Current Topics in Peptide & Protein Research
Vol. 18, 2017

Original Communication

Quantification of a novel anti-breast cancer cyclic peptide (AFPep) in serum using liquid chromatography coupled with mass spectrometry and its application in a pharmacokinetic study

Jinghua Zhu[1], Wasila Mansouri[2], Thomas T. Andersen[2], James A. Bennett[3], Douglas Cohn[4], Paul Feustel[5] and Qishan Lin[1,*]

[1]Proteomics and Mass Spec Facility, Center for Functional Genomics, University at Albany, Rensselaer, NY 12144; [2]Departments of Molecular and Cellular Physiology; [3]Immunology and Microbial Disease; [4]Animal Resource Facility; [5]Department of Neuroscience and Experimental Therapeutics, Albany Medical College, Albany, NY 12208, USA.

ABSTRACT

We previously designed a cyclized nine-amino acid peptide, AFPep, to resist the hydrolysis of exopeptidases. A major finding was that AFPep inhibited estrogen-induced development and growth of experimental breast cancers. However, no detailed pharmacokinetic study has ever been performed to understand the fate and bioavailability of the peptide in animal models. The present study aims to develop a liquid chromatography configured with tandem mass spectrometry (LC-MS/MS) method to quantify the AFPep in mammalian serum. Our method was validated in terms of accuracy, precision, selectivity, sensitivity, stability and reproducibility. The developed method was found to be accurate and precise with a low limit of quantification (LLOQ) and a low limit of detection (LLOD) of 1.96 ng/ml and 0.65 ng/ml, respectively; method selectivity was confirmed by the absence of any matrix interference with the analytic peak. The constructed calibration curve was linear in the range of 2-5,000 ng/ml, with a regression coefficient of 0.998. Average recovery of AFPep from fortified samples (n = 6) was 98% with a relative standard deviation (RSD) of 5.3%. In addition, the peptide was found to be functionally stable in mouse serum at room temperature for 24 hrs. Overall, the established method provides rapid, sensitive, rugged, and robust LC-MS analysis for the quantitative determination of AFPep in biological matrices. This methodology was applied to establish a preliminary pharmacokinetic profile of AFPep following its intravenous administration to mice, dogs and monkeys.

KEYWORDS: cyclic peptide, AFPep, peptide pharmacokinetic, alpha-fetoprotein.

1. INTRODUCTION

Breast cancer is a leading cause of morbidity and mortality worldwide [1]. Estrogen promotes the growth of most breast cancers [2, 3]. Alpha-fetoprotein (AFP) is a fetal protein that is anti-estrogenic and has been found to inhibit the growth of experimental breast cancers [4-6]. Mesfin *et al.* [7] reported that within the 590-amino acid alpha-fetoprotein (AFP) molecule, there is an 8-amino acid sequence (EMTPVNPG, amino acids 472-479 in AFP) which retained all of the anti-estrotrophic activity found in full-length AFP. This peptide inhibited the estrogen-dependent growth of immature

*Corresponding author: qlin@albany.edu

mouse uteruses as well as the estrogen-dependent proliferation of human breast cancer cells [7]. To increase the stability of EMTPVNPG, the 8-mer peptide was redesigned. Substituting hydroxyproline for proline and lysine for methionine increased hydrophilicity, while adding an asparagine residue allowed for cyclization as a 9-mer peptide. This resulted in the increase of molecular stability [7, 8]. Using standard amino acid abbreviations, the 9-mer AFPep contains the sequence *cyclo* (EKTOVNOGN), where O is hydroxyproline. Cyclization provided rigidity, resulting in improved shelf-life, and also resistance to hydrolysis by exopeptidases due to the lack of both amino and carboxyl termini. In general, cyclic peptides also show better biological activity compared to their linear counterparts due to their conformational rigidity [9]. AFPep has been shown to arrest the growth of human breast cancer xenografts growing in severe combined immunodeficiency (SCID) mice [10] and prevent the development of carcinogen-induced mammary cancer in rats [11]. Clearly, further investigation into the medicinal developments of AFPep will be an area of keen interest. In the context of therapeutics, peptide-based drug candidates display both advantages and disadvantages in comparison to their small molecule counterparts. Peptides, while frequently less toxic than small molecules, when administered intravenously and displaying higher selectivity for specific biological functions, do not traverse cell membranes and are prone to proteolytic degradation, resulting in short plasma half-lives [12].

An important task in the development of drug therapeutics is to understand the pharmacokinetic (PK) behavior, as well as the PK/pharmacodynamics (PD) relationship. However, no pharmacokinetic information is available for AFPep, perhaps because PK investigations have been hindered by a lack of sensitive analytical methodology. AFPep has no good chromophores. There is no antibody available to the peptide.

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that couples high-resolution chromatographic separation with sensitive and specific mass spectrometric detection. LC-MS is a powerful technique that has very high sensitivity and selectivity. In recent years, LC-MS/MS has become a gold standard for the PK/PD study of drugs in the pharmaceutical industry [13]. Because of the high degree of selectivity routinely provided by LC-multiple-reaction-monitoring (LC-MRM), bioanalytical method development time for quantitative determinations of one or several analytes has been reduced to a few days or less.

In the present study, a sensitive and reliable liquid chromatography configured with tandem mass spectrometry (LC-MS/MS) method was developed to quantify AFPep in mammalian serum. The stability of AFPep was assessed *in vitro* and a preliminary PK profile of AFPep was determined *in vivo* following its intravenous administration to mice, dogs and monkeys. Knowledge obtained from this study will be useful for the evaluation of AFPep as a therapeutic agent for the treatment and prevention of breast cancer.

