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Short communication

High-performance liquid chromatographic analysis of Peptide T in rabbit plasma with on-line column enrichment

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Abstract

The present paper describes the development of a simple and sensitive analytical method for quantification of Peptide T (PT) in rabbit plasma, using standard analytical equipment and on-line column enrichment, without prior extraction, clean-up or derivatization. The analytical procedure was found to be accurate, precise and linear. The accuracy was 100% (range 97–103%) and the mean precision was 8% (range 3–14%) for all ($n=6$) concentrations (0, 15, 50, 100 and 200 ng/ml). The total recovery was found to be approximately 80%, and it was found to be dependent upon the injection rate onto the extraction column. The correlation between added and found concentrations was 0.9982, and the limit of detection was estimated to be around 5 ng/ml. The method is therefore found to be suitable for bioavailability studies, involving Peptide T, in rabbits.

Keywords: Peptide T

1. Introduction

(D-Ala[1])-Peptide T Amide (PT) is a synthetic octapeptide with the amino acid sequence D-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr-NH_2. PT was originally developed as an anti-viral drug for the treatment of acquired immunodeficiency syndrome (AIDS). Clinical trials, however, have not demonstrated any anti-viral activity, but the peptide is still thought to have potential efficacy in the treatment of autoimmune diseases such as psoriasis, rheumatoid arthritis and connective tissue disease, due to its immuno-modulatory properties.

Since many peptide or protein drugs are active in plasma at concentrations far lower than those of conventional drugs, analytical sensitivity in the nanogram to picogram range and beyond is often required. Development of sensitive and specific methods for plasma analysis for in vivo studies is one of the greatest challenges in peptide or protein drug research [1,2]. To complicate the issue further, the biological matrix contains numerous interfering substances that are themselves proteins.

To obtain high sensitivity, isotopically labelled drugs or immunological assays such as RIA or ELISA can be used. These methods, however, may have a questionable specificity, especially if not combined with HPLC [3]. Detection with HPLC–MS [4,5] is normally specific and sensitive when combined with a clean-up procedure, but it is expensive and the analytical procedure requires highly trained personnel.

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A few HPLC-fluorescence methods based on pre- or post-column derivatization have been described [2,6]. The sensitivity of these methods is approximately 1 ng/ml, but the procedures are rather complicated and the capacity is only two samples per hour. With laser fluorescence detection it may be possible to lower the detection limit by two or three orders of magnitude in comparison to conventional fluorescence detection [7]. Unfortunately, laser fluorescence detectors are not readily commercially available. Additionally, the laser fluorescence detection technique is dependent on the availability of a derivatization agent with a maximum at the excitation wavelength of the laser, in order to exploit the full gain of sensitivity compared to the conventional fluorescence detection.

Fluorescence detection is normally more specific and selective than the equivalent UV absorbance methods and represents a good alternative for the analysis of peptides or proteins. Using solid-phase extraction and HPLC–UV, a detection limit of about 500 ng/ml in plasma has been obtained [8].

There are several reasons for choosing a single-step analytical method for quantification of peptides at low concentrations: non-specific adsorption of peptides or proteins, which are often electrically charged, to the surface of different containers, solid material or other utensils is minimised, the analytical procedure will be cheaper and less time consuming and it can be performed by most laboratory personnel with only little training.

The purpose of the present paper is to describe a simple and sensitive analytical method for detection of PT in plasma using standard analytical equipment and on-line column enrichment, with no prior extraction, clean-up or derivatization. Quantification of PT was carried out by utilising the naturally fluorescent tyrosine residue by standard fluorescence detection. The method is expected to be suitable for bioavailability studies of PT in rabbits, requiring a detection limit of 10 ng/ml or lower.

