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Research paper

Bioanalytical method development and validation for determination of fibroblast growth factor peptide and its application to pharmacokinetic studies

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ABSTRACT

Fibroblast growth factor peptide (FGF-P) is a polypeptide analog of FGF-2 that could be a potential mitigation and treatment agent for radiation syndromes. Prior to conducting preclinical pharmacokinetics, we developed and validated the LC-MS/MS bioanalytical method for determination of FGF-P in rat plasma for the first time. FGF-P was extracted from rat plasma using the protein precipitation technique followed liquid-liquid extraction using dichloromethane as a solvent. The mobile phases consisted of two components: (a) 0.1% formic acid in water; and (b) acetonitrile: 0.1% formic acid in water (95:5) under gradient elution. The validated method was also successfully applied to a pharmacokinetic study of FGF-P (10 mg/kg, intravenous) in Wistar rats. The method proved to be specific, accurate, precise, and linear over the concentration range of 2–500 ng/mL with coefficient of determination greater than 0.99 in all validation batches. The within-run and between-run accuracy was 87.97–115.00% with a precision of less than 14%. The mean recoveries ranged from 88.14% to 101.73%. The stability of the compound in plasma samples was proven under various storage conditions. After intravenous administration of FGF-P (10 mg/kg) the C_0 was 70.4 μg/mL and the AUC was 86.2 μg*min/mL.

1. Introduction

FGF-P is a polypeptide structure-modified analogue of fibroblast growth factor-2 (FGF-2) comprised of 17 amino acids with the sequence illustrated in Fig. 1. Our previous studies showed that FGF-P mitigates various radiation syndromes following total-body irradiation (TBI) [1,2]. Both *in vitro* and *in vivo* studies have demonstrated its ability to improve small bowel barrier function, cell proliferation of irradiated human keratinocytes, and wound healing, such as cutaneous β burns in a mouse model [3]. We also found that FGF-P promotes bone marrow recovery after irradiation by increasing the percentages of stem cells, progenitors, and differentiated leukocytes in NIH Swiss mice after TBI (6 Gy) [4]. Indeed, irradiated bone marrow cells from BALB/c mice exposed to different concentrations of FGF-P exhibited a dose-dependent enhancement in progenitor cells [5]. FGF-P has the following advantages over the full-length peptide: (1) it is stable under severe conditions; (2) it has long shelf life; (3) it can be self-administered via intramuscular or subcutaneous injection; and (4) it can be synthesized in large quantities with high purity at a substantially lower cost than native peptide [6]. Due to the presence of low-affinity inhibitory receptors, high doses of FGF-P lose their benefit; therefore, determining the optimal dose, schedule, and mode of delivery as well as converting treatment schedules from animals to humans requires high-quality pharmacokinetic analysis.

In drug development, quantitative analysis of therapeutic proteins and peptides is an important tool for pharmacokinetic studies. The development and validation of quantitative methods for the determination of proteins and peptides in biological fluids in toxicokinetic and pharmacokinetic studies can be very complex [7–14]. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) and immunoassays are the predominant techniques used to quantify proteins and peptides in a biological matrix. An immunoassay is an analytical tool that requires minimal sample preparation and offers a high-throughput platform. However, immunoassays are limited by poor selectivity between structurally and chemically similar proteins or peptides [15]. In recent years, LC-MS/MS has been used for quantification of proteins and peptides, such as a chlorotoxin-like peptide [13], angiotensin peptide [16], bivalirudin [11], monoclonal antibodies [8], apolipoproteins [17], salmon calcitonin [18], and tenecteplase [14]. The LC-MS/MS assay provides high sensitivity and specificity for target

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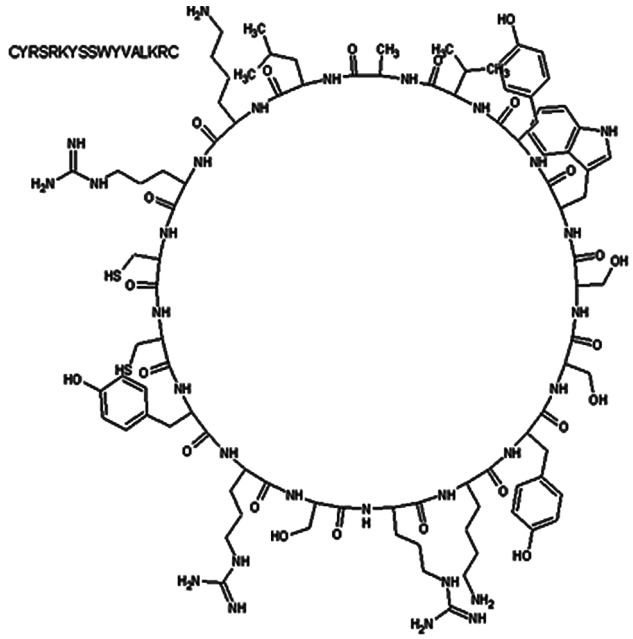

Fig. 1. Chemical structure of oxidized FGF-P. The linear reduced form has a MW of 2211.

analytes without the need for antibodies. Moreover, this technique can be developed and implemented more quickly than an immunoassay.

Stability, adsorption, and sample preparation are all challenges of peptide and protein analysis. Peptides can undergo both chemical and enzymatic degradation, including deamidation, oxidation, reduction, hydrolysis, β-elimination, aggregation, and denaturation [19–21]. The amphipathic structure of proteins or peptides can be prone to absorb to many surfaces, including laboratory glassware, plasticware, and other containers [22,23]. These stability and adsorption issues may lead to a poor response, inaccurate quantification of peptides, and incorrect interpretation of results. Due to their chemical similarity with target peptides, it is difficult to eliminate endogenous proteins in biological fluids, especially in serum and plasma. Techniques, such as protein precipitation (PPT), liquid-liquid extraction (LLE), and solid-phase extraction (SPE), are used to extract target analytes, and a combination of sample extraction techniques can be used to improve efficiency [16].

To date, there is no analytical method for quantification of FGF-P in a biological matrix. In order to conduct preclinical or biopharmaceutics and clinical pharmacology studies, a quantitative bioanalytical method is required. In this study, we developed and validated a sensitive bioanalytical method using LC-MS/MS, in accordance with United States Food and Drug Administration (FDA) guidance, to quantify FGF-P in rat plasma. The validated method was further used to determine FGF-P concentrations in a pharmacokinetic study.

2. Materials and methods

2.1. Chemicals and reagents

FGF-P was synthesized through a standard solid-phase method with 95.6% purity (Genemed Synthesis, Inc., TX, USA). Vancomycin hydrochloride (internal standard, IS) with 99.6% purity was purchased from Sigma-Aldrich (MO, USA). Drug-free Wistar rat plasma with sodium ethylenediaminetetraacetic acid (EDTA) was purchased from Innovative Research, Inc. (Novi, MI, USA) and stored at −20 °C. Halt™ protease inhibitor cocktail was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Low-retention microcentrifuge tubes (1.5 mL and 2.0 mL) (Fisher Scientific, PA, USA) were used throughout the study. All other reagents, such as acetonitrile and formic acid, were high-performance liquid chromatography (HPLC) grade (Thermo Fisher Scientific).

