buffer to remove impurities and plasma components. MT-1 was then eluted with 250 μl of 30% (v/v) acetonitrile in 0.1 *M* phosphate buffer solution. This was injected (100 μl) onto the column. As MT-1 was observed to bind avidly to polypropylene Eppendorf tubes and glass vials, silanized tubes and vials were used to minimize non-specific adsorption.

2.5. Standard curves

Samples in cell culture transport buffer containing 100, 200, 400, 600, 800 and 1000 ng/ml of MT-1 were prepared. Standard curves were constructed using peak areas of MT-1. The linearity of the curves was verified by means of correlation and regression analysis.

2.6. Validation—accuracy and precision

Validation was accomplished by assaying six different concentrations of MT-1 for five consecutive days. Since non-specific adsorption of MT-1 to glass vials and polypropylene tubes was observed during storage, fresh standards were prepared daily and used within 2–4 h of preparation. This eliminated adsorption to glass vials as a confounding variable during the course of the between-day validation study. Consequently, the relative error (R.E.) could not be assessed for between-day measurements. However, within-day R.E. was assessed for two concentrations based on the standard curve generated for that day. The coefficient of variation (C.V.) was determined for between-day measurements using peak areas.

2.7. Peak identity

The peak of interest was collected in a silanized Eppendorf tube. To eliminate the buffer components from the eluted peak, solid-phase extraction was performed. This was accomplished by loading 1.5 ml of the sample onto C_8 SPE Bond-Elut cartridges (Varian, Harbor City, CA, USA) previously conditioned with 100% acetonitrile followed by 0.1% (v/v) TFA in water (pH 2.5). The sample was then washed with 3 ml of water to remove the phosphate buffer components. MT-1 was then eluted with 500 μl of 30% (v/v) acetonitrile–70% (v/v) 0.1% TFA in water. The eluted sample was diluted with 50% (v/v) methanol–water with 1% acetic acid. This was injected onto the mass spectrometer (Finnigan MAT TSQ 7000) using the electrospray technique.

2.8. Stability-indicating procedure

To demonstrate the stability-indicating nature of this assay MT-1 was intentionally degraded at an elevated temperature. A 20 μg/ml MT-1 solution in 0.1 *M* phosphate buffer (pH = 7.3, $\mu = 1.5$ *M*) was placed in an 80°C incubator and aliquots were collected at frequent intervals for 4.5 half-lives. The aliquots were then diluted and assayed using the conditions described above.

2.9. Transport study

In order to demonstrate the practical application of the assay, the transport of MT-1 was

evaluated in the Caco-2 cell monolayer model and the concentrations were determined by HPLC using the conditions described above. The Caco-2 model is now widely accepted, both for assessing intestinal permeability and for elucidating the transport mechanisms of drug molecules [4–6]. The details of the cell culture and transport study procedures are described elsewhere [7]. Briefly, on the day of an experiment, the Transwells were equilibrated with the transport buffer (HBSS with 25 m*M* HEPES, pH 7.4) for 30 min at 37°C. The buffer was then replaced with MT-1 solution (100 μg/ml). An aliquot of 50 μl was withdrawn from the apical side in order to determine the initial concentration of MT-1. A 100-μl volume was withdrawn from the basolateral side at frequent intervals. After sampling from the basolateral side, an equal volume of the buffer was added to maintain a constant volume. The samples were immediately transferred to a −80°C refrigerator and stored at this temperature until analysis. The duration of storage was typically less than 2 weeks. Studies in our laboratory have shown that non-specific adsorption of MT-1 to the siliconized polypropylene Eppendorf tubes was insignificant at this temperature during storage for over a month.

2.10. Column care

For overnight storage, the column was washed with distilled water for 20 min, followed by a wash with acetonitrile–water (50:50, v/v) for 15 min. Finally, the column was washed with acetonitrile–water (95:5, v/v) for 20 min to remove any remaining adsorbed materials. The flow-rate used was 1.0 ml/min.

3. Results

Representative chromatograms of MT-1 in the cell culture transport buffer using this method are shown in Fig. 2. The peptide peak was well resolved from other compounds present in the transport buffer. Peak identity was verified by mass spectrometry. Other features of this method include a relatively short retention time and elimination of sample clean-up prior to injection.

An attempt was made to extend this assay to the determination of MT-1 in human plasma. Although good separation was achieved, the recovery was low (about 50%) and the sensitivity was about one-tenth of that achieved with the cell culture transport buffer.

The specificity of the method was determined by intentionally degrading MT-1 in 0.1 *M* phosphate buffer ($\mu = 1.5$ *M*) at pH 7.3 at 80°C. Fig. 3 shows the comparison of chromatograms generated from the initial sample (i.e., $t = 0$ h) and after 4.5 half-lives (i.e., $t = 288$ h). It is clear that MT-1 was completely resolved from its degradation products. The identity of the major degradation products has not yet been determined.