2. MATERIALS AND METHODS

2.1. Synthetic peptides

AFPep peptides including *cyclo* [Glu-Lys-Thr-Hyp-Val-Asn-Hyp-Gly-Asn], (see Figure 1) its stable isotope-labelled version *cyclo* [Glu-Lys-Thr-Hyp-Val-Asn-Hyp-Gly($^{13}C_2$, 2,2-D_2, ^{15}N)-Asn], and a linear form of AFPep TOVNOGNEK were purchased from AmbioPham, Inc. (North Augusta, SC). The purity of the peptides was confirmed as 96% by analytical high performance liquid chromatography (HPLC) and electrospray ionization (ESI)-time of flight mass spectrometry. The lyophilized peptides were weighed, aliquoted and stored at -80 °C. After solublization in water, the peptide concentrations were confirmed utilizing ultraviolet-visible spectrophotometry.

2.2. Materials and reagents

Acetonitrile (HPLC-grade) was obtained from EMD Millipore (Billerica, MA, USA). Methanol (HPLC-grade) was obtained from Sigma (St. Louis, USA). Ammonium formate was purchased from Sigma (St. Louis, USA). In-house Milli Q water was used throughout the analysis. Drug-free mouse plasma and serum were purchased from Sigma (St. Louis, USA). All the other reagents were of analytical grade.

2.3. Serum samples

All animals were housed in facilities certified by the American Association for the Accreditation of Laboratory Animal Care. The animal studies were

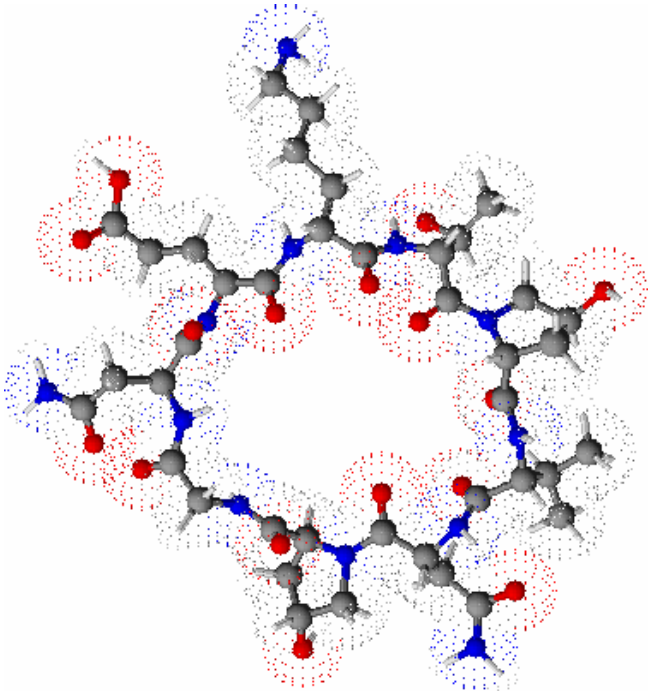

Figure 1. AFPep is cyclo(EKTOVNOGN) where O is hydroxyproline. AFPep was synthesized commercially as described in reference [8]. The C-terminus to N-terminus peptide bond obviates the need for disulfides and eliminates exopeptidase activity.

carried out in adherence to the guidelines established in the *Guide for the Care and Use of Laboratory Animals* with approval of the Albany Medical College Animal Care and Use Committee. Swiss Webster mice, weighing around 25 grams, were purchased from Taconic Biosciences. The animals were maintained under experimental conditions of room temperature (20-23 °C), humidity (50 ± 10%), light (12-h light/dark cycle), and had free access to water and food. They were allowed to acclimatize for 1 week before starting the experiment. Blood sample collection from mice was performed at multiple time points after administration of AFPep. Bleeding to euthanasia was through the retro-orbital plexus of each deeply anesthetized mouse. Mice were anesthetized with a ketamine (100 mg/kg)/ xylazine (10 mg/kg) cocktail, injected intraperitoneally (i.p.), and blood samples were obtained through the retroorbital plexus. Blood was collected into a 1.3 mL Vacutainer tube containing the chelating agent ethylene diamine tetra-acetic acid (EDTA) (Kent Scientific Corp., Torrington, CT). The tube was centrifuged at 2,000 rpm for 10 minutes. The supernatant (plasma sample) was aliquoted and stored at -20 °C until analysis. No sample underwent more than two freeze-thaw cycles prior to LC-MS/MS analysis.

Sexually mature female beagle dogs were ordered from Marshall Farms, North Rose, NY and were singly housed in large indoor pens. All animals were on a 12-hr/12-hr light/dark cycle and fed food and water *ad libitum*. Dogs weighing approximately 10 kg each were injected with 4 mg/kg of AFPep through an indwelling catheter in the cephalic vein. The cephalic vein was cannulated with a 20-gauge catheter and blood samples were obtained by the veterinary staff at Albany Medical College. At the end of the canine studies, the dogs were adopted by local families.

Male rhesus monkeys (*Macaca mulatta*) weighing approximately 10 kg were physically immobilized using the squeeze-back mechanism of the cage, then subjected to atropine (0.05 mg/kg) injected into either the biceps femoris or the quadriceps muscle (IM) using a 25-gauge needle. The squeeze-back mechanism is then released, and 10 minutes later the monkey is immobilized again and injected

IM with midazolam (0.2-0.5 ml; 0.05-0.15 mg/kg) mixed with ketamine 5-15 mg/kg IM. Once recumbent, the animals were intubated and given isoflurane (1-5%), oxygen (30%) and nitrous oxide (70%). An intravenous catheter was placed in the cephalic vein and a baseline blood sample was obtained. AFPep (4 mg/kg) was administered i.v. through the same site. Heart rate and SP_{O2} were monitored *via* a pulse oximeter clasping the finger, while body temperature was monitored with a rectal thermometer. Blood samples were collected at several time points post-injection for pharmacokinetic analyses. Samples analyzed for blood chemistry and complete blood count variables were taken prior to AFPep administration and 24 hours after administration. After 4 hours, the catheter was removed and the monkeys were allowed to recover from anesthesia in the home cage. The following morning, animals were sedated for a short time in order to obtain a 24-hour blood sample.