2.2. Equipment

The LC-MS/MS system consisted of a Perkin Elmer series 200 autosampler and pump (Waltham, MA, USA) coupled to an API 4000 triple quadrupole mass spectrometer with a turbo interface (AB SCIEX, Framingham, MA, USA). We utilized a Centrifuge5810R (Eppendorf AG, Hamburg, Germany) for sample preparation and a refrigerated Sorvall Legend Micro 21R Microcentrifuge to separate plasma samples (Thermo Fisher Scientific).

2.3. Liquid chromatographic conditions

Chromatographic separation was performed at ambient temperature (22 °C – 25 °C) using a Polaris C18-A, 5 µm, 50 × 3.0 mm HPLC column (Algilent Technologies, CA, USA), coupled with an Agilent Polaris C18-A, MetaGuard, 3 µm, 2.0 mm (Algilent Technologies). The mobile phases consisted of (a) 0.1% formic acid in water; and (b) acetonitrile: 0.1% formic acid in water (95:5) under gradient elution started from 5 to 50% mobile phase B at a flow rate of 0.4 mL/min (Table 1). The injection volume was 20 µL.

2.4. Mass spectrometer conditions

Positive ion electrospray tandem mass spectrometric analysis was carried out in multiple reaction monitoring (MRM) mode. Table 2 outlines the mass transitions for FGF-P and IS (Vancomycin). The ion source parameters were optimized and set as follows: ion spray voltage 4000 V, curtain gas 10 psi, collision gas 10 psi, temperature 400 °C, TurboIonSpray nebulizer gas (GS1) 35 psig, TurboIonSpray heater gas (GS2) 45 psig, collisionally activated dissociation gas (CAD) 10.

2.5. Preparation of standard solution, calibration samples, and quality control samples

FGF-P stock solution was prepared at a concentration of 1000 µg/mL in water. Working solutions for calibration samples were prepared at concentrations of 40, 100, 200, 1000, 2000, 6000, and 10000 ng/mL by diluting FGF-P stock solution with a mixture of acetonitrile and 0.1% formic acid in water (20:80 v/v). Three concentration levels of FGF-P working solution (120, 4000, and 9000 ng/mL) were prepared for quality control (QC) samples. Internal standard (IS) stock solutions were prepared at 1000 µg/mL in methanol and water (50:50 v/v).

Calibration samples and QC samples were prepared by spiking 5 µL of each corresponding working solution to 95 µL of drug-free rat plasma. The nominal concentrations were 2, 5, 10, 50, 100, 300, and 500 ng/mL for calibration samples and 6, 200, and 450 ng/mL for QC samples.

2.6. Sample preparation

FGF-P was extracted from rat plasma by protein precipitation using

Table 1
Mobile phase gradient elution program, a is 0.1% formic acid in water and b is acetonitrile:0.1% Formic acid in water (95:5 v/v).

Time (min)	a (%)	b (%)
0	95	5
1	95	5
2	50	50
3	50	50
4	95	5
12	95	5

Table 2
Mass spectrometer parameters.

	Transition *m/z*	Declustering potential (Volt)	Entrance potential (Volt)	Collision energy (Volt)	Collision exit potential (Volt)
FGF-P	553.2 → 136.4	87	12	43	9
	553.2 → 72.2	90	12	62	5
	443.0 → 136.4	52	10	35	12
IS	725.5 → 144.4	63	7	21	9

acetonitrile containing 2% formic acid followed by LLE using dichloromethane as an extraction solvent. Here dichloromethane used as an LLE solvent. All sample preparation was conducted on ice. Briefly, 100 μL of the plasma sample was precipitated with 400 μL of acetonitrile containing 2% formic acid. The sample was then mixed for 30 s and placed on ice for 20 min. The mixture was centrifuged at 4000 rpm at 4 °C for 10 min to remove precipitated proteins. Thereafter, 400 μL of supernatant was transferred to a new 2.0 mL microcentrifuge tube, 50 μL of IS (600 ng/mL) was mixed in, and 800 μL of dichloromethane was added. The mixture was vortexed for 2 min and centrifuged again at 4000 rpm at 4 °C for 10 min. The aqueous phase in the upper layer was taken and mixed with acetonitrile to make the final sample containing 20% of acetonitrile. A 20 μL of sample solution was injected into LC-MS/MS system for analysis.

2.7. Adsorption study

Common glass and plastic containers were used to measure adsorption of FGF-P to various surfaces. The FGF-P solution (45 ng/mL) was prepared in a mixture of acetonitrile and 0.1% formic acid at a ratio of 20:80 by volume. The remaining FGF-P in the mixture was measured after allowing it to stand at room temperature for 1 h in the container. The adsorption of FGF-P to the container was calculated by comparing the amount of FGF-P at 1 h with the amount at 0 min.

2.8. Stability of FGF-P in solution

The FGF-P stock solution was diluted with acetonitrile, methanol, isopropanol, and a mixture of acetonitrile and 0.1%formic acid (20:80 v/v) and kept at 4 °C. FGF-P stability was determined by comparing the amounts of FGF-P in the solution at 3 and 24 h to fresh solution.

2.9. Stability of FGF-P in plasma

2.9.1. Effect of temperature

Plasma samples containing low and high concentrations of FGF-P (6 and 450 ng/mL) were incubated on ice (4 °C), at room temperature, and at 37 °C in a water bath. After incubation, plasma samples were taken from each incubated tube at predetermined time points (0, 0.25, 0.5, 1, 2, 4, 6 h) and the FGF-P was immediately extracted. The stability of FGF-P was calculated against the initial amount of FGF-P at each temperature condition.

$$\%\text{Stability} = \frac{\text{Amount of FGF}-\text{P at each time point}}{\text{Amount of FGF}-\text{P at initial time point (T = 0)}} \text{x100} \quad (1)$$

2.9.2. Effect of enzyme inhibitor

Several protease inhibitors, such as sodium fluoride (NaF), phenylmethyl sulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and Halt™ protease inhibitor cocktail, were tested to stabilize FGF-P. A single protease inhibitor or combination of protease inhibitors were added into the plasma before it was spiked with FGF-P. Stability time profiles of plasma spiked with FGF-P at a concentration of 450 ng/mL were conducted at room temperature. Samples were evaluated after the designated incubation time.

2.9.3. Effect of organic solvent

In plasma, peptides or proteins are susceptible to degraded by different enzymes (protease or peptidase). We investigated how the addition of acetonitrile with 2% formic acid to the plasma sample inhibited enzyme activity. A 100 μL of plasma spiked with FGF-P (6 and 450 ng/mL) was immediately mixed with 400 μL of acetonitrile with 2% formic acid; thereafter, the samples were processed and analyzed by LC-MS/MS.

2.10. Method development and validation

Using LC-MS/MS, we developed a bioanalytical method for the determination of FGF-P, which was then validated as per the documentation of selectivity, specificity, linearity, limit of quantification (LOQ), accuracy, precision, dilution integrity, recovery, matrix effect, and stability criteria proposed by the US-FDA.

