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Research Article

Characterization and Quantification of an Fc-FGF21 Fusion Protein in Rat Serum Using Immunoaffinity LC-MS

Fengping Li,[1,5] Yan Weng,[2] Guodong Zhang,[3] Xiaogang Han,[3] Dongmei Li,[4] and Hendrik Neubert[1]

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ABSTRACT. The fusion of an Fc moiety to a therapeutic protein is widely applied as a half-life extension strategy. However, unlike monoclonal antibodies, Fc-fusion proteins have been shown to be more susceptible to protease-mediated catabolism. The resultant catabolites may still be pharmacologically active and therefore require characterization. We combined intact protein LC-MS and digestion LC-MS/MS methods to both characterize the biotransformation of the fusion protein, Fc-FGF21, and quantify the intact molecule and its major catabolites in rat serum. The digestion LC-MS/MS assay and intact protein LC-MS assay determined that there were four major catabolites formed *in vivo*: one amino acid (dC1), two amino acids (dC2), or three amino acids (dC3) clipped off from the C-terminus, and a truncated fragment. By 72 h post dosing, only 66% of the intact protein remained. The digestion method was developed with a sensitivity of 20 ng/mL—10 times more sensitive than the intact protein method at 200 ng/mL. While the digestion approach proved more sensitive, the intact LC-MS method was primarily employed for understanding the biotransformation of the Fc-FGF21 fusion protein in the rat *in vivo* study.

KEY WORDS: digestion; Fc-FGF21; immunoaffinity; intact protein; LC-MS.

INTRODUCTION

Fibroblast growth factor (FGF) 21 is an important metabolic regulator of glucose and lipid homeostasis (1,2). It is an endocrine hormone generated in the pancreas, liver, skeletal muscle, and brown adipose tissue (1). Administration of FGF21 in preclinical obese and diabetic animal models has shown promising pharmacological effects, such as improved insulin sensitivity, improved glucose tolerance, and reduced hepatic and serum triglyceride level (1). Therefore, FGF21 may have therapeutic potential for the treatment of obesity, diabetes, and fatty liver disease. Native FGF21 is a 19-kDa unglycosylated protein and the recombinant FGF21 has an *in vivo* half-life of only 30 min in mice and 2 h in monkey (3). In order to extend its half-life, FGF21 was fused to a human fragment crystallizable region (Fc). The total molecular weight of Fc-FGF21 (PF-06645849) is about 90 kDa with two FGF21 molecules fused to one FC via a peptide linkage shown in Fig. 1 (sequence available in Supplemental Table 1). Glycans are on the C-terminal asparagine of each engineered FGF21 molecule. Through optimization of the structure and sequence, the *in vivo* half-life of FGF21 has been significantly increased to about 72 h in rats and 200 h in cynomolgus monkeys (1). However, the half-life of active Fc-FGF21 is still much shorter than what might be expected from this class of biotherapeutics, suggesting additional clearance mechanisms that include the potential for *in vivo* catabolism.

Catabolism at the C-terminus of FGF21 has been reported to affect the bioactivity of Fc-FGF21 *in vivo* (4,5). Relevant catabolites from full-length Fc-FGF21with varying bioactivity are those that have one amino acid (dC1), two amino acids (dC2), or three amino acids (dC3) clipped off from the C-terminus. Thus, it is important to understand how much of these Fc-FGF21 forms are generated *in vivo*. This imposes bioanalytical challenges to develop a clear understanding of the protein biotransformation and to ensure the correct active forms are measured for correlation with efficacy and safety endpoints. Characterization of the biotransformation of a similar Fc-FGF21 RG (L98R and P171G) fusion protein was reported previously (4). Quantification

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[1] BioMedicine Design, Pfizer Inc., 1 Burtt Road, Andover, Massachusetts 01810, USA.
[2] BioMedicine Design, Pfizer Inc., 1 Portland St, Cambridge, Massachusetts 02139, USA.
[3] Pharmacokinetics, Dynamics and Metabolism, Pfizer Inc., 280 Shennecossett Road, Groton, Connecticut 06340, USA.
[4] Cardiovascular, Metabolic, and Endocrine Diseases (CVMED) Research Unit, Pfizer Inc., 1 Portland St, Cambridge, Massachusetts 02139, USA.
[5] To whom correspondence should be addressed. (e–mail: fengping.li@pfizer.com)