MT-1 peak areas were plotted versus MT-1 concentrations and least-squares linear regression was performed. Table 1 summarizes the calibration curves for analysis in the transport buffer. Plots were observed to be linear over the concentration range (100–1000 ng/ml) tested with correlation coefficients (r) typically ≥ 0.994. Statistical analysis showed that the intercepts were not significantly different ($p < 0.01$) from zero. The between-day and the within-day precision and accuracy, as measured by C.V. and R.E., respectively, are summarized in Tables 2 and 3. These results indicate that the assay was both accurate and reproducible. The quantification limit for this assay in cell culture transport buffer was 50 ng/ml using the criterion that the signal from the minimum quantifiable peak should be ≥three times the baseline noise level.

An application of this assay to study the transport of MT-1 in the Caco-2 cell monolayer model is shown in Fig. 4. The concentrations obtained at each time point for a particular insert were normalized to the initial concentration (i.e., at $t = 0$ min) at the apical side and the data expressed as a percentage. The concentrations analyzed ranged from a low of 68.93 ng/ml at 60 min up to 342.21 ng/ml at 200 min. The coefficient of variation was generally within 9.0% except for the 60 min time point which had a C.V. of 15.3%. The apparent permeability coefficients

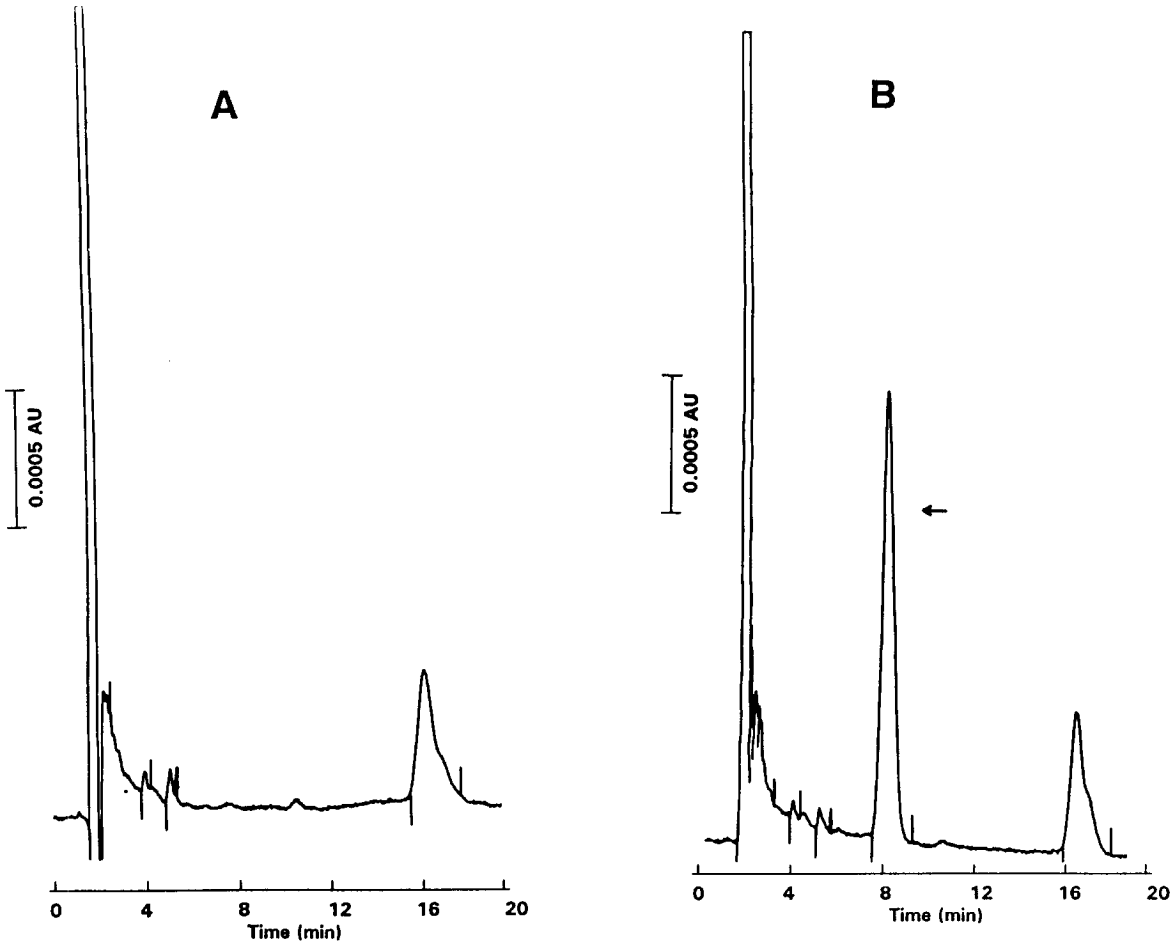

Fig. 2. Chromatograms of MT-1 in cell culture transport buffer (Hank's Balanced Salt Solution with 25 m*M* HEPES and 5.6 m*M* glucose, pH = 7.4). (A) Control (i.e., buffer alone), and (B) buffer spiked with 1 μg/ml MT-1. The arrow on the chromatogram refers to the elution position of MT-1.