2.4. Preparation of calibration standards and QC samples

Two fresh stock solutions of AFPep peptide were prepared independently in water at a concentration of approximately 1 mg/ml and stored at -20 °C. One solution was used to spike the plasma calibration samples and the other was used to prepare the quality control (QC) samples. The stock solutions were diluted further with methanol to obtain working solutions on the day of use. A dilution series of AFPep was prepared in methanol at thirteen levels of concentrations (0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5,125, 250, 500, 1,000, 2,000, and 5,000 ng/ml). QC samples were prepared in a similar way, resulting in concentrations of 2 (LLQ), 2.5 (low), 50 (medium), and 500 ng/ml (high). A stable isotope labeled AFPep (5 ng/ml-final) was used as an internal standard.

2.5. Fragment analysis of AFPep

For product ion characterization by tandem MS at nominal mass, molecular ions were isolated by quadrupole 1 (Q1) and subjected to collision-induced dissociation (CID) in Q2. Q3 was operated in ion trap scanning mode from m/z 100 to m/z 1000. Conditions of multiple reaction monitoring (MRM) were optimized by auto-tuning during infusion of AFPep solution (50 ng/ml). Collision energy was optimized to achieve the best sensitivity.

2.6. Sample preparation

For extraction of AFPep, 20 µl of the internal standard (IS) solution (containing approximately 5 ng/ml AFPep in water) was added to 50 µl of plasma. After vortex-mixing for 10 s, 100 µl of methanol was added. The resulting sample mixture was vortexed vigorously followed by centrifugation at 14,000 rpm for 10 min at 4 °C. The supernatant (160 µl) was removed and dried by SpeedVac™. Then the dried samples were reconstituted with 100 µl of 0.1% formic acid. The mixture was vortexed thoroughly. A sample of 60 µl was transferred to an auto-sampler vial for LC-MS/MS analysis and 10 µl was injected.

2.7. LC-MS/MS experiment

LC-MS/MS analysis was performed on a 6500 QTRAP mass spectrometer (ABSCIEX, Framingham, MA, USA) configured with a Shimadzu LC-30AD pumping system, a SIL-30AC auto-sampler, and a CTO-30AC column oven (Shimadzu, Columbia, Maryland, USA). The system was operated under Analyst 1.63 control. The chromatographic separation of compounds was achieved on an Ascentis® Express C18 with a guard cartridge (90 Å, LC column 50 × 2.1 mm ID column packed with 2.0 µm particles). The mobile phase consisted of solvent A (0.1% formic acid + 0.05% ammonium hydroxide solution) and solvent B (100% methanol + 0.1% formic acid). The samples were eluted at a flow rate of 0.50 ml/min with a gradient as follows: 0-1.1 min, 2% B; 1.1-2.4 min, 10% B; 2.4-2.5 min, 90% B; 2.5-3.7 min, 90% B; 3.7-3.8 min, 2% B. The total run time was 7 min. The column oven temperature was kept at 40 °C and the sample injection volume was 10 µl. Two injections were performed for each sample. The MRM transition for AFPep was m/z 485.2 > 841.1. The transition for internal standard (IS) was m/z 487.7 > 846.1. The instrument was operated in a positive ion mode at unit-mass resolution with a turbo V electrospray source. The parameters for the operation were as follows: curtain gas, 25 psi; heated nebulizer temperature, 450 °C; ion spray voltage, 4500V; gas 1, 60 psi; gas 2, 50 psi; entrance potential (EP), 10 V and collision gas, high. The de-clustering potential (DP), collision energy, and collision cell exit potential (CXP) were as follows: 56, 23 and 54, respectively. The dwell time for each transition was set for 100 msec.

Data acquisition and analysis were performed with Analyst 1.63 or MultiQuant™ 2.1 software. Calibration curves were plotted using linear regression with a weight factor of 1/x.

2.8. AFPep stability study

The peptide samples were incubated in both phosphate-buffered saline and mouse serum at room temperature. Aliquots were taken at 1, 2, 4, 6, 8, 10, and 24 hrs, followed by protein precipitation and extraction. The remaining peptide concentrations after the incubations were measured by LC-MS/MS analysis. In the other set of experiments, the biological activity of AFPep after incubation was measured by uterine growth inhibition assay as described elsewhere [7, 10, 14].

2.9. Calibration curves

A calibration curve for the peptide was prepared by spiking the AFPep at twelve concentrations into 50 μL mouse serum. The peptide was extracted and analyzed by LC-MS/MS. Peak area ratios to the corresponding IS were plotted against peptide concentrations. A linear regression with 1/x (where x = concentration) weighting was used for calibration curve fitting. In order to pass our acceptance criteria, no more than two standard levels or two adjacent standard levels in the calibration curve could fail [15].

2.10. Spike-and-recovery, matrix effect, and stability assessment

Spike recoveries were determined by comparing the measured concentration of peptide in fortified samples to the actual fortified concentration. The recovery of AFPep from plasma was determined by spiking aliquots of intermediate standard solutions (5 μl) plus IS into 25 μl plasma solution. Fortified samples were allowed to equilibrate 15 min prior to extraction as mentioned above. Several end spikes at the lowest, medium, and highest concentrations of the standard curve were evaluated to determine the loss of instrument sensitivity due to a matrix effect. To determine the presence of a matrix effect, solution standards were injected in parallel with matrix standards (corresponding to blank plasma samples fortified at the end of the sample preparation). Spike or extraction recovery was determined using the following formula: peak area ratio of spiked AFPep vs IS/peak area ratio of standard peptide vs. IS directly injected on to LC-MS × 100%. Peptide stability was assessed by reinjecting the fortified samples that had been stored at 8 °C in the auto-sampler for multiple days.