We evaluated the specificity and selectivity of the method by comparing the six different chromatograms of the blank extracted plasma samples with the chromatogram of the FGF-P and IS solution. Each validation run and sample analysis run consisted of the system suitability test samples, calibration curve samples, blank sample, zero blank samples, and seven non-zero points and QC samples. The lower limit of quantitation (LLOQ) was established as the lowest concentration of the calibration curve. Within-run precision and accuracy were determined using five replicates of QC samples at the low, medium, and high levels; between-run precision and accuracy were determined for six runs. Accuracy was calculated by comparing observed concentrations to the corresponding theoretical concentration.

$$\text{Accuracy}(\%) = \frac{\text{Observed concentration}}{\text{Theoretical concentration}} \text{x100} \quad (2)$$

$$\text{CV}(\%) = \frac{\text{Standard deviation}}{\text{Mean observed concentration}} \text{x100} \quad (3)$$

Relative recoveries of FGF-P from plasma sample were determined by comparing peak areas of FGF-P obtained from the extracted plasma sample with those of the extracted standard solution at the three QC levels.

$$\text{Recovery (\%)} = \frac{\text{Peak area of FGF}-\text{P from extracted plasma sample}}{\text{Peak area of FGF}-\text{P from extracted standard solution}} \text{x100} \quad (4)$$

Dilution integrity was performed as a part of method validation in order to analyze samples above ULOQ. Dilution integrity was determined by diluting supernatant obtained from FGF-P-spiked plasma samples with supernatant obtained from the drug-free plasma samples. The dilution was performed at three different dilution factors: 2, 20, and 50 times. At each level of dilution, samples were prepared in triplicate. The accuracy and precision percentages were calculated.

The matrix effect was evaluated by comparing the peak response of FGF-P and IS in the standard solution with the peak response of FGF-P and IS after blank plasma samples were spiked. The matrix effect was determined at low and high QC concentrations.

FGF-P stability was assessed after precipitation of the plasma samples at low and high concentrations with acetonitrile containing 2% formic acid. We compared specimens stored under various conditions

with the freshly prepared calibration curve. The post-preparative samples were kept in the autosampler for 24 h and were reinjected to determine the autosampler stability. For short-term stability, supernatant was left on ice for 9 h. For freeze-thaw stability, the supernatant samples were stored at −80° C for 24 h and thawed unassisted on ice. When completely thawed, the samples were refrozen for 12 to 24 h under the same conditions. The freeze and thaw cycles were repeated two more times. The remaining FGF-P was analyzed on the first, second, and third cycle. Long-term stability was determined by storing four aliquots of each of the supernatant separated from QC samples at −80 °C for 1 month; thereafter, they were thawed on ice and analyzed. We then determined the stability of the FGF-P stock and working solutions as well as the IS solution. The FGF-P standard solution and the IS solution were diluted, analyzed, and compared to the freshly prepared solution at the same concentration.

2.11. Pharmacokinetics study in Wistar rats

A single-dose pharmacokinetic study was performed in Wistar rats (n = 3). FGF-P was dissolved with sterile normal saline and administered to rats by IV tail-vein injection (10 mg/kg). Blood samples were collected at 2, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, and 300 min after IV injection. Blood collected at the desired time point was immediately centrifuged to separate the plasma; thereafter, 100 μL of separated plasma was combined with 400 μL of acetonitrile containing 2% formic acid, and 400 μL of supernatant was separated and stored at 4° C until analysis.

2.12. Data analysis

All data acquisition and quantitation were performed with Analyst software version 1.4.2 (AB SCIEX). The calibration curves were plotted using the peak area ratio (FGF-P/IS) against nominal concentration by least-squares regression analysis with a weighting factor of 1/x. The slopes, intercepts, and coefficient of determination of the regression line were used to determine the concentration of FGF-P in the samples.

Non-compartmental analysis was employed to calculate the pharmacokinetic parameters using Phoenix 64 software (Pharsight, Mountain View, CA, USA). The time-range fit method was used with uniform weighting to determine the elimination rate constant.

3. Results

3.1. Adsorption study

FGF-P exhibited high adsorption to flint and borosilicate glass tubes but low adsorption to polystyrene and low-retention polypropylene microcentrifuge tubes. After 1 h of storage at room temperature, 9.58% and 33.66% of FGF-P was lost in polypropylene and polystyrene microcentrifuge tubes, respectively. These results reveal that FGF-P has lower binding to polypropylene tubes compared with polystyrene tubes and other materials.

3.2. Stability of FGF-P in solution

As shown in Table 3, FGF-P was stable in the tested solvent. After storage at 4 °C, the stability of FGF-P in acetonitrile and a mixture of acetonitrile and 0.1% formic acid (20:80 v/v) was within ± 15% of initial level. In methanol, FGF-P decreased to 75% after 24 h of storage.

3.3. Stability of FGF-P in plasma

Preliminary studies conducted with the serum and plasma (anticoagulant used heparin and EDTA) revealed similar stability levels. As shown in Fig. 2, FGF-P rapidly degraded at 37 °C and at room temperature; however, the degradation rate was much slower at 4 °C compared to the other two conditions. At 37 °C and room temperature, FGF-P disappeared within 2 h; however, at 4 °C, 60% remained after 2 h with minimal additional degradation for the remaining 6 h. These results suggest that FGF-P exists in at least 2 states in plasma at 4 °C, including a heat labile state that confers stability. As shown in Table 4, the addition of a single or a combination of enzyme inhibitors to plasma did not effectively prevent the degradation of FGF-P. Moreover, the combination of two or more inhibitors adversely affected the sensitivity of the method. Finally, in plasma, direct denaturation of the enzyme activity by adding acetonitrile containing 2% formic acid more effectively stabilized FGF-P compared to the protease cocktail.

Table 3
Stability of FGF-P in solvents (Mean ± SD, n = 3).

Solvent	Stability (%)		
	0 h	3 h	24 h
Acetonitrile	100	87.40 ± 4.36	109.17 ± 6.22
Methanol	100	92.51 ± 5.52	75.60 ± 0.93
Acetonitrile:0.1% Formic acid (20:80 v/v)	100	95.55 ± 4.76	104.01 ± 7.74

3.4. Method development

To develop the bioanalytical method using LC-MS/MS, we first tuned the FGF-P compound to determine the mass spectrum and optimize the mass spectrometer parameters; thus, an ESI probe with a positive mode was applied. The ion spray voltage, curtain gas, collision gas, temperature, GS1, GS2, and CAD were optimized in the MRM mode using built-in software Analyst 1.4.2 (AB SCIEX). Fig. 3 shows the resulting FGF-P mass spectrum. FGF-P exhibited five parents *m/z* corresponding to the protonated form of the compound with the molecular weight of 2210: $[M + 5H]^{+5} = 443.0$, $[M + 4H]^{+4} = 553.5$, $[M + 3H]^{+3} = 737.7$, $[M + 2H]^{+2} = 1105.5$, and $[M + 1H]^{+1} = 2211$ (peak not shown). The prominent *m/z* of 553 and 443 were chosen, and collision energy was applied to obtain product ions. Three major mass transitions (553.2 → 136.4, 553.2 → 72.2, and 443.0 → 136.4) were selected for quantification of FGF-P. Optimized mass spectrometer parameters were applied to each pair of mass transitions to determine the highest ion pair abundance and signal response (Table 2). The total ion count mode achieved with these three mass transition pairs was used to determine the amount of FGF-P.