(P_{app}) were calculated according to the following equation:

$$P_{app} = V_R \cdot (dQ/dt)/A \cdot C_o \cdot 60$$

where V_R is the volume of the basolateral side (2.5 ml), dQ/dt is the permeability rate (in ng ml^{-1} min^{-1}) under sink conditions (i.e., before >10% was transported), A is the surface area of the membrane (4.71 cm^2) and C_o is the initial concentration of MT-1 in ng ml^{-1} min^{-1}. P_{app} was determined to be $3.49 \cdot 10^{-8} \pm 0.43$ cm/s.

4. Discussion

The method reported here represents the first analytical HPLC procedure that is suitable for quantitation of MT-1 in biological matrices. The Caco-2 cell monolayer model is an in vitro system consisting of Caco-2 cells (human cells of adenocarcinoma origin) grown as a monolayer on microporous membrane supports. This model has been shown to mimic the intestinal absorptive cells (enterocytes) and is being used to assess the transport properties (membrane permeabili-

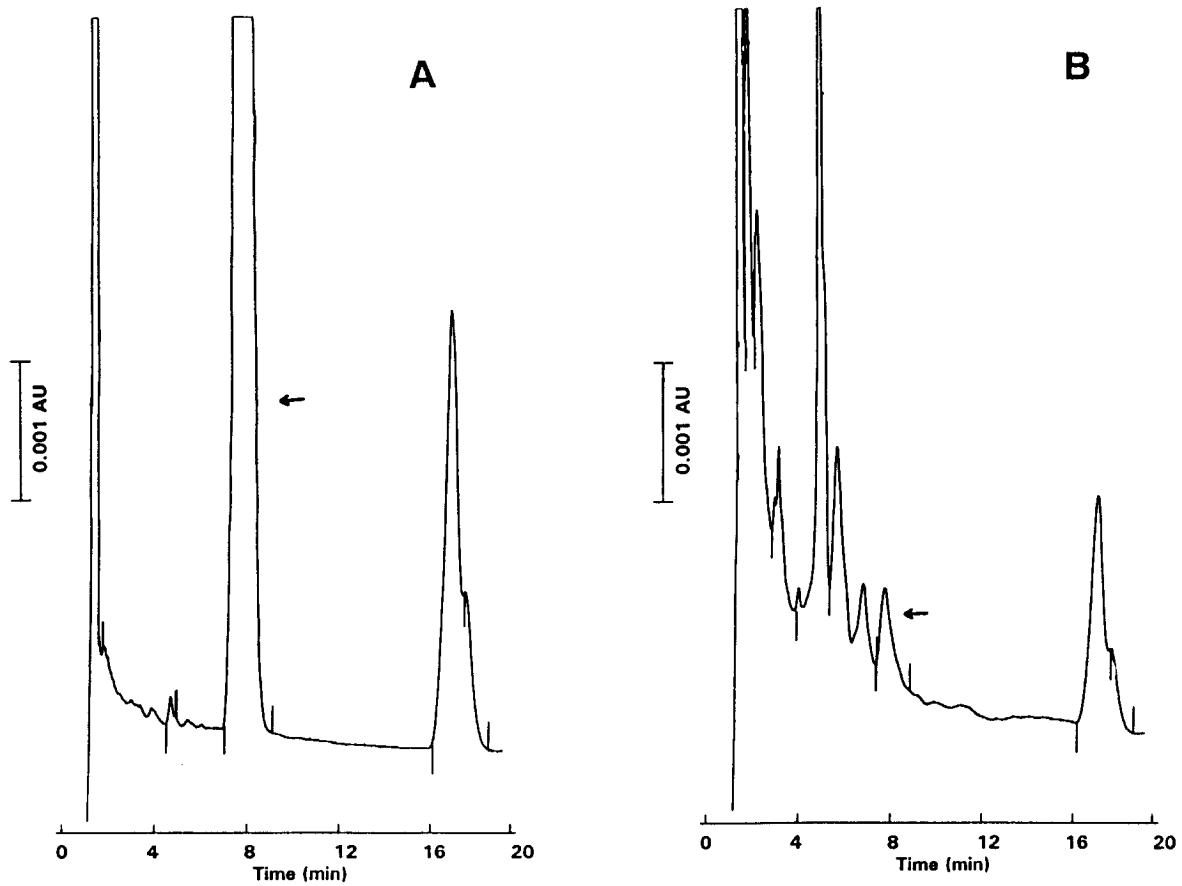

Fig. 3. Chromatograms demonstrating the stability-indicating nature of the HPLC assay. A 20 μg/ml MT-1 solution in 0.1 *M* phosphate buffer, pH = 7.3, and $\mu = 1.5$ *M* at (A) $t = 0$ h (i.e., initial sample), and (B) $t = 288$ h at 80°C (4.5 half-lives). The arrows on the chromatograms refer to the elution position of MT-1.