2.11. Precision and accuracy

Inter-batch precision (coefficient of variation (CV), %) and accuracy (deviation from nominal concentration) were assessed by analyzing the calibration curves prepared on four separate days. Intra-batch precision and accuracy also were assessed by analyzing fortified samples at different levels in triplicate, as described by Hu's group [15].

2.12. Application to pharmacokinetic study

The validated method was used to determine the plasma concentration of AFPep in a pharmacokinetic study. All animals received a single dose of 4 mg/kg AFPep intravenously. Mice received AFPep in a volume of 0.2 ml saline injected i.v. through the tail vein. Dogs and monkeys received AFPep in a volume of 1 mL through an indwelling catheter placed in the cephalic vein. Blood samples were collected at 5, 10, 20, 30, 60, 120, 240, and 1440 minutes after administration. Samples were centrifuged, separated, and stored at -20 °C prior to analysis. Pharmacokinetic data analysis was carried out using WinNonlin software (version 5.2, Pharsight, Mountain View, CA) with a non-compartmental model and one compartment first order, on lag time and first order elimination. The area from 0 to 24 h under the AFPep concentration-time curve was used to determine the AUC. All the other parameters including Cmax, Tmax and $t_{1/2}$ were determined with WinNonlin software as well.

The anti-estrotrophic activity of AFPep was determined using the immature mouse uterine growth assay as described by Bennett *et al.* [8, 10]. As described in references [8] and [10], intraperitoneal (i.p.) injection of 0.5 ug of 17β-estradiol (E2) into 13-15-day-old female mice has been shown to double the uterine weight in 24 hours with a corresponding increase in mitotic figures. Adhering to that protocol, Swiss/Webster female mice (13-15-day old, Taconic Farms, Germantown, NY, USA) were weighed and distributed into treatment groups typically of five mice each such that groups contained mice of comparable weight ranges. Each group received two sequential injections spaced 1 hour apart. The first injection contained AFPep

at 4 mg/kg in 0.2 mL normal saline or saline control. The second injection contained 0.5 ug E2 or vehicle (0.2 mL). Twenty-two hours after the second injection, the mice were euthanized, weighed and uteri dissected, trimmed free of mesenteries, and immediately weighed. Uterine weights were normalized to body weights (mg uterine weight per g of body weight) to compensate for differences between the body weights of littermates. The percent inhibition of E2-stimulated uterine growth was calculated from the average values of five replicate mice for each group using the following equation:

% Growth inhibition =
100 x (Full E2 stimulation – E2 stimulation in test group)/
(Full E2 stimulation – No E2 stimulation).

3. RESULTS AND DISCUSSION

The goal of this study was to establish a rugged, robust HPLC-MS assay to analyze AFPep in biological samples (serum, plasma or urine matrix). This assay should be able to withstand the test of time during the longest phase of clinical development. Thus, the requirements and adherence to specificity, selectivity, and stability become very important.

3.1. Determination of MS/MS parameters

LC-MS/MS electrospray conditions were optimized by tuning with a 10 ng/µl solution of AFPep, which was fed into the mass spectrometer with a syringe pump at 10 µl/min while 500 µl/min of 25% acetonitrile solution was added *via* a T-union. The combined stream was introduced into the electrospray interface. The standard solution (10 ng/ml) of AFPep peptide was infused into the Turbo V™ source at the positive ion mode for MS/MS optimization. In all cases, $[M+2H]^{2+}$ was selected as the precursor ion due to its specificity for peptide. The most abundant transition with consideration of product ion m/z > precursor was selected for quantitation. The collision energy was optimized to be 17 ev so as to achieve the highest sensitivity. Optimized values for other parameters are indicated in the "Materials and Methods" section. Figure 2 shows the resulting MS and MS/MS spectra. Both singly and doubly charged forms of peptides were observed in the spectrum. The mass spectra obtained are dominated by a doubly charged peak m/z 485.20 and a significant singly charged fragment peak m/z 841.10. This transition was used in the LC eluent to construct a calibration curve for all the experiments.

3.2. Optimization of chromatographic separation

No methods for the analysis of AFpep were found in the literature. Chromatographic conditions were developed through a systematic analysis of several parameters that included column efficiency and selectivity [16]. Optimization of eluent composition plays an important role in LC-MS/MS method development, as sorbents possess differing selectivity

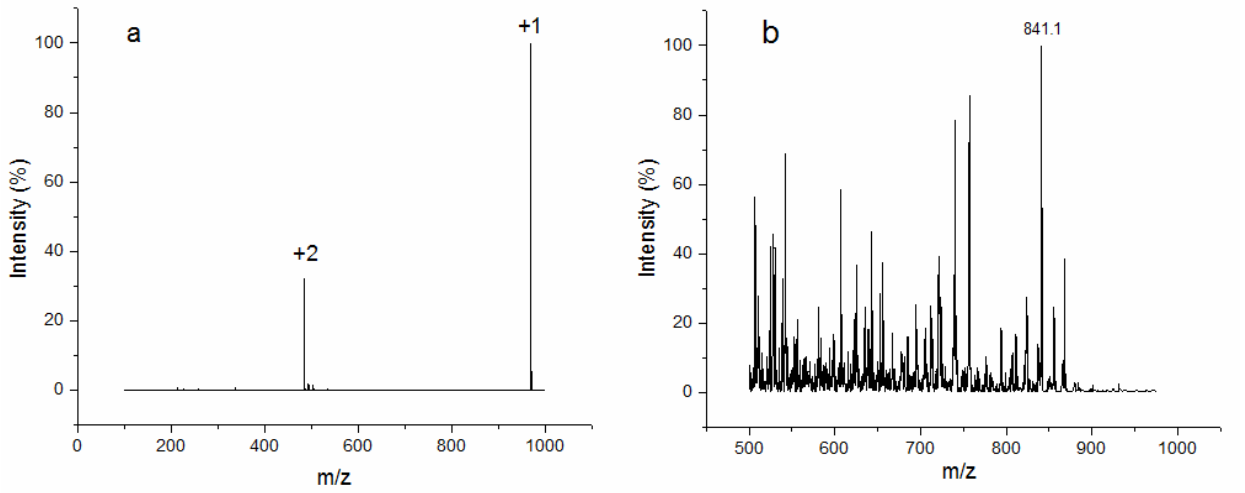

Figure 2. MS and MS/MS spectra of AFPep 1277 (a) MS spectrum; (b) MS/MS spectrum of AFPep (parent m/z 485.2, doubly charged) with product ion (m/z 841.1) used for quantitation.