2. Experimental

2.1. Apparatus

The HPLC system was from Merck (Darmstadt, Germany) and consisted of a Hitachi 655A-11 pump and a Hitachi LaChrom L-7480 fluorescence detector connected to a Hitachi D-2500 Chromato-Integrator (plot attenuation=4). The injector was a Rheodyne model 7125 (Berkeley, CA, USA), equipped with a 14 mm×3.9 mm I.D. on-line enrichment column in place of the injection loop. The enrichment column was packed in a ratio 1:1 with Bondesil C_{18} (40 μm) from Analytical Instruments (Værløse, Denmark) and Sep-Pak C_{18} (55–105 μm) from Waters. This combination has, in pilot studies, been found to be optimal with respect to maximum recovery and reduced blank values and blocking. The coarser particles (Sep-Pak C_{18}) were placed closest to the injection port, primarily to reduce blocking.

2.2. Chromatography

The column was a 250 mm×4 mm I.D. LiChrosorb RP-18 and the guard column was a LiChroCART 4-4, both from Merck. The mobile phase (pH 2.5) consisted of an aqueous solution of 30 m*M* phosphoric acid, 0.15 *M* sodium sulphate and 7.5% acetonitrile. The mobile phase was filtered through a 0.45 μm filter and degassed ultrasonically. The flow-rate was 1.0 ml/min. The excitation wavelength was 220 nm and emission was measured at 302 nm.

2.3. Reagents and glassware

Peptide T×HCl was from Peptech (Europe) (Hillerød, Denmark). Demineralized water was used throughout the test. Acetonitrile (far UV) was purchased from Romil Chemicals (Leicester, UK). Sodium sulphate and phosphoric acid were of analytical grade and obtained from Merck. Rabbit blood was collected in heparin-Li coated tubes (WHP-19) and plasma was stored at −20°C in microcentrifuge tubes (TEN-15), both types from Bie and Berntsen (Copenhagen, Denmark).

2.4. Procedure

Prior to injection, the plasma was diluted 1:1 with water. Before each plasma injection, the enrichment column was washed with 2 ml 95% acetonitrile in water followed by 2 ml water. Next, 500 μl of sample was applied followed by 2 ml water. The sample and the subsequent water were injected with flow-rates of 0.6 and 1.2 ml/min, respectively. Fig. 1

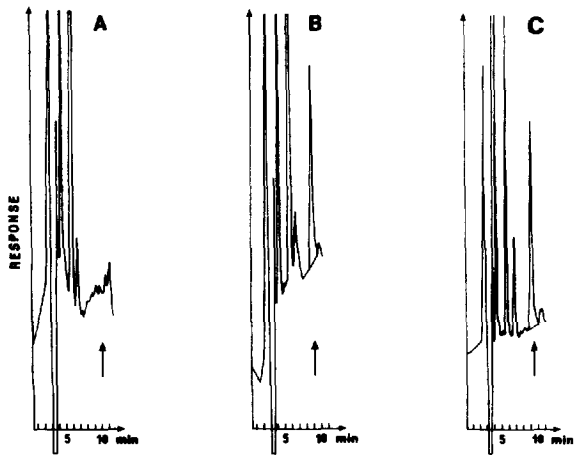

Fig. 1. Typical chromatograms at recording range 100 of: (A) blank from rabbit plasma, (B) rabbit plasma spiked with 50 ng/ml Peptide T×HCl and (C) rabbit plasma 15 min after intranasal application of PT. The retention time for PT was approximately 9 min.

shows typical chromatograms at recording range 100 of blank from rabbit plasma, rabbit plasma spiked with 50 ng/ml Peptide T×HCl and rabbit plasma 15 min after intranasal application of PT, respectively.

2.5. Testing of the analytical procedure

The accuracy, precision and linearity of the method were determined using spiked rabbit plasma ($n=6$) at five levels of concentration, 0, 15, 50, 100 and 200 ng/ml. The test procedure was evaluated on two successive days. All data were corrected for blank values, and the concentrations were calculated based on the average of plasma standards (200 ng/ml), injected after every 5 samples as a one-point calibration. The total recovery of PT was determined as the response of analysed plasma sample relative to the response of 50 μl of an aqueous solution of PT (200 ng/ml) directly injected, without the subsequent 2 ml water wash.

The plasma blank inter- and intra-rabbit variation was determined for 6 individuals. From each rabbit, two blanks were taken with an interval of one hour. Statistical analyses of inter- and intra-variation were performed using one way analysis of variance and Students t-test for paired data, respectively.