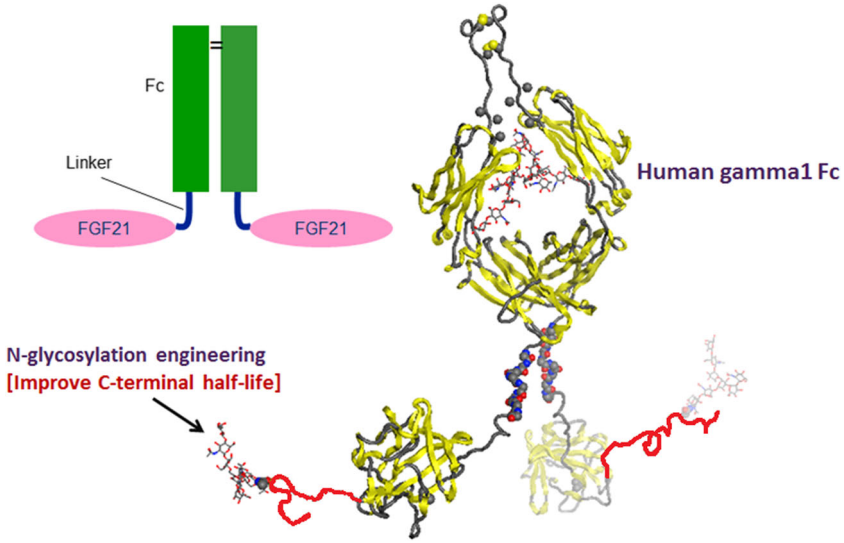

Fig. 1. Schematic, three-dimensional structure of Fc-FGF21. Two FGF21 molecules are fused to Fc through peptide linkers highlighted in red

results from three different LBA methods were compared and methods were applied to a native FGF21 fused to Fc and a mutant FGF21 that had been engineered to reduce aggregation and increase proteolytic stability. Triple mutation RGE (L98R, P171G, and A180E) of Fc-FGF21 further protected the protein from the carboxypeptidase-like proteolysis (6). In addition, LC-MS was used to characterize the reduced proteins and enzymatically derived peptides (4,6).

Bioanalytical approaches that couple immunoaffinity enrichment with liquid chromatography-mass spectrometry (IA-LC-MS) detection provide high measurement specificity and wide dynamic range for detecting and quantifying peptides and proteins. In such an assay format, an antibody with reasonable selectivity is used to enrich the protein analyte. The assay then combines HPLC separation with MS detection of specific *m*/*z* values of either the protein analyte or enzymatically derived signature peptides. The analysis of signature peptides from the digested protein typically improves assay performance, such as sensitivity, compared with the intact protein due to better ionization of the smaller peptides in the mass spectrometer (7–9). In addition, fragment ions in tandem mass spectrometry of the signature peptides provide additional selectivity. LC-MS for the analysis of intact proteins without enzymatic digestion has been used mainly to characterize proteins and their catabolites (4,6,10). However, some recent pivotal studies also showcase the use of intact protein LC-MS for quantification of high molecular weight (MW) proteins (11–13).

In this report, an IA-LC-MS method was used to characterize Fc-FGF21 and its major catabolites as well as to quantify the intact Fc-FGF21 from rat serum. Since one Fc-FGF21 molecule contains two FGF21 domains attached to one humanized Fc fragment, Fc-FGF21 was reduced at the hinge region to produce a protein of about 45 kDa. Therefore, the following intact protein LC-MS analysis refers to the reduced Fc-FGF21 (amino acids 1–414). The second assay, an IA-LC-MS/MS method, measures enzymatically derived peptides as surrogates for the parent molecule and its catabolites. The method was developed in rat serum to quantify the C-terminal peptides generated from Asp-N digestion of full-length Fc-FGF21 and its C-terminal catabolites.

MATERIALS AND METHODS

Materials and Reagents

Rat serum was purchased from BioIVT (Westbury, NY). Organic solvents and chemicals were purchased from Fisher Scientific (Hampton, NH). All mobile phases were prepared with LC-MS-grade solvents. PNGase F and Dynabead MyOne Streptavidin T1 were from Thermo Fisher Invitrogen (Carlsbad, CA). Asp-N was from Roche (Indianapolis, IN). Biotinylated anti-human Fc mAb was purchased from Jackson ImmunoResearch (West Grove, PA). The C-terminal isotope-labeled peptide (DSTGR^SP^SYA^S, R^=R+10, P^=P+6, and A^=A+4) was synthesized at New England Peptide (Gardner, MA). Phospho/total ERK1/2 whole cell lysate kit was from Meso Scale Diagnostics (Rockville, MD) and DMEM/F12 culture media were from Thermo Fisher Scientific (Waltham, MA). Fc-FGF21, anti-Her2 mAb, and *in vivo* rat samples were obtained from Pfizer.

