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Quantitation of a 36-amino-acid peptide inhibitor of HIV-1 membrane fusion in animal and human plasma using high-performance liquid chromatography and fluorescence detection

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Abstract

Selective extraction of a 36-amino-acid peptide (DP-178, T20, pentafuside) from the protein matrices of animal and human plasma was achieved using acetonitrile containing 1% trifluoroacetic acid and 1% *n*-nonyl-β-D-glucopyranoside. The peptide concentration of the extract was measured using reversed-phase high-performance liquid chromatography (RP-HPLC) and fluorescence detection. The eluent was excited at 280 nm and the intrinsic fluorescence signal was collected at 350 nm. Recovery of T20 from the plasma matrices was 75% (mouse), 60% (rat), 50% (cynomolgus monkey), and 55% (human) based on parallel-processed aqueous T20 standard solutions. The fluorescence peak area vs. concentration of T20 was linear in the range 4–160 ng/ml based on the final solute concentration in the HPLC vial, corresponding to original plasma concentrations of 100–4000 ng/ml. Experiments with truncated analogs of T20 demonstrate that this assay offers the advantage of detecting metabolites attributable to bio-transformation degradation processes differing by as little as one amino acid from the original peptide. © 1998 Elsevier Science B.V.

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1. Introduction

Chromatography-based techniques for determining the in vivo concentration of a drug are pivotal in the drug development process. This paper illustrates the general use of fluorescence detection and reversed-phase high-performance liquid chromatography (RP-HPLC) for quantifying the in vitro concentration of pentafuside in different plasma matrices. Pentafuside (hereafter, T20) is a 36-residue peptide whose sequence (Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2) is derived from the HIV-1 transmembrane fusion protein gp41 [1,2]. T20 has been shown to block virus-mediated cell-to-cell fusion and de novo HIV-1 infection of T-cells with 50% effective concentration values of 1 and 80 ng/ml, respectively [1]. In a phase I/II clinical trial, administration of this peptide, which targets a different mechanism of action than current HIV therapies, reduced viral load to undetectable levels with no drug-associated side effects observed [3]. To aid in the preclinical and clinical studies which require detection and quantitation of the concentration and integrity of T20 in animal and human plasma, we

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have developed an HPLC-fluorescence assay which has a low limit of detection (~50 ng/ml). Besides demonstrating the identity of the drug product, the method also allows the detection of metabolites arising from in vivo bio-degradation processes. The HPLC–fluorescence assay is applicable to a wide range of peptides derived from the fusion proteins of other viruses, such as respiratory syncytial virus, which we are developing to inhibit membrane fusion in other viral systems [4].

HPLC methods have been employed for the detection of a variety of drug substances in biological fluids [5]. Most of these drug substances are composed of small molecules which elute at high organic concentration, thus making their separation from the plasma proteins a relatively easy task. The separation of T20 from plasma proteins present in different species provided a challenge to this project, primarily due to the nature of this peptide. For instance, to extend the linear range into the low ng/ml range, we needed to prevent the adhesion of T20 to sample tubes. This adhesion presumably arises from the interaction of the two hydrophobic regions on the N- and C-peptide termini with surfaces. Another challenge was to prevent T20 from adhering to the residual free silanol groups on the HPLC column. Both tasks needed to be accomplished without increasing the amount of background fluorescence.

The typical UV-absorption detection method employed with standard HPLC experimentation has a relatively high quantitation limit (~1 μg/ml) which precludes its application in this instance. Fluorescence detection is well suited for this application, due to the gain in sensitivity of approximately three orders of magnitude over UV absorption detection. This increase in sensitivity affords detection of the peptide concentration in plasma within an order of magnitude to that of immunochemical-based assays [6].

2. Experimental

2.1. Materials

Acetonitrile and water were of HPLC grade and obtained from Burdick and Jackson (Muskegon, MI, USA) and J.T. Baker (Phillipsburg, NJ, USA), respectively. Trifluoroacetic acid (TFA) was obtained from Acros (Pittsburg, PA, USA). Arginine hydrochloride and the detergent *n*-nonyl-β-D-glucopyranoside were obtained from Sigma (St. Louis, MO, USA).