Liquid chromatography conditions, such as mobile phase composition, flow rate, and column were optimized. The aqueous mobile phase containing 0.05–2% v/v formic acid and 0.5–10 mM of ammonium formate was evaluated. When 0.1% formic acid was added to the aqueous phase, the result featured an easily interpreted high-intensity peak. For the organic phase, acetonitrile provided a better peak shape than methanol. Elution of both FGF-P and IS was achieved under gradient elution starting from 5% in the organic phase and increasing to 50% (Table 1). Various HPLC columns of different lengths, diameters, and particle sizes were tested to separate FGF-P and IS from the endogenous interference in extracted plasma samples. The Polaris C18-A, 5 μm, 50 × 3.0 mm column provided good peak shape, high peak response, consistent retention time for FGF-P and IS, and a shorter run time that was suitable to use as a separation phase for FGF-P when compared with other HPLC columns. The separation was performed with a flow rate of 0.4 mL/min. The run time of the chromatographic method was 12 min with retention times of approximately 6.3 and 6.0 min for FGF-P and IS, respectively (Fig. 4-B). A mixture of acetonitrile and water (50:50 v/v) was used as a needle wash solution to minimize carry-over.

We compared the PPT, LLE, and SPE extraction techniques to determine the best method for sample preparation. The criteria used to judge the most effective method were: (1) consistent recovery of the FGF-P extracted from plasma samples; (2) the LLOQ level. Table 5 details FGF-P recovery after rat plasma extraction with each technique. The LLE

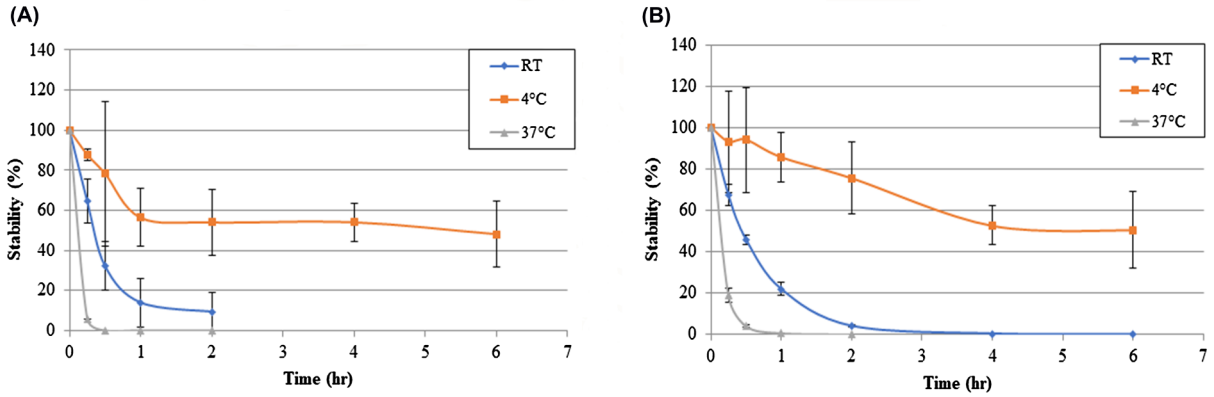

Fig. 2. Effect of temperature on stability of FGF-P in plasma at (A) low concentration, 6 ng/mL and (B) high concentration, 450 ng/mL.

Table 4
Stability of FGF-P in plasma under various conditions (Mean ± SD, n = 3, Mean).

Condition	Stability (%)						
	0 h	0.25 h	0.5 h	1 h	2 h	4 h	6 h
FGF-P (6 ng/mL) without inhibitor							
4 °C	100	87.77 ± 2.94	78.33 ± 36.02	56.35 ± 14.52	53.97 ± 16.53	54.02 ± 9.54	48.14 ± 16.66
Room temperature	100	64.43 ± 10.96	32.04 ± 12.17	13.81 ± 12.02	9.22 ± 9.48		
37 °C	100	5.89					
FGF-P (450 ng/mL) without inhibitor							
4 °C	100	93.13 ± 24.39	94.02 ± 25.32	85.65 ± 12.02	75.54 ± 17.41	52.68 ± 9.37	50.53 ± 18.32
Room temperature	100	67.35 ± 5.21	45.81 ± 2.31	21.85 ± 3.09	4.07 ± 0.68	0.29 ± 0.03	
37 °C	100	18.90 ± 3.18	4.09 ± 0.55	0.59 ± 0.03			
FGF-P (450 ng/mL) at room temperature with inhibitor							
Halt™ protease inhibitor cocktail	100	81.76	44.86	40.70	60.15		
Halt™ protease inhibitor cocktail + 1 mM PMSF	100	80.38	71.52	60.92			
1 mM PMSF	100	61.74	31.66	9.83			
1 mM PMSF + 5 mM NaF + 10% TCA	100	39.54	24.40	14.42			
1 mM PMSF + 5 mM NaF + 2 mM EDTA	100	92.23	50.07	18.10			
2 mM PMSF	100	51.53	26.04	12.59			
5 mM NaF + 2 mM EDTA	100	52.85	23.19	4.84			
Sodium citrate	100	36.19	26.91	19.99			
Precipitation with acetonitrile containing 2% formic acid							
4 °C, 7 days	98.63 ± 2.03% (6 ng/mL)				96.47 ± 11.36% (450 ng/mL)		

technique had an inadequate recovery level of < 1%, whereas SPE ranged from 3 to 50%; however, recovery with SPE was concentration dependent. Moreover, drying caused increased variability with both the LLE and SPE methods. Extraction and variability were better with the PPT method, and PPT was more efficacious with acetonitrile than with methanol, acetone, hydrochloric acid, or trichloroacetic acid. Extraction efficiency was then improved by optimizing the ratio of precipitant to plasma and pH. The best recovery was seen with acetonitrile containing 2% formic acid as a precipitating solvent, which resulted in ≈65% of FGF-P with minimal concentration sensitivity. Supernatant was taken and extracted using LLE technique comprise of dichloromethane as an organic solvent. This procedure of PPT with LLE helped in achieving LLOQ at 2 ng/mL. Therefore, the present study relied upon PPT using acetonitrile containing 2% formic acid followed by dichloromethane extraction for sample preparation. The entire extraction procedure was conducted at 4 °C to avoid degradation. IS choice being a very critical step in the method development. Ideally stable labeled isotope or structural analogue is essential for the bioanalysis. However, in the early discovery preclinical study, it is difficult to obtain stable label isotope. In the absence of stable isotope labeled or structural analogue, several compounds were tried as IS in initial stage of drug discovery method development. Finally, vancomycin (glyopeptide antibiotics) was selected as an IS because of ionization efficiency, extraction behavior, and other chromatographic similarities.

3.5. Method validation

The developed method was fully validated according to US-FDA guidance.

3.5.1. Selectivity

Selectivity of the method was evaluated by analyzing six blank plasma sample of different appropriate biological sources. The chromatograms in Fig. 4 show that FGF-P and IS could be detected separately from endogenous compounds. There were no significant interference peaks at the same retention time as FGF-P or IS.

3.5.2. Carry over

Injection of the blank sample after the highest concentration of calibration sample did not lead to a significant peak at the same retention time as FGF-P or IS.

3.5.3. Linearity and sensitivity

Six validated runs were performed with the calibration curve concentration range of 2–500 ng/mL FGF-P. All calibration curves showed good linearity with an average coefficient of determination (r^2) is 0.9966 ± 0.0024 (Fig. 5). The LLOQ for FGF-P (defined as the lowest concentration used for the calibration curve) was achieved at 2 ng/mL with signal to noise (S/N) ratio 12.