Table 1
Summary of HPLC calibration curves for Melanotan-1

Determination	Slope[a]	Standard error	Intercept[b]	*r*
1	0.00469	±0.00009	−0.137	0.999
2	0.00661	±0.00034	−0.507	0.994
3	0.00612	±0.00029	−0.330	0.995
4	0.00617	±0.00025	−0.502	0.997
5	0.00491	±0.00030	−0.237	0.994
Mean	0.00570		−0.342	
S.E.M.	±0.0003		±0.06	

[a] Slopes were significantly different from zero at $p < 0.005$.
[b] Intercepts were not significantly different from zero at $p < 0.01$.

Table 2
Between-day variability for MT-1 analysis in cell culture transport buffer

Concentration (ng/ml)	Peak area[a] (in thousands)	C.V.[b] (%)
100	32.10 ± 3.94	12.27
200	80.34 ± 7.11	8.74
400	180.32 ± 10.10	5.60
600	302.17 ± 18.45	6.10
800	437.88 ± 21.31	4.86
1000	586.52 ± 47.14	8.03

[a] Mean ± S.D., $n = 5$.
[b] Coefficient of variation.

Table 3
Within-day variability for MT-1 analysis in cell culture transport buffer

Concentration added (ng/ml)	Measured concentration[a] (ng/ml)	C.V.[b] (%)	R.E.[c] (%)
400	420.04 ± 11.03	2.62	−5.01
800	782.19 ± 37.07	5.00	3.75

[a] Mean ± S.D., $n = 5$.
[b] Coefficient of variation.
[c] Relative error (%) = (true concentration − measured concentration)/(true concentration) · 100.

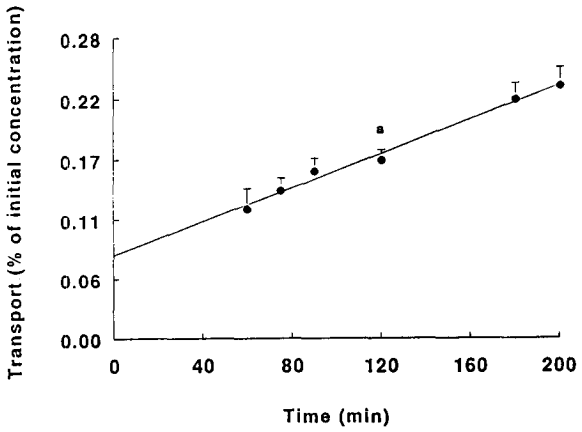

Fig. 4. MT-1 transport in the Caco-2 cell monolayer. An application of the HPLC assay. All values represent means ± S.D., $n = 3$; a = for this time point $n = 2$.

ty) of drug molecules. MT-1 has been shown previously to be transported in the Caco-2 cell model and an in situ rat model [4]. In addition, in the same study, it was shown that the enhancement of MT-1 absorption is possible with the co-administration of aprotinin (a protease inhibitor). Nevertheless, the precise mechanisms of transport (active vs. passive, transcellular vs. paracellular, etc.) have not yet been elucidated. The HPLC method developed here can potentially be utilized to study the different mechanisms of MT-1 transport in this cell monolayer model. The degradation kinetics of MT-1 in aqueous solution was investigated in our laboratory using this stability-indicating HPLC method. The details of this study will be reported at a later date. Briefly, based upon the calculated parameters, the shelf life of MT-1 at room temperatures was estimated to be 40 days. In addition, this peptide appears to be stable under acidic conditions, ionic strengths up to 1.5 *M* and phosphate buffer concentrations ranging from 0.05 to 0.1 *M*. Delivery systems of MT-1 for administration via parenteral and enteral routes have been formulated recently in our laboratory. The method developed here is currently being used to characterize and evaluate these formulations. Finally, it is recommended that silanized vials and tubes be used during the course of sample preparation of this peptide since it has been observed to bind avidly to glass and polypropylene tubes after relatively short (i.e., less than 24 h) contact times.

Acknowledgements

We thank Dr. Thomas D. McClure for assistance with mass spectrometry analysis of Melanotan-1.

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