Collection of Rat Serum

Two rats were dosed with 10 mg/kg of Fc-FGF21 intravenously (IV). Serum samples were carefully collected at 0.25, 2, 24, 48, and 72 h and were stored at −80°C until used for analysis. All animal studies were conducted in accordance with animal care and use protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Pfizer, Inc.

In Vitro Activity Assay of Fc-FGF21 Catabolites

Phospho/Total ERK1/2 assay was performed with human adipocyte stem cells (hASC). Fc-FGF21 catabolites were tested across three hASC culture plates and pERK/tERK

plates (Meso Scale Diagnostics K15107D). Biological replicates were 3 per dose and no technical replicates. Compounds were serially diluted in culture media DMEM/F12 with 0.1% BSA. Each well has two assay spots (antibody spots, phosphor ERK and total ERK), and the results are the percentages of phosphorylated ERK on total ERK.

Preparation of Standards and Quality Controls

In the digestion method, Fc-FGF21 stock solution was spiked into rat serum and standards were prepared at 0.02, 0.04, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/mL. Four QC levels were prepared at 0.02, 0.06, 0.6, and 8 μg/mL of Fc-FGF21 spiked into naive rat serum.

In the intact protein method, standards were prepared at 0.2, 0.5, 1, 2, 5, 10, 20, and 40 μg/mL of Fc-FGF21. Five quality control (QC) levels were prepared at 0.2, 0.6, 1.5, 8, and 40 μg/mL. All of the standards and QCs were prepared in naive rat serum.

Sample Preparation for Analysis of Fc-FGF21 C-Terminal Peptides

Rat serum samples (5 μL) were diluted 50-fold with rat control serum and 50 μL of the diluted sample was used for analysis. Biotinylated anti-human-Fc mAb at 5 μL of 1 mg/mL was used per sample in a plate. PBS (100 μL) was added to each sample before plate incubation at RT for 1 h with shaking.

Dynabeads MyOne Streptavidin T1 were washed with PBST and PBS separately. Then, 35 μL of the washed beads was used per sample. The sample plate was incubated at RT for 40 min with shaking. The beads were washed with 300 μL of PBST (× 1) and PBS (× 2) separately, and then reconstituted in 50 μL PBS. For on-bead degylcosylation of Fc-FGF21, 3.0 μL of PNGase F was added per sample. The plate was incubated at 37°C for 2 h. Asp-N was dissolved in deionized water to make a 0.01 μg/μL solution, and 5 μL of the solution was added per sample for on-bead digestion. The incubation was at RT for 1 h with shaking. After the incubation, the beads were settled on a magnet and the supernatant was transferred to a new plate. Stable isotope-labeled C-terminal peptide used as an internal standard was prepared in deionized water to 10 μg/mL and 10 μL was added per sample. Twenty-five microliters of the final solution was injected onto LC-MS for analysis.

Peptide Quantification Using LC-MS/MS

Peptide quantification using selected reaction monitoring (SRM) was performed on an Acquity UPLC system (Waters, Milford, MA, USA) coupled to an AB Sciex API 5500 for one of the accuracy and precision runs, or on an Agilent 1200 HPLC coupled to an API 6500 QTrap mass spectrometer equipped with an electrospray ionization source for two accuracy and precision runs and sample analysis. Peptides were separated on an Echelon C18 column, 1.7-μm particle size, 50 × 2.1 mm at 60°C. The mobile phases were 0.2% DMSO in 0.1% formic acid in water (A) and 0.2% DMSO in 0.1% formic acid in acetonitrile (B). The HPLC flow rate was 300 μL/min. Gradient conditions were as follows: 99% mobile phase A decreasing to 80% at 2 min, to 40% at 4 min, and to 10% at 5 min. Optimal conditions of mass spectrometric settings were ionspray voltage (IS) 1000, temperature 150°C, ion source gas (GS1) 60, and ion source gas (GS2) 60. Peptide MS/MS conditions are listed in Supplemental Table 2. The C-terminal peptide SRM transitions were identified using Skyline. The data were processed using MultiQuan 3.0.2 with a linear regression weighting of $1/x^2$.