and performance based on gradient shape and choice of acidic modifier. The chromatographic variables were optimized to achieve adequate separation among the analytes without interference from the background and with short retention times as well as good peak shapes. Specificity is established by the lack of interference peaks at the retention time of IS and the analyte. For this purpose, the composition of the chromatographic column and mobile phase were investigated. AFPep is a very polar peptide with poor retention in a regular reverse-phased column when acetonitrile is used as the organic solvent. It has been shown that the elution strength of methanol is weaker than that of acetonitrile in reverse-phased columns [17]. In this study, the use of methanol kept the peptide retained. In addition, a short fused-core column (Ascentis Express 2.0 μm C18) was chosen. The fused-core particle provides a thin porous shell of high-purity silica surrounding a solid silica core. According to the manufacturer (https://www.sigmaaldrich.com/united-states.html), this particle design exhibits very high column efficiency due to the shallow diffusion paths in its 0.4-micron-thick porous shell. This has resulted in small molecular applications with reduced run times and increased throughput without the need for advanced new ultra-high performance liquid chromatography (UHPLC).

3.3. Sample preparation

Sample preparation is a crucial step in removing interfering compounds from the biological matrix. To avoid time-consuming and tedious liquid-liquid extraction or expensive solid-phase extraction procedures, protein precipitation was first chosen to set up the assay [18]. As there is a risk that endogenous compounds or other drugs may interfere in the LC-system, methanol was chosen as the extraction solvent. Methanol is generally a preferred solvent amongst organic solvents as it can produce a clear supernatant, making it appropriate for direct injection into LC-MS/MS. The extraction capability of methanol was reflected by the recovery and accuracy as indicated in the “Materials and Methods” section. As shown in Figure 3, no interfering chromatography peaks were found at the LLOQ when negative control sera were analyzed.

3.4. Method validation

The validation of linearity, accuracy (precision and trueness), sensitivity, and selectivity was performed according to the US Food and Drugs Administration (FDA) guidelines for industry bioanalytical method validation [19].

3.5. Linearity, LLOD and LLOQ

A calibration curve for the peptide was generated by plotting the peak area ratio of the analyte to the IS against the nominal concentration (x) of the analyte in the standards by 1/x weighted linear regression. The validated concentration ranged from 2 to 2000 ng/ml. The individual percent relative error value was < 7% for standards at all levels (n = 6). The calibration curve of the validation run was described with the equation $y = 0.00178x + 0.00214$. The correlation coefficient of the calibration curve was 0.99912. The lower limits of detection (LLOD) and quantification (LLOQ) for the peptide were 0.65 ng/ml and 1.96 ng/ml, respectively. LLOD is defined as $3\sigma/S$, and LLOQ is defined as $10\sigma/S$ where σ is the standard deviation of the intercept and S the slope of the calibration curve [20].

3.6. Precision and accuracy

The precision of the method was evaluated by examining intra- and inter-day variations of the peak response for replicate assays of multiple QC levels (low QC, middle QC, and high QC; n = 6). A batch consisting of a standard curve in duplicate and the three QCs were run 4 times on four consecutive days. The intra-day precision, inter-day precision and accuracy of the peptide were validated, and the results were within the acceptable criteria (RSD% < 15%; RE% ± 15%). Inter-day and intra-day precision and accuracy were shown to be well within bioanalytical validation guidelines (Table 1).

3.7. Extraction recovery and matrix effect

Development of an efficient LC-MS analytical method requires maximizing the signal-to-noise ratio while achieving identical responses for a given amount of analyte in standard diluent (the standard curve) and sample matrix (biological sample + sample diluent). Similar to an immunoassay [21], the LC sample matrix may contain components that affect the LC assay response to the analyte differently than the standard diluent. A spike-and-recovery