3. Results and discussion

The analytical procedure for PT in rabbit plasma was found to be accurate and precise. The overall accuracy was 100% (range 97–103%), calculated as the percentage found on the basis of the plasma standards (Table 1). The mean precision, expressed as the coefficient of variance (C.V.%), was found to be 8% (range 3–14%) for all concentrations. At the lowest concentration level tested (15 ng/ml), the precision was 14%.

The analytical procedure was linear within the concentration range studied, since the deviation of accuracy from 100% was smaller than 5% and smaller than the precision at all concentration levels tested. The correlation between added and found concentrations was 0.9982. The blank values for the rabbit plasma tested, were found to be 2 ng/ml. These blanks were equal to those found in other individual rabbits ($n=6$), where no inter- or intra-variation was observed ($p>0.05$). Based on this observation, the limit of detection was estimated to be around 5 ng/ml, which is about two times the blank value. As mentioned, the precision at this concentration level is relatively low. One-point calibration was used for testing the quality of the method. For routine use, an adequate set of low and high plasma standards should be used.

The sample injection time was chosen to be 50 s (0.6 ml/min), as the total recovery for PT in plasma relative to PT in water, directly injected without the washing procedure, was dependent upon the injection rate onto the enrichment column. The total recovery was found to be 59, 81 and 85% for injection times of 5, 50 and 150 s, respectively. The analytical capacity for the method was found to be 4 samples per hour.

Table 1
The accuracy and precision of the analytical procedure for Peptide T×HCl ($n=6$) in rabbit plasma

Added (ng/ml)	Found (ng/ml)	Accuracy (found%)	Precision (C.V.%)
15	15	99	14
50	52	103	9
100	97	97	6
200	204	102	3
Mean		100	8

The sensitivity of the method may be improved by use of derivatization [2,6] and/or use of laser fluorescence detection [7]. However, the potential advantages of these more complicated methods may be limited without further removal of plasma blank interference. The reason for this is that blanks could also be derivatized and that the response from blanks is normally proportional to the sensitivity of the detector.

Even though the presented on-line extraction method is relatively simple, a detection limit of about 50 ng/ml was obtainable by use of UV absorbance detection at 214 nm, which is ten-fold more sensitive than the method described by Surendran et al. [8]. To our knowledge, the method presented is the most sensitive HPLC–UV analysis for peptides in plasma. As demonstrated, the limit of detection is another ten-fold lower by use of a fluorescence detector.

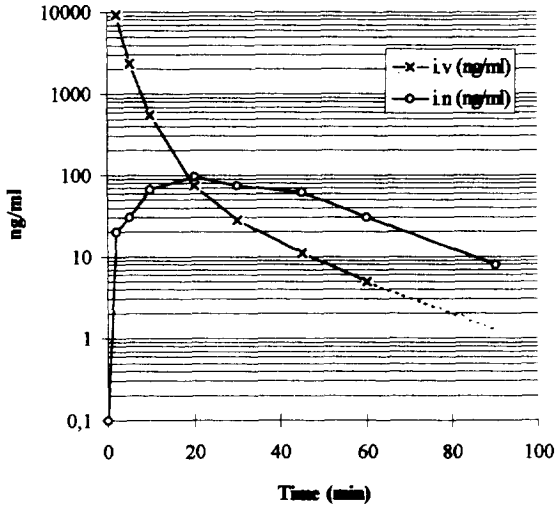

Fig. 2. Example of semilogarithmic plasma concentration profiles following intravenous and intranasal dosage of 2.0 and 4.8 mg Peptide T×HCl, respectively, to rabbits.

It may be concluded that the described HPLC/fluorescence system, with on-line column extraction is a simple, accurate, precise and relatively sensitive method for determination of Peptide T in rabbit plasma. Detection of PT concentrations as low as 5 ng/ml, makes the method suitable for bioavailability studies (Fig. 2). It is likely that a modification of the method may be applicable to the determination of other peptides containing naturally fluorogenic amino acids.

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