Sample Preparation for Analysis of Reduced Fc-FGF21

For intact protein quantification and characterization of Fc-FGF21 catabolism, 5-μL aliquots of the rat serum samples were diluted 10-fold using control rat serum. To 50 μL of the diluted samples was added 50 μL of 2 μg/mL of anti-Her2 mAb in PBS as an internal standard (IS) for quantification. Fc-FGF21 and humanized anti-Her2 mAb are both captured by the anti-human Fc mAb, and thus the IS is incorporated in the IA step in the response normalization. After reduction, the reduced Fc-FGF21 and the mAb heavy chain coelute from a capillary C4 column; hence, both are analyzed in the same deconvolution spectrum window. PNGase F (10 units/μL) was diluted in PBS and final 3 units of PNGase F in 50 μL PBS were then added to each sample. The sample plate was incubated at 37°C for 2 h with shaking.

Following deglycosylation, 5 μL of 1.0 mg/mL of biotinylated anti-human Fc mAb was added to each rat serum sample. The plate was incubated at RT for 1 h with gentle shaking. MyOne Streptavidin T1 Dynabeads were prewashed with PBST and PBS, and then reconstituted in PBS. Then, 35 μL of the washed beads was used per sample. The plate was incubated at RT for 40 min with shaking. The beads were then washed with 300 μL of PBST (× 2), PBS (× 2), and deionized water (× 1) separately using a STAR liquid handling robot (Hamilton, Reno, NV). The elution was accomplished with 35 μL of acetonitrile:water (15:85, *v*/*v*) containing 0.6% (*v*/*v*) formic acid per sample for 15 min at RT with gentle shaking. The beads were then settled on a magnet and the supernatant was transferred to a clean LoBind plate (Eppendorf, Hauppauge, NY). Reduction was performed using 5 μL of 200 mM TCEP per sample with a 20-min incubation at RT and 5 μL was injected onto LC-MS.

Capillary UPLC-TOF System

Analysis of the intact proteins was performed on an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Waters Xevo G2 quadrupole time-of-flight (QTOF) mass spectrometer. Proteins were separated on an Acquity BEH 300 C4 column, 1.7-μm particle size, 300 μm × 50 mm at 85°C. Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 12 μL/min. Gradient conditions were as follows: 95% mobile phase A was maintained for 0.8 min, then decreased to 75% at 3 min, and to 10% at 7 min.

Signal collection time on the Xevo G2 QTOF (Waters, Milford, MA, USA) was 3.0 to 6.0 min. The *m*/*z* range was 725–1600. The TOF was under sensitivity mode and scan time was 1 s. The MS tune parameters were 3.3 kV capillary voltage, 55 sampling cone, 80 source offset, 150°C source temperature, and 280°C desolvation temperature.

TOF Data Processing

The TOF MS data were deconvoluted using Biopharmalynx 1.3.3 at the time window of 3.85–5.0 min. Background threshold