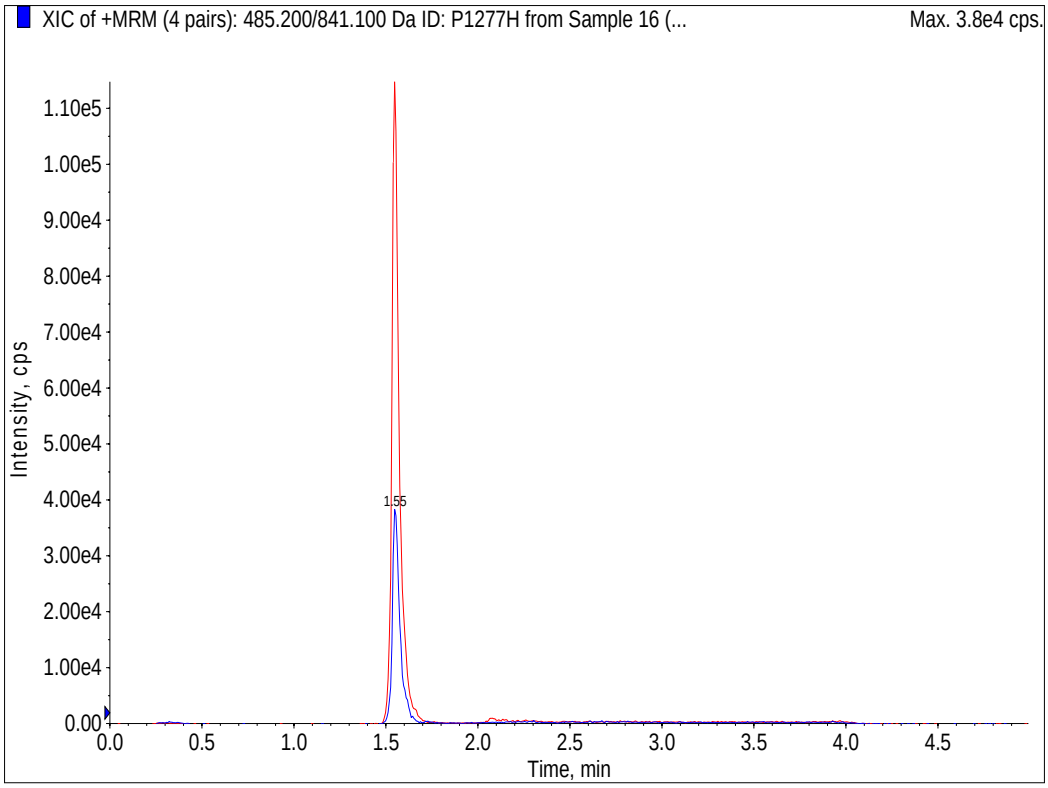

Figure 3. Multiple reaction monitoring chromatograms (quantifier: m/z 485.2-841.1 (red trace); IS: 487.2-847.1 (blue trace)) of a serum sample containing 5 ng/ml AFPep. No interfering chromatography peaks were found for the internal standard which co-elutes with native AFPep in double blank samples.

Table 1. AFPep QC results. Inter-day and intra-day precision and accuracy over 4 days for peptide in mice serum (RE, relative error; RE (%) = (O-T)/T ×100. O, overserved value; T, true value).

QC level	Day	N	Mean	STD DEV	Percent CV	Accuracy (RE %)
2.5 ng/ml	1	6 of 6	2.35	0.152	6.61	-6.0
	2	6 of 6	2.47	0.104	4.21	-1.2
	3	6 of 6	2.36	0.125	5.30	-5.6
	4	6 of 6	2.51	0.148	5.92	0.4
	Average				5.51	
50 ng/ml	1	6 of 6	51.9	1.25	2.41	3.8
	2	6 of 6	51.2	1.35	2.64	2.4
	3	6 of 6	50.2	1.28	2.55	0.4
	4	6 of 6	51.8	1.36	2.63	3.6
	Average				2.56	
500 ng/ml	1	6 of 6	510.0	20.5	4.04	2.0
	2	6 of 6	500.2	9.48	1.90	0.04
	3	6 of 6	513.5	12.50	2.43	2.7
	4	6 of 6	472.8	8.99	1.90	-5.4
	Average				2.57	

experiment was designed to assess this difference in the assay response. The mean extraction recovery of the peptide was more than 95% at different concentration levels (Table 2). The use of the isotopically labelled internal standard can compensate for the bias caused by the matrix effect and experimental losses. The matrix effect of the peptide ranged from 95% to 105% at 3 concentration levels (data not shown). No significant matrix effect for the peptide was observed.

3.8. Assay stability assessment

As analyses are usually not performed directly after the collection of biological samples but after they have been processed and stored, it is essential that analyte stability be maintained throughout relevant storage conditions to ensure that the obtained concentration results adequately reflect those directly after sampling. For this reason, stability assessment has always been an important part of bioanalytical method validation. The auto-sampler stability of AFPep was examined by the re-injection of the extracts stored in the auto-sampler at 10 °C for 24 hours. After 24 hrs of storage in the autosampler, the concentration measured for the AFPep at 3 QC levels deviated within ± 10%. In addition, the stability of AFPep in mouse plasma at room temperature for 24 hrs was also evaluated at the 3 QC levels. The relative error of the measurement ranged from -11.5 to 13.8% (n = 3) (Table 3). Additionally, the biological activity of AFPep was examined using a common screening assay (inhibition of estrogen-stimulated growth of immature mouse uterus) [10]. There was no loss in the biological activity or physical integrity of AFPep after 24 hours of storage in serum or buffer (Figure 4), indicating that the analyte could be detected accurately in blood samples obtained

Table 2. Recovery of AFPep from fortified serum samples and negative control.

Sample	Average % Recovery ± % RSD (n)
Control	Not confirmed (n = 4)
Methanol blank	Not detected (n = 2)
2 ng/ml fortified serum	96.5 ± 12.3 (n = 6)
5 ng/ml fortified serum	95.9 ± 8.1 (n = 6)
500 ng/ml fortified serum	97.8 ± 7.0 (n = 6)
3,500 ng/ml fortified serum	98.0 ± 5.3 (n = 6)

Table 3. Stability of AFPep in mouse plasma at room temperature. The remaining peptide concentration was measured by LC-MS/MS after the incubation (n = 3).

Incubation time	Samples	Relative error %	Precision % RSD
Hour 2	5 ng/ml in serum	6.4	8.4
	50 ng /ml in serum	-1.2	4.0
	200 ng/ml in serum	-8.2	3.9
Hour 4	5 ng/ml in serum	-1.7	2.0
	50 ng /ml in serum	4.9	4.1
	200 ng/ml in serum	-5.2	3.4
Hour 8	5 ng/ml in serum	-5.7	13.8
	50 ng /ml in serum	-7.9	7.8
	200 ng/ml in serum	-5.5	1.5
Hour 24	5 ng/ml in serum	6.4	8.4
	50 ng /ml in serum	-3.5	3.0
	200 ng/ml in serum	-11.5	2.3