Human plasma (fresh-frozen, in heparin) was obtained from the American Red Cross (Durham, NC, USA). Heparinized cynomolgus monkey, rat and mouse plasma was obtained from Lampire Biological (Pipersville, PA, USA). All plasma samples were unfiltered and nonsterile.

The peptides T20, T919, T920 and T924 were synthesized on a Rainin (Woburn, MA, USA) Symphony Multiplex multiple peptide synthesizer using standard solid-phase synthesis techniques and 9-fluorenylmethoxycarbonyl (FMOC) protected amino acids [7,8]. T20 was blocked with acetyl and amide groups at the N- and C-termini, respectively. The peptides T919 and T920 were not blocked on the C-terminus and T924 was not blocked on the N-terminus to simulate the result of a biodegradation process. Cleavage from the resin (Rink amide-MBHA resin obtained from Novabiochem, La Jolla, CA, USA) with concomitant removal of sidechain blocking groups was performed using TFA in the presence of thioanisole, water, ethanedithiol and phenol as carbocation scavengers [9]. After cleavage, the peptides were precipitated with cold diethyl ether and the precipitate lyophilized and purified. Peptides were purified by reversed-phase HPLC using a Waters (Milford, MA, USA) DeltaPak C_{18} column (300×25 mm, 15 μm particles) using a water and acetonitrile gradient containing 0.1% (v/v) TFA. The peptide was >95% pure as monitored by analytical HPLC; sample identity was confirmed with electrospray mass spectrometry (Protein Structure Facility, University of Michigan, Ann Arbor, MI, USA).

2.2. Preparation of standards

A stock solution of T20 (0.8 mg/ml) was prepared in water. Ammonium hydroxide (0.1%) was added dropwise to solubilize the peptide and produce a clear solution (final pH 8). Peptide concentration was determined by the Edelhoch method [10]. Standard solutions of T20 in human plasma were prepared by serial dilutions.

2.3. Extraction of T20 from plasma

Extraction of T20 from animal and human plasma was performed by the addition of 50 μl of T20-containing plasma to 450 μl of ACN containing 1% TFA and 1% *n*-nonyl-β-D-glucopyranoside (ACN extraction mixture) in a PTFE microfuge tube (Savillex, MN, USA). This solution was vortexed for 15 s, then centrifuged at 12 000 *g* for 5 min. The supernatant was then diluted with HPLC water (supernatant–water, 40:60, v/v) for injection onto the column. All steps were performed at room temperature.

2.4. Chromatographic system

Analysis was performed on a Waters liquid chromatography system incorporating an autosampler (717 plus), multisolvent delivery system (600 E), photodiode array absorbance detector (996), and a scanning fluorescence detector (474). The Kromasil column (25 cm×4.6 mm I.D., 5 μm particles, 100 Å pore size), purchased from MODcol Corporation (St. Louis, MO, USA) was held at 28°C. Reversed-phase separations were carried out using a 30–60% ACN gradient, run at 1 ml/min. Running buffers were prepared with 0.1% TFA and 1% arginine hydrochloride, employing a 70% ACN–30% water cosolvent for the organic buffer. Fluorescence detection was performed with excitation and emission wavelengths of 280 and 350 nm, respectively. These wavelengths were chosen to coincide with the maxima of the absorbance and emission spectra of the native peptide which are dominated by the 3 to 1 ratio of tryptophan to tyrosine residues in the peptide. Fluorescence detector gain was set at 1000, with an attenuation setting of 1 and employed a standard detector response time.

2.5. Recovery

The recovery of the peptide from plasma using the extraction procedure was assessed by parallel processing of samples of identical concentrations of T20 in the various plasma matrices vs. an aqueous standard solution containing 1% *n*-nonyl-β-D-glucopyranoside.

3. Results and discussion

3.1. Comments on the extraction procedure

Several organic solvents in addition to acetonitrile were examined as candidates for the extraction of T20 from the plasma matrices, including methanol, ethanol, isopropanol, 1-butanol and t-butanol. These latter solvents did not sufficiently precipitate plasma components which fluoresce strongly and elute under conditions similar to T20. The addition of 1% TFA to the extraction mixture precipitated interfering components from the plasma matrices that ACN alone did not affect.