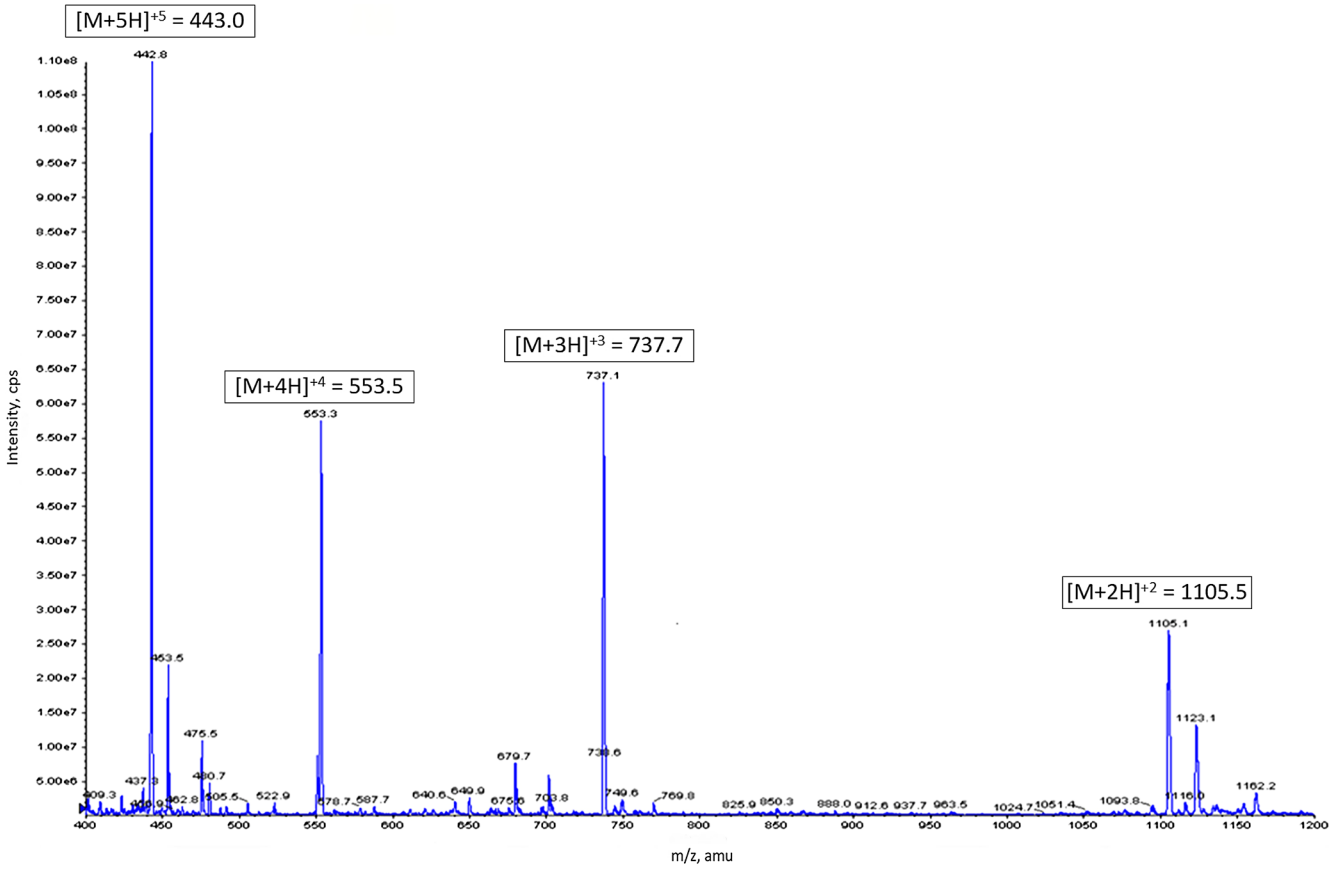

Fig. 3. Mass spectrum of FGF-P.

3.5.4. Accuracy, precision, and recovery

The within-run and between-run accuracy and precision were determined at LLOQ and with three different QC samples (n = 5 each concentration) for 6 runs. Table 6 summarizes the within-run and between-run accuracy and precision of this bioanalytical method. The within-run accuracy and %CV of the method were 87.97–111.00% and 2.38–12.85%, respectively. At the LLOQ level, the accuracy was 94.53–115.00% with a %CV less than 14%. The between-run accuracy was 97.35–107.15% with a %CV less than 11% in all concentrations. The mean recoveries of FGF-P extracted from plasma for QCL, QCM, and QCH were 88.14, 99.95, and 101.73%, respectively (Table 7). The results indicate that the method exhibited acceptable within-run and between-run accuracy and precision.

3.5.5. Dilution integrity

The accuracies of samples after 2, 20, and 50-fold dilution were 91.88 ± 11.47, 94.28 ± 8.86, and 106.73 ± 7.40%, respectively. The precision was less than 12.5% at all dilution factors. Thus, method was sufficiently validated to analyze FGF-P concentration above 500 ng/mL.

3.5.6. Matrix effect

No significant matrix effect was observed in all of the six lots of plasma at tested QC levels. The matrix effect of the method was within acceptable ± 15% at the QCL and QCH concentration levels.

3.5.7. Stability

The stability of FGF-P was evaluated using triplicates of low-QC and high-QC samples (QCL and QCH) under various storage conditions.

For short-term stability, acetonitrile containing 2% formic acid was added into QC samples and the supernatant was separated and stored on ice for 9 h. As shown in Table 8, the short-term stability of FGF-P was 106.77 and 85.80% for QCL and QCH, respectively. The long-term storage at 4 °C for 7 days and 1 month was between 96.47 and 98.63%. The stability of supernatant after storage at −80° C for 1 month was between 86.13 and 102.67%. Following, three freeze/thaw cycles, FGF-P samples had a stability within ± 15% of the nominal concentrations. After FGF-P samples were extracted and left in the autosampler up to 24 h, stability was 89.00%. FGF-P stock solution at a concentration of 1000 µg/mL in water and IS stock solution at 1000 µg/mL in 50% methanol were stable up to 1 month after storage at 4° C compared to the freshly prepared stock solutions. The stability of FGF-P and IS stock solution were 104.00 ± 7.82% and 93.22 ± 2.18%, respectively. The working solution stored at 4° C was stable up to 1 month with 97.00% stability. These findings not only reveal the optimum stability for the prepared stock and working solutions throughout the period intended for routine use but also indicate that precipitated FGF-P plasma samples can be stored at −80 °C without leading to stability-related problems for the duration of the study.

3.6. Pharmacokinetic study

Fig. 6 shows the mean plasma concentration-time profile of FGF-P after IV administration. The concentration of FGF-P dramatically decreased in the first period; thereafter, the drug concentration gradually decreased and could be quantified up to 300 min. Table 9 outlines the pharmacokinetic parameters calculated by non-compartmental analysis. The initial concentration (C_0) was 70.4 ± 16.9 µg/mL. The AUC and CL were 86.2 ± 16.1 µg*min/mL and 119.0 ± 24.4 mL/min/kg, respectively. The half-life was 13.3 ± 2.4 min.