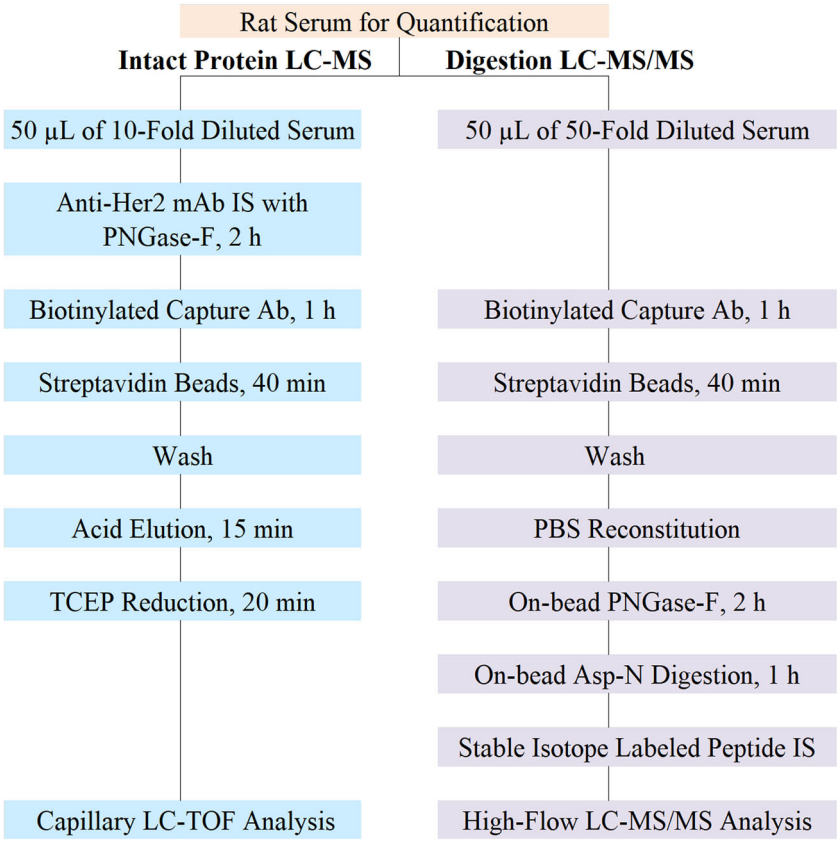

Fig. 2. Flowchart of intact protein and digestion LC-MS methods for Fc-FGF21 analysis

was 10%. Background polynomial was set to 10. Spectrum smooth was 3 channels and 3 smooths using Savitzky Golay. Deconvoluted peak intensities were recorded and peak intensity ratios to the IS were used for quantification in GraphPad Prism version 7.04. Line standard curve model was used to interpolate the data with a weighting of $1/y^2$.

RESULTS

The workflows for the intact protein and digestion LC-MS methods are summarized in Fig. 2. These two methods used different internal standards for normalization: a humanized anti-Her2 mAb for the intact protein method and a stable isotope labeled peptide for the digestion method. Acid elution was used for the intact protein method, while the peptide method used on-bead digestion.

Development of Digestion LC-MS/MS Method

The sample preparation of the digestion method was optimized to be within 5 h before LC-MS/MS analysis. Adding 0.2% DMSO in mobile phases enhanced the peptide signals by ~2-fold (Supplemental Fig. 1). Dithiothreitol (DTT) and iodoacetamide (IAA) were not used for disulfide reduction and alkylation in the final assay since the C-terminal peptide sequences did not contain any cysteine and adding DTT and IAA did not increase the peptide signal (Supplemental Fig. 2). RapiGest did not improve digestion efficiency and was not used in the experiment (Supplemental Fig. 3). Asp-N was a better enzyme than Glu-C in generating C-terminal dC0 peptide (Supplemental Fig. 4).

Different incubation times (1, 2, 4, or 20 h) and incubation temperatures (RT or 60°C) were tested (Supplemental Figs. 5 and 6). One-hour incubation at RT yielded the best result when compared with other conditions. The intensity of the C-terminal peptide was lower when PNGase F and Asp-N were added simultaneously (Supplemental Fig. 7) rather than separately. Results from on-bead or off-bead digestion were also compared (Supplemental Fig. 8). The data showed that after IP, on-bead digestion presented a stronger signal than off-bead digestion. Biotin-Ab IP first followed by deglycosylation and digestion yielded the best results (Supplemental Fig. 8). Overall, for the digestion LC-MS/MS method, the best conditions were to exclude DTT reduction and IAA alkylation, but include 0.2% DMSO in each mobile phase, PNGase F deglycosylation first for 2 h followed by Asp-N digestion at room temperature for 1 h. The assay flowchart is shown in Fig. 2.

Characterization of Catabolites by Digestion LC-MS/MS Method

C-terminal peptides were generated from Asp-N digestion. They are specific to the fusion protein Fc-FGF21 and do not occur anywhere else in the rat proteome. The peptide dC0 [404–414] was used as a surrogate for the intact, unmodified protein. Other catabolized peptides including dC1 [404–413], dC2 [404–412], and dC3 [404–411] were monitored simultaneously in this method.

Extracted ion chromatograms of C-terminal peptides with or without catabolism are shown in Fig. 3 for the rat serum sample taken 72 h post dosing. The peptides dC0 [404–414], dC1 [404–413], and dC2 [404–412] had similar retention times. However, the catabolized peptide [404–411] seemed to be more polar resulting in quicker column elution.