The extension of the linear range of this assay into the ng/ml range was possible only by preventing the adhesion of T20 to sample tubes, which is particularly problematic at low concentrations of T20, where a greater percentage of the total T20 in solution is lost to surfaces. The use of 1% *n*-nonyl-β-D-glucopyranoside in the ACN extraction mixture prevents T20 from adhering to the sample container. Several other detergents were examined for this use, including sodium dodecyl sulfate, reduced Triton X-100, polyoxyethylene sorbitan monolaurate, polyoxyethylene 9-lauryl ether and polyoxyethylene 8-decyl ether. However, these detergents were unsuitable for this use because each had a strong fluorescent background which interfered with the detection of T20.

Signal linearity was achieved at the lowest injection concentrations of T20 (4–20 ng/ml) by the addition of 1% arginine hydrochloride to the HPLC buffers. We presume that the action of this material is to prevent the adhesion of T20 to the minute concentration of free silanol groups which are present on the column. Several other approaches were used to eliminate nonspecific adsorption of T20 to column materials. These approaches included the addition of (1) detergents (2) tetramethyl ammonium tetrafluoroborate or (3) triethylamine hydrochloride to the TFA running buffers and (4) triethylammonium phosphate running buffers. None of these approaches proved useful, as they either did not improve the linearity (1, 2 and 3) or generated a fluorescent background (4).

The use of PTFE microfuge tubes for the extraction is necessary because we found that the ACN

extraction mixture can remove plasticizers from typical eppendorf tubes which contaminate the fluorescence background. For the same reason, glass HPLC vials were used in place of the typical plastic inserts to hold the solution before HPLC injection.

3.2. Recovery

Fig. 1 presents five HPLC traces demonstrating the processing and recovery of T20 at a concentration of 4 μg/ml from water containing 1% detergent and four types of plasma (mouse, rat, monkey, and human) with the ACN extraction mixture. T20 elutes at approximately 44% ACN and the signal is baseline-resolved from any plasma protein peaks. Comparison of the fluorescence peak areas of T20 extracted from the various plasma matrices to that of the parallel-processed aqueous standard provides a measure of the recovery of T20 from the various biological fluids: 75%, mouse plasma; 60%, rat plasma; 50%, monkey plasma; and 55%, human plasma.

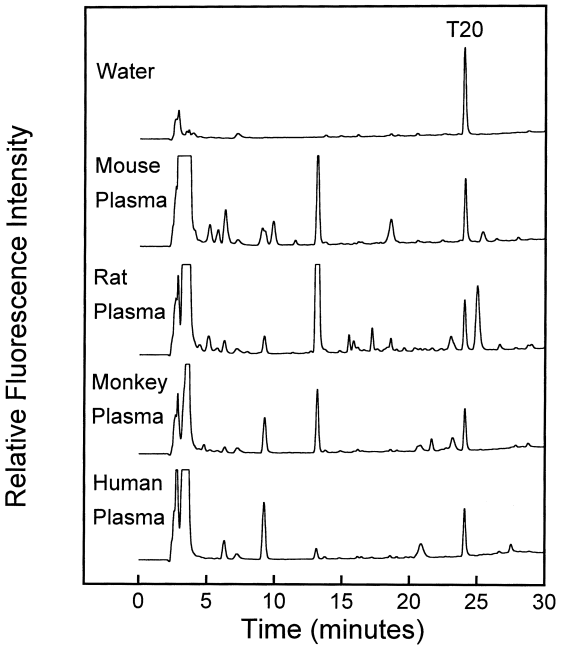

Fig. 1. Reversed-phase chromatograms of T20 extracts of mouse, rat, monkey and human in vitro plasma samples spiked with 4 μg/ml compared to the extract from an aqueous standard solution. The peptide T20 elutes at approximately 44% ACN (24 min) and is depicted in the first HPLC trace.

3.3. Linearity and limit of quantitation with T20 standard solutions

Standard solutions of T20 in human plasma (100–4000 ng/ml) were used to estimate the quantitation limit and to demonstrate extraction and detection linearity. Fig. 2 presents the linear relationship between fluorescence peak area and T20 concentration, arising from an experiment with three independent series of dilutions. The peak areas of the triplicate injections differ by an average of 10%. Linear regression data for this curve are as follows: slope=2260 area units/ng/ml; y-intercept=0; r^2= 0.9961. Four standard curves prepared over a 4-month period yielded an average slope of 2140±82 area units/ng/ml (coefficient of variation=3.8%). One measure of accuracy of the method is given by the average difference between the measured fluorescence peak areas and the peak areas predicted by the standard curve for the set of standard solutions. By this measure, the accuracy of the method is 94% over the range of the standard curve (T20 plasma concentrations of 100–4000 ng/ml).