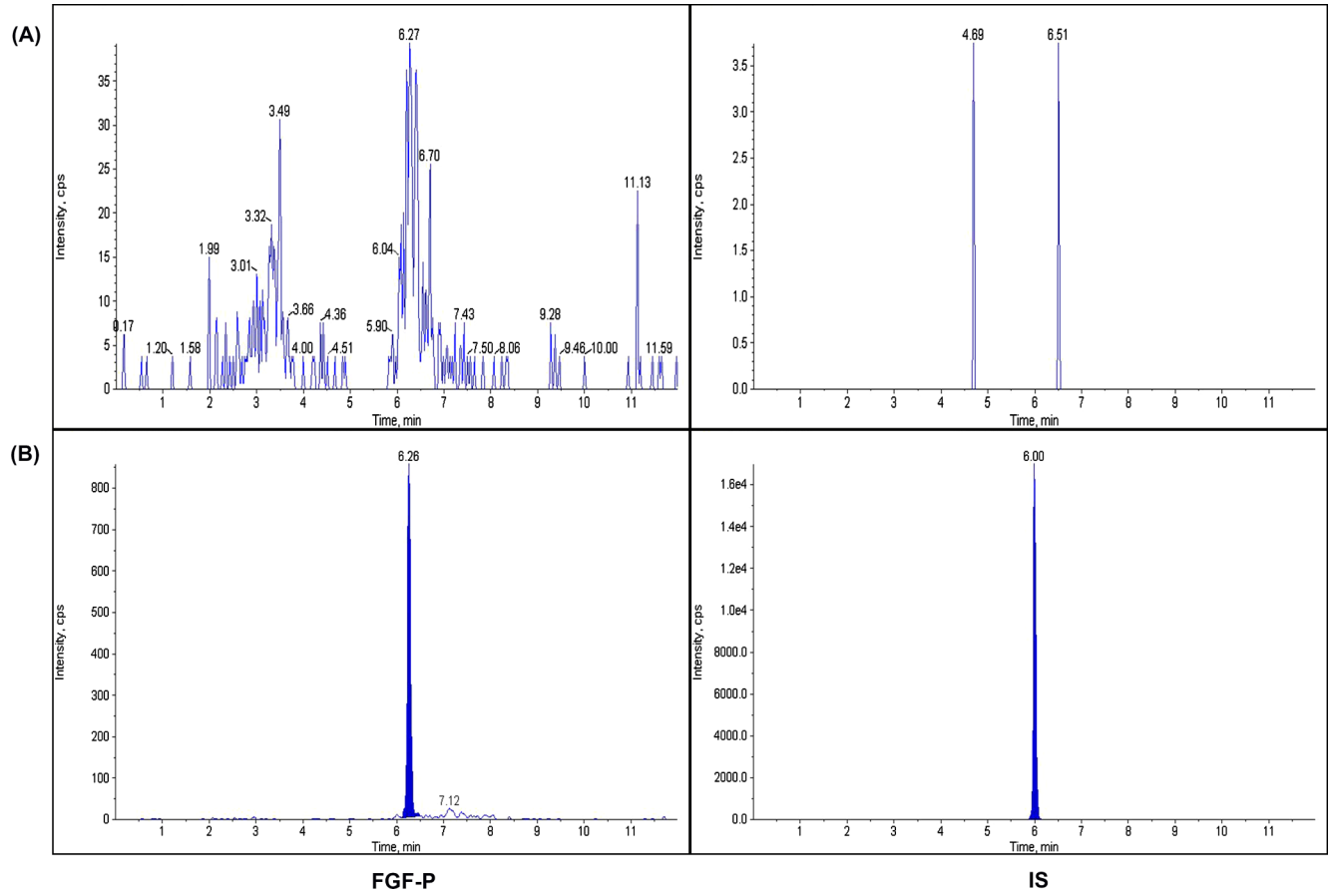

Fig. 4. Representative chromatogram of (A) extracted blank plasma sample and (B) extracted spiked plasma sample, FGF-P 50 ng/mL and IS 62.5 ng/mL (retention time of FGF-P 6.26 min, IS 6.00 min).

Table 5
Summary of sample extraction methods for FGF-P.

Extraction method	Result
LLE	
Ethyl acetate	< 1% Recovery
Toluene	< 1% Recovery
Hexane	< 1% Recovery
SPE	
C8 (Bond elute C8, Algilent Technologies)	~50% Recovery
C18 (Bond elute C18, Algilent Technologies)	~20–30% Recovery
C18 HLB (Oasis HLB, Waters)	~25 %Recovery
Polymeric (Strata X, Phenomenex)	~45% Recovery
Cationic (Strata-XC, Phenomenex)	~30% Recovery
Weak Cationic (Oasis WCX, Waters)	~3% Recovery
Mixed mode ion exchange (Oasis MCX, Waters)	~20% Recovery
PPT	
Methanol, acetone, hydrochloric acid, trichloroacetic acid	Very low recovery
Acetonitrile	~3% Recovery
Acetonitrile containing formic acid (1–10%)	~0–60% Recovery
Acetonitrile containing ammonium solution (2%)	~8% Recovery
Acetonitrile containing 2% formic acid followed by dichloromethane extraction	~65% Recovery

4. Discussion

4.1. Adsorption study

Based on inconsistent observations seen in animal experiments, we were concerned that FGF-P might adsorb to container materials, thereby leading to inadequate dosing. Indeed, there was adsorption, but we used low-retention polypropylene tubes to minimize this effect. In a previous study, Goebel-Stengel also found that polypropylene tubes were superior or equal to polystyrene for all peptides studied. Higher recovery was observed for peptide YY, glucagon-like peptide-1, and sulfated cholecystokinin-8 in polypropylene tubes [24]. Moreover, no evidence of adsorption of bivalirudin to polypropylene vials was detected during a series of transfers [11]. Low-retention polypropylene microcentrifuge tubes not only minimized the adsorption effect throughout these FGF-P studies but also allowed us to overcome inconsistencies in our animal studies.

4.2. Stability of FGF-P in solution

We found that FGF-P was more stable in organic solvent and solvent mixtures in an acidic environment. FGF-P was less stable in methanol. These results indicate the effect of solvents on the stability of FGF-P. The selected solvent mixture of acetonitrile with 0.1% formic acid had no stability issues.

4.3. Stability of FGF-P in plasma

Various methods have been used to stabilize peptides in plasma, including using low temperatures, using enzyme inhibitors (protease and phosphatase inhibitors) [15,25–27], adding organic solvent to quench enzyme activity, and adjusting the pH of the sample to acidic conditions [7]. In this study, enzyme inhibitors did not prevent the degradation of FGF-P. We chose these protease inhibitors based on potential protease cleavage sites identified using the PeptideCutter tool provided by ExPASy, SIB Swiss Institute of Bioinformatics [28]. Based on that analysis, we identified at least fourteen enzymes that can cleave