Characterization of Catabolites Using Intact Protein LC-MS Method

In rats, catabolites of Fc-FGF21 with cleavage of C-terminal amino acids were observed in deconvoluted spectra from LC-TOF data acquisition (Fig. 4). The control sample spiked with Fc-FGF21 had only one major peak at 45,052 Da [1–414]. In rats with 10 mg/kg of intravenous (IV) dosage, catabolized proteins were observed with one amino acid cleaved [1–413] (44,967 Da, Δmass 85), two amino acids cleaved [1–412] (44,898 Da, Δmass 154), and a truncated catabolite [1–384] (42,081 Da, Δmass 2971 Da). As determined in an activity assay, the catabolite [1–413] retains approximately 50% bioactivity of the intact Fc-FGF21, 8% for [1–412], and no bioactivity for [1–411] and the truncated form [1–384] (Supplemental Fig. 9). The catabolite [1–411] was not observed in these rat samples likely because it was below the limit of detection of the assay.

The percentage of each catabolite was calculated based on its peak intensity in the deconvoluted spectra. At 72 h post dosage, the intact molecule was 50.9% of the total catabolites, 12.4% for [1–413], 6.4% for [1–412], and 30.4% for the truncated catabolite [1–384]. This is a relative measurement because the ionization efficiency of each

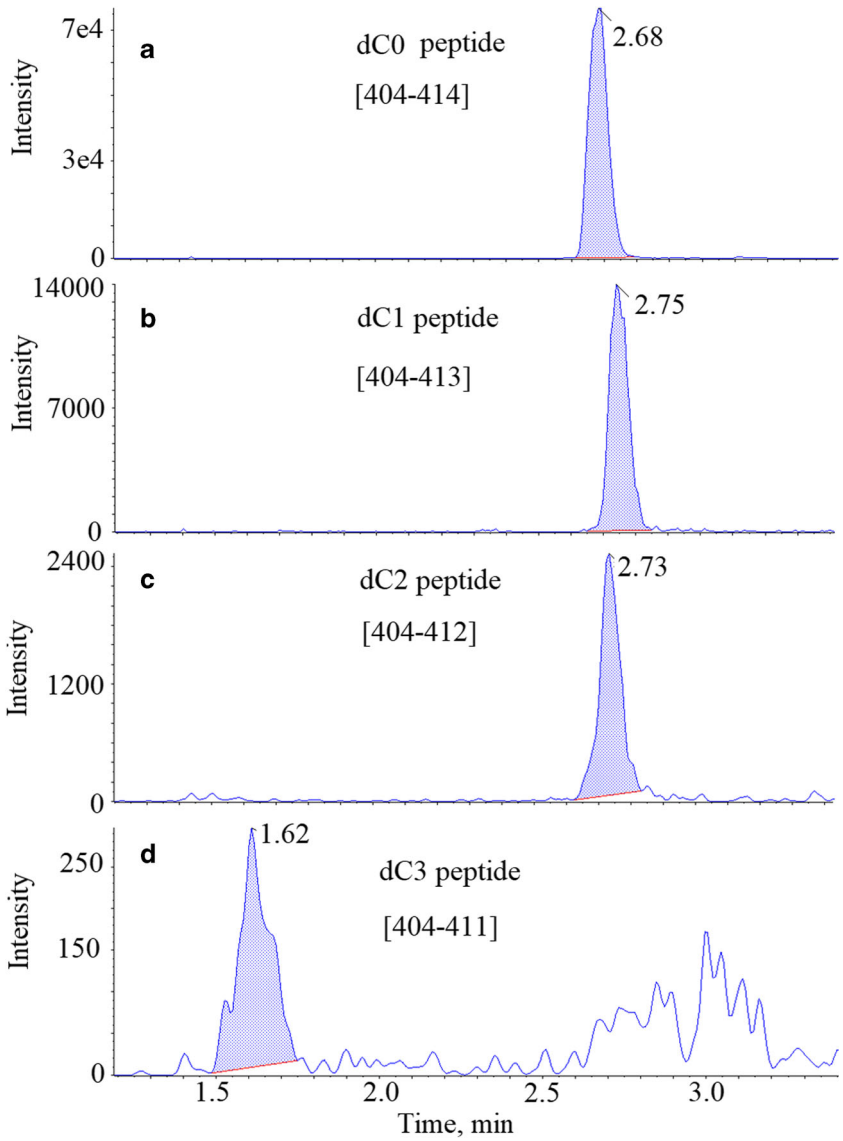

Fig. 3. Extracted ion