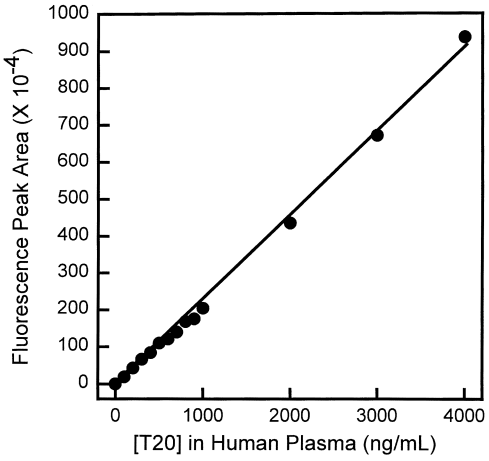

Fig. 2. Demonstration of extraction and detection linearity in the range from 100 to 4000 ng/ml T20 (original human plasma concentration), corresponding to a linear range of 4 to 160 ng/ml (injected solution concentration). Each point represents an average of three independent data measurements. Linear regression data: slope=2260 area units/ng/ml; y-intercept=0; r^2=0.9961.

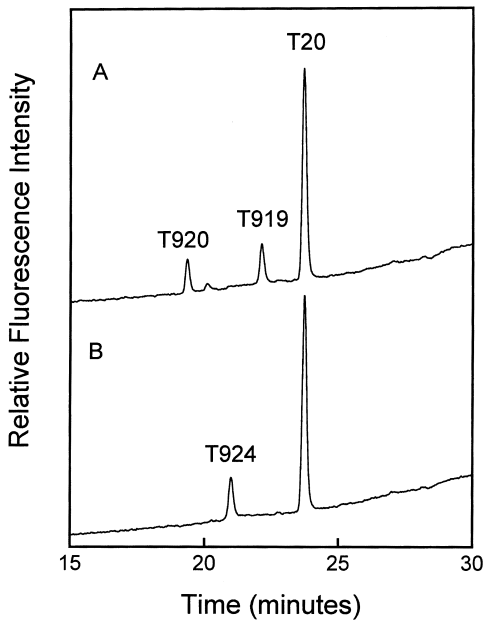

Fig. 3. Reversed-phase chromatograms of mixtures of T20 with possible biodegradation products illustrating the facile separation of these products from the parent peptide T20. (A) 40 ng/ml T20, 10 ng/ml T919 (T20 minus F-NH_2) and 10 ng/ml T920 (T20 minus WF-NH_2). (B) 40 ng/ml T20, and 10 ng/ml T924 (T20 minus Ac-YT).

3.4. Detection of T20 and truncated analogs

We investigated the utility of this assay for determining the possible degradation products which could arise from in vivo proteolysis of T20 in animals or humans. Fig. 3 presents two HPLC traces of solutions containing (A) 40 ng/ml T20 with 10 ng/ml T919 (T20 minus F-NH_2) and 10 ng/ml T920 (T20 minus WF-NH_2), and (B) 40 ng/ml T20 with 10 ng/ml T924 (T20 minus Ac-YT). The peptide corresponding to T20 minus Ac-Y elutes very close to T924. The clear separation of the degradation analogs from the parent T20 peak demonstrates the usefulness of this assay in detecting possible biotransformation products.

4. Conclusions

We have developed a simple, reliable and sensitive extraction/HPLC method for the detection and quantitation of a 36-residue peptide from animal and human plasma samples. The quantitation limit of this method is 4 ng/ml (injected concentration), corresponding to 100 ng/ml of T20 in the original plasma sample. We have demonstrated the linearity of this method from 4 ng/ml to 160 ng/ml (injected concentrations), corresponding to original in vitro plasma concentrations of 100–4000 ng/ml. This assay is particularly useful since it can be applied to detect biodegradation products resulting from in vivo enzymatic activity on T20. This assay has been readily applied to many other similar peptides derived from viral fusion proteins and containing aromatic residues and has proven useful in a variety of in vivo pharmacokinetic studies.

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