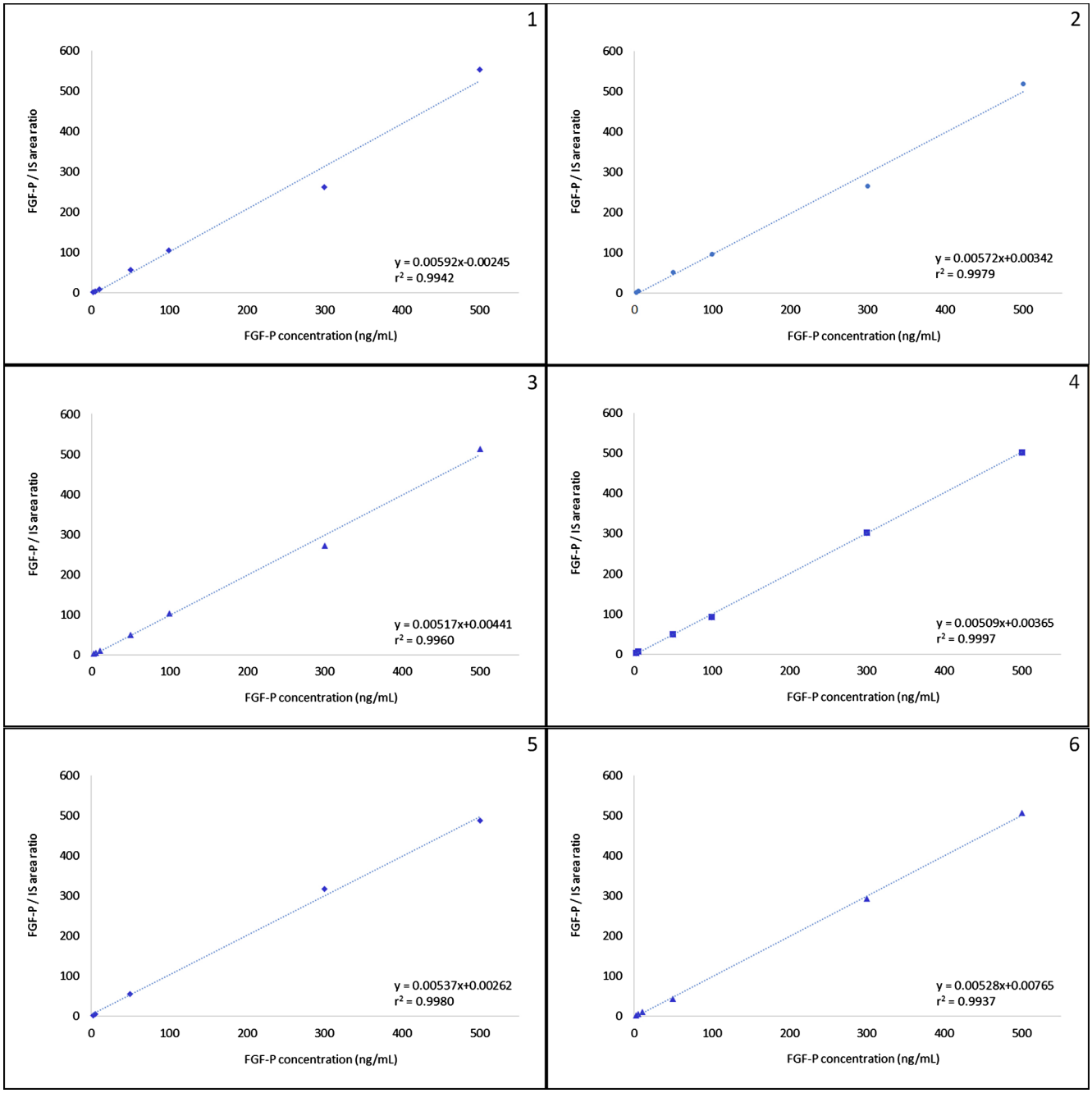

Fig. 5. Calibration curve of six validation batches.

FGF-P; thus, complete inhibition of protease activity is unlikely using any cocktail of specific inhibitors. Indeed phenylmethylsulfonyl fluoride (PMSF), a commonly employed serine protease inhibitor, requires at least 1 h for maximum effect, during which time substantial degradation has already occurred [29]. In this study, precipitating plasma samples with acetonitrile containing 2% formic acid effectively stabilized FGF-P. The supernatant stability test demonstrated that FGF-P was stable in the supernatant during sample preparation and storage. As with most protein and peptide studies, samples left on ice (4 °C) are more stable than samples left at room temperature [15,27].

4.4. Method development

LC-MS/MS has been used extensively as a quantitative technique in the analysis of protein and peptide compounds with high sensitivity and selectivity [8,9,11,16–18]. LC-MS/MS generally provides higher specificity than ELISA and can better distinguish the peptide from its fragmentation or degradation products [15]. It is also difficult to produce a specific antibody to FGF-P given its short length and minimal 3D structure.

Chromatographic conditions, including various combinations of acetonitrile/methanol, and acid additives on different reverse phase columns to optimize the liquid chromatographic condition were tested. In the present method, acetonitrile was used as an organic mobile phase due to its superior elution and peak shape. In the aqueous phase, formic acid is a common mobile phase modifier for LC-MS/MS analysis. Although the combination of formic acid and ammonium formate has been reported to improve peptide separation and load [30], we found that adding formic acid (0.1%) increased the response signal of FGF-P while ammonium formate did not. A Polaris C18 HPLC column showed

Table 6
Within- and between-run accuracy and precision of bioanalytical method at different concentrations of FGF-P (n = 5 for within-run and n = 30 for between-run).

	2 ng/mL	6 ng/mL	200 ng/mL	450 ng/mL
Within-run				
Run 1				
Mean ± SD	2.19 ± 0.14	6.08 ± 0.33	198.33 ± 21.13	450.00 ± 28.58
Accuracy (%)	110.00	100.28	99.00	99.87
CV (%)	6.56	5.36	10.55	6.32
Run 2				
Mean ± SD	1.89 ± 0.11	6.68 ± 0.16	185.60 ± 10.88	415.00 ± 27.73
Accuracy (%)	94.53	111.00	92.82	92.23
CV (%)	5.93	2.38	5.82	6.64
Run 3				
Mean ± SD	2.09 ± 0.29	6.36 ± 0.22	197.40 ± 14.36	440.20 ± 31.16
Accuracy (%)	104.67	106.00	98.74	97.84
CV (%)	14.08	3.77	7.37	7.18
Run 4				
Mean ± SD	2.21 ± 0.26	5.94 ± 0.34	192.25 ± 12.82	450.80 ± 45.81
Accuracy (%)	110.30	98.93	96.05	100.20
CV (%)	11.79	5.57	6.64	10.30
Run 5				
Mean ± SD	2.15 ± 0.21	6.01 ± 0.67	184.00 ± 9.54	412.00 ± 21.93
Accuracy (%)	107.37	100.08	92.10	91.60
CV (%)	9.74	10.98	5.29	5.29
Run 6				
Mean ± SD	2.30 ± 0.15	5.28 ± 0.68	220.00 ± 10.44	450.67 ± 33.50
Accuracy (%)	115.00	87.97	109.67	100.10
CV (%)	6.79	12.85	4.50	7.29
Between-run				
Mean ± SD	2.14 ± 0.22	6.04 ± 0.57	195.26 ± 16.31	438.09 ± 33.83
Accuracy (%)	107.15	100.57	97.58	97.35
CV (%)	10.30	9.29	8.26	7.74

Table 7
Relative recovery of FGF-P (Mean ± SD, n = 3).

	FGF-P area (extracted plasma sample)	Average FGF-P area (extracted standard solution)	Recovery	
			(%)	Mean ± SD
6 ng/mL	1850	2235	82.77	88.14 ± 8.53
	1870		83.67	
	2190		97.99	
200 ng/mL	60,500	63,067	95.93	99.95 ± 3.54
	64,700		102.59	
	63,900		101.32	
450 ng/mL	130,000	135,000	96.30	101.73 ± 5.20
	138,000		102.22	
	144,000		106.67	

Table 8
Stability of FGF-P under various conditions (Mean ± SD, n = 3).