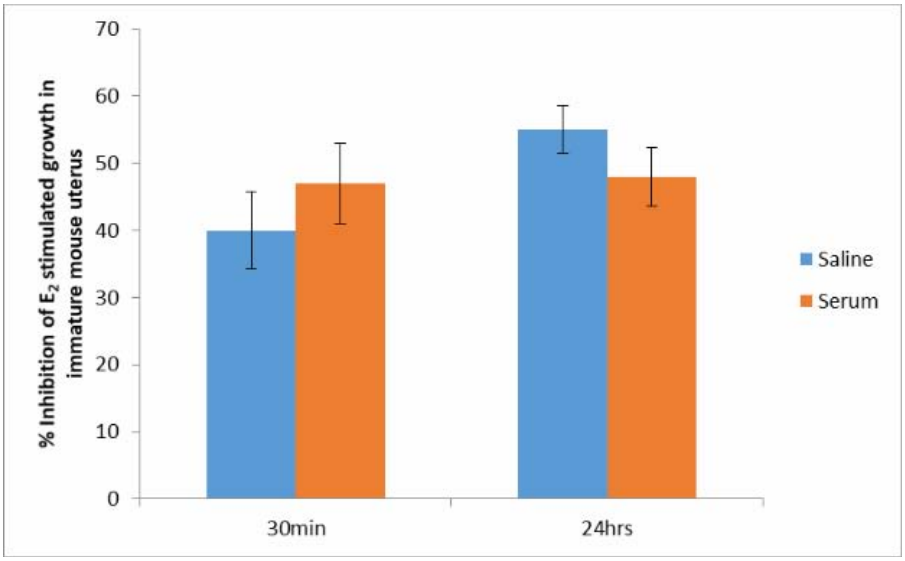

Figure 4. Biological activity of AFPep is stable after storage in saline or serum**.** AFPep was incubated in phosphate-buffered saline or in mouse serum for the indicated times, then subjected to a bioassay that measures the ability to inhibit estrogen-stimulated growth of the uterus in immature mice.

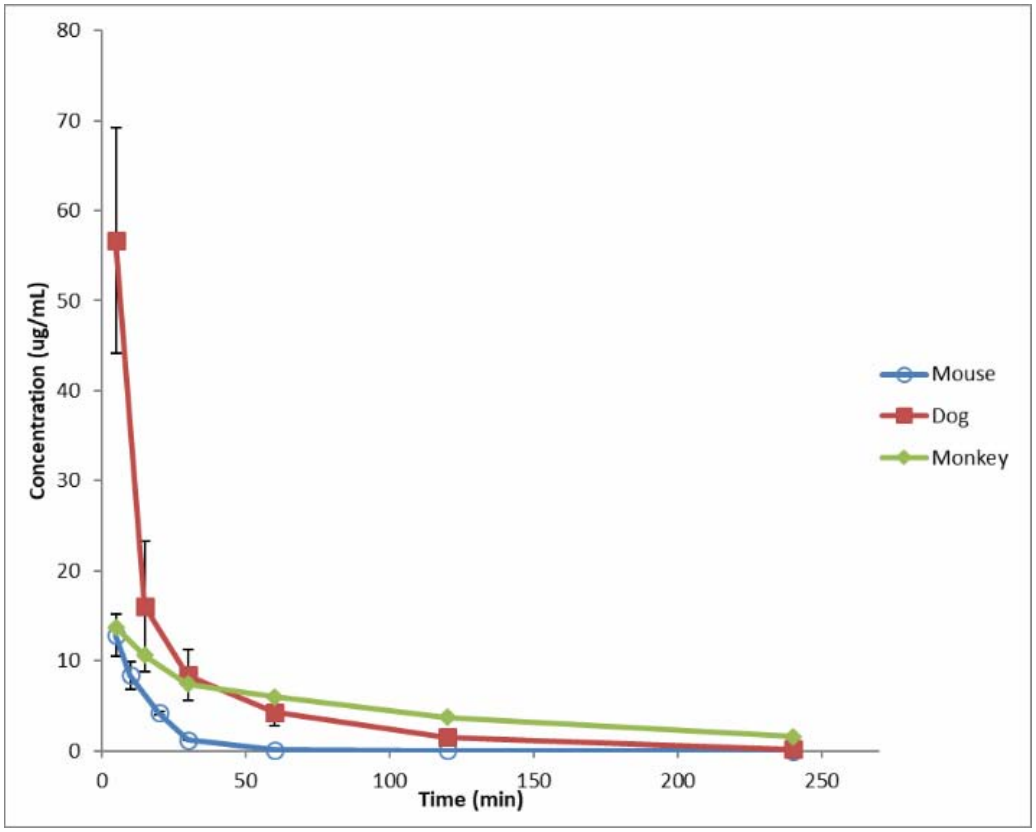

Figure 5. Plasma AFPep level versus time profile in mice, dogs and monkeys. Formulation was administered by the intravenous route to mice, dogs and monkeys at a dosage of 4 mg/kg. Data are shown as mean ± SD (n = 3).

Table 4. PK Parameters of AFPep (4 mg/kg) given i.v. in three species.

	Mouse	Dog	Monkey
Cmax (µg/mL)	12.8 ± 2.3	77.7 ± 27.3	13.7
Tmax (min)	5	5	5
T1/2 (min)	11 ± 2	37 ± 7	86
Vd (mL)	6.4 ± 0.7	1393 ± 108	461.6
AUC (min)(µg/mL)	252 ± 28	1223.3 ± 202	2150.5

during PK studies. Although the cyclic AFPep molecule should not be a substrate for exopeptidases, it was thought that trypsin-like endopeptidases might cleave the molecule at Lys, which would yield the linear peptide TOVNOGNEK. LC-MS/MS analysis of samples incubated at room temperature yielded little or no TOVNOGNEK. Similarly, TOVNOGNEK was not detected in any of the serum samples from the PK studies described below.