Condition	Duration	Stability (%)	
		6 ng/mL	450 ng/mL
Plasma sample			
Freeze-thaw cycles	3rd cycle	110.67 ± 1.53	94.18 ± 8.71
Autosampler	24 h	89.32 ± 8.04	88.80 ± 11.06
Short-term on ice	9 h	106.77 ± 7.35	85.80 ± 4.83
Long-term at 4 °C	1 month	96.43 ± 10.61	96.63 ± 2.96
Long-term at −80 °C	1 month	102.67 ± 9.29	86.13 ± 8.85
Standard stock solution			
FGF-P at 4 °C	6 weeks	104.93 ± 7.82	
IS at 4 °C	6 weeks	93.22 ± 2.18	
Working solution			
FGF-P at 4 °C	1 month	97.00 ± 3.61	

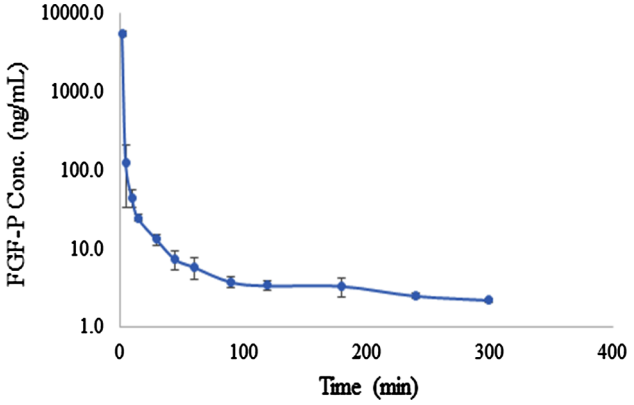

Fig. 6. Plasma concentration-time profile of FGF-P after 10 mg/kg, IV administration (mean ± SD, n = 3).

Table 9
Non-compartmental pharmacokinetic parameters of FGF-P after 10 mg/kg, IV administration (n = 3).

Parameter	10 mg/kg, IV dose
Elimination rate constant (1/min)	0.054 ± 0.011
$T_{1/2}$ (min)	13.3 ± 2.4
C_0 (μg/mL)	70.4 ± 16.9
$AUC_{(0-t)}$ (μg*min/mL)	86.2 ± 16.1
$AUC_{(0-\infty)}$ (μg*min/mL)	86.2 ± 16.1
CL (mL/min/kg)	119.0 ± 24.4
Vd (L/kg)	2.3 ± 0.7

the best sensitivity, efficiency, and peak shape. Thus, for overall method development, gradient elution with a mixture of 0.1% formic acid and acetonitrile using a 0.4 mL/min flow rate was the most effective liquid

chromatography condition for FGF-P analysis.

The sample preparation process remains a major challenge that can inhibit the determination of proteins and peptides in biological matrices. Certainly, the abundance of endogenous proteins and peptides in plasma and serum is difficult to eliminate due to the similarity of target analytes. Thus, we systemically compared the LLE, SPE, and PPT techniques. For LLE technique, we used different polarity index (PI) solvents (ethyl acetate PI = 4.4, toluene PI = 2.4, hexane PI = 0.1). Since FGF-P is a hydrophilic compound, low partition of FGF-P into the organic phase led to a low recovery. After optimization of each step, the best SPE cartridge was the C8 cartridge, which provided a recovery of up to 50%, but with great variability between runs. For the PPT technique, we evaluated many organic solvent compositions. Other groups have reported protein precipitation efficiency using acids, metal ions, organic solvent, and salts in rat plasma [31]. We observed high-protein precipitation efficiency when trichloroacetic acid was used as a precipitant. Acetonitrile was found to be a superior organic solvent at low volume ratios, whereas methanol and ethanol exhibited similar protein precipitation efficiency at volume ratios (plasma: precipitant) more than 1:2. Moreover, acetonitrile exhibit the lowest ionization suppression when using pure mobile phase solvent. While acetonitrile was the best precipitant, the recovery of FGF-P from rat plasma at the LLOQ was inefficient. Low recovery of FGF-P after precipitation with acetonitrile may be attributable to FGF-P binding to the precipitated protein pellet, particularly albumin [32]. A previous study found that optimization of protein precipitation effectively removed albumin when a combination of organic solvents and formic acid or trichloroacetic acid were used [33]. We used formic acid to improve FGF-P recovery. In order to achieve a low LLOQ, we used the PPT technique and concentrated the extracted sample with centrifuge evaporation or nitrogen evaporation. However, the recovery of FGF-P was low due to the exposure of the analyte to high temperatures during the drying process. The LLE with dichloromethane after PPT allowed us to concentrate the sample without exposing it to high temperatures. Thus, PPT followed by dichloromethane extraction was the most suitable sample preparation method for FGF-P analysis.

4.5. Method validation

Our validation results indicated that the method had good specificity and selectivity to quantify FGF-P in plasma without significant interference. The calibration curve ranged from 2 to 500 ng/mL of FGF-P with good linearity. The dilution integrity at 2, 20, and 50-fold dilution showed good accuracy and precision within acceptable criteria. Therefore, the samples with higher concentrations of FGF-P than the calibration range can be diluted to quantify the amount of FGF-P with good accuracy and precision. The stability results showed that FGF-P was stable in supernatant for at least 1 month when samples were stored at either 4 °C or −80 °C. Both the standard and working solutions were stable for at least 1 month. Moreover, the autosampler stability results revealed that processed samples were stable at 24 h, indicating that FGF-P should not exhibit stability problems in the final samples and should be stable for reinjection at 24 h.

4.6. Pharmacokinetic study

After IV injection, FGF-P reached systemic circulation and decreased very quickly with a short half-life and rapid elimination. Similarly, we discovered that FGF-P had poor *in vitro* stability of FGF-P in plasma. The likely cause is proteolytic hydrolysis by protease or peptidase enzymes, but there may also be adsorption onto target or non-target tissues.

5. Conclusions

The LC-MS/MS analytical method for determination of FGF-P was developed and validated and met the acceptance criteria in terms of selectivity, specificity, linearity, accuracy and precision, recovery, matrix effect and stability according to US-FDA bioanalytical guidance. The best container material for FGF-P handling was polypropylene, which reduced adsorption. The mixture of acetonitrile and water containing 0.1% formic acid at a ratio of 20:80 v/v was utilized to avoid the non-specific binding of FGF-P in matrix-free solution to the container. For stability of FGF-P in plasma, temperature ≤4 °C and precipitation with acetonitrile containing 2% formic acid prevented degradation of FGF-P. Addition of a single or a combination of enzyme inhibitors to plasma did not effectively prevent the degradation of FGF-P. The present method was specific, sensitive, precise, and accurate for the determination of FGF-P in rat plasma samples obtained from the pharmacokinetic study. The methods were validated in a rat plasma and confirmed the utility of the method for evaluation of FGF-P pharmacokinetics. FGF-P rapidly eliminated from the plasma after IV administration in the rat. Furthermore, the developed method is able to quantify concentration above 500 ng/mL with maximum of 50-fold dilution. In conclusion, this method should be useful for the determination of FGF-P in other subject species or determination of other similar peptides in the future.

Acknowledgements

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