3.9. Application to a pharmacokinetic study

The method described in this study was developed to accommodate pharmacokinetic studies. Figure 5 and Table 4 demonstrate that AFPep can be detected in the serum of mice, dogs and monkeys to which AFPep had been administered by tail vein/saphenous vein injection. Ample quantities of AFPep were detected by our LC-MS/MS method at all time points measured following a 4 mg/kg dose of AFPep. The chemical half-life of the drug is relatively short in mice (11 min), which is not uncommon for peptides, but surprisingly the half-life is much longer in dogs (37 min) and monkeys (86 min), yielding a larger area under the curve for those species. The volume of distribution of the drug is quite reasonable in all three-species suggesting that AFPep can move out of the plasma compartment and distribute effectively into tissue. In studies reported elsewhere, ng/mL concentrations of AFPep were readily measured in the blood of animals receiving lower doses of AFPep [22].

4. CONCLUSION

We developed and validated a new method for the separation and quantification of AFPep in mammalian plasma. This method highlights the use of methanol for elution to overcome the characteristic hydrophilic challenge of AFPep. A fused-core column was chosen to improve productivity (from efficient, fast separations) as well as detection sensitivity (from the sharper peaks). The method was found to be accurate, precise, selective and highly sensitive with an LLOD of 0.65 ng/ml and an LLOQ of 2 ng/ml. The stability data indicated that no significant degradation of AFPep occurred in mouse plasma stored at room temperature for 24 hours. In addition, AFPep exhibited a good, stable shelf-life. The developed method was used effectively for the PK study in experimental animal models.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

This study was supported in part by grants from CDMRP BCRP W81XWH-15-1-0242 and the National Institute of Health 1S10 RR017812-01A. We thank Yufan Lin for critically reading and editing the manuscript.

ABBREVIATIONS

AFPep, alpha fetoprotein peptide; UHPLC, ultra-high pressure liquid chromatography; PK, pharmacokinetic; LC-MS/MS, liquid chromatography configured with tandem mass spectrometry; ESI, electrospray ionization; IS, internal standard; QC, quality control; RSD, relative standard deviation; LLOQ, low limit of quantification; LLOD, low limit of detection.

REFERENCES

1. DeSantis, C. E., Fedewa, S. A., Goding Sauer, A., Kramer, J. L., Smith, R. A. and Jemal, A. 2016, CA: A Cancer Journal for Clinicians, 66, 31.

2. Pike, M. C., Spicer, D. V., Dahmoush, L. and Press, M. F. 1993, Epidemiologic Reviews, 15, 17.
3. Ravdin, P. M., Cronin, K. A., Howlader, N., Berg, C. D., Chlebowski, R. T., Feuer, E. J., Edwards, B. K. and Berry, D. A. 2007, The New England Journal of Medicine, 356, 1670.
4. Aussel, C., Lafaurie, M., Masseyeff, R. and Stora, C. 1981, Steroids, 38, 195.
5. Bennett, J. A., Zhu, S., Pagano-Mirarchi, A., Kellom, T. A. and Jacobson, H. I. 1998, Clinical Cancer Research: An Official Journal of the American Association for Cancer Research, 4, 2877.
6. Soto, A. M. and Sonnenschein, C. 1980, Proceedings of the National Academy of Sciences of the United States of America, 77, 2084.
7. Mesfin, F. B., Bennett, J. A., Jacobson, H. I., Zhu, S. and Andersen, T. T. 2000, Biochimica et Biophysica Acta, 1501, 33.
8. DeFreest, L. A., Mesfin, F. B., Joseph, L., McLeod, D. J., Stallmer, A., Reddy, S., Balulad, S. S., Jacobson, H. I., Andersen, T. T. and Bennett, J. A. 2004, The Journal of Peptide Research: Official Journal of the American Peptide Society, 63, 409.
9. Roxin, A. and Zheng, G. 2012, Future Medicinal Chemistry, 4, 1601.
10. Bennett, J. A., Mesfin, F. B., Andersen, T. T., Gierthy, J. F. and Jacobson, H. I. 2002, Proceedings of the National Academy of Sciences of the United States of America, 99, 2211.
11. Parikh, R. R., Gildener-Leapman, N., Narendran, A., Lin, H. Y., Lemanski, N., Bennett, J. A., Jacobson, H. I. and Andersen, T. T. 2005, Clinical Cancer Research: An Official Journal of the American Association for Cancer Research, 11, 8512.
12. Muheem, A., Shakeel, F., Jahangir, M. A., Anwar, M., Mallick, N., Jain, G. K., Warsi, M. H. and Ahmad, F. J. 2016, SPJ: The Official Publication of the Saudi Pharmaceutical Society, 24, 413.
13. Ahmed, T. and Shashank, R. 2012, Journal of Pharmacy Research, 5, 2514.
14. Mesfin, F. B., Andersen, T. T., Jacobson, H. I., Zhu, S. and Bennett, J. A. 2001, The Journal of Peptide Research: Official Journal of the American Peptide Society, 58, 246.
15. Schacherer, L. J., Owens, M. A. and Hu, T. X. 2017, Anal. Methods, 9, 2821.
16. Girard, M., Hannah, K., Menzies, J. A., Solomonraj, G. and Whitehouse, L. W. 1994, Journal of Pharmaceutical and Biomedical Analysis, 12, 833.
17. Neue, U. D. 2005, Journal of Chromatography A, 1079, 153.
18. Chen, Y., Liu, Y. and Wang, C. 2009, Anal. Lett., 42, 3004.
19. U.S. Food and Drug Administration (FDA). 2014, Guidiance for Industry: Bioanalytical Method Validation.
20. Shrivastava, A. and Gupta, V. 2011, Chronicles of Young Scientists, 2, 21.
21. Tech Tip: Linearity and spike-and-recovery experiments for ELISA, 2015 http://tools.thermofisher.com/content/sfs/brochures/TR0058-Spike-and-Recovery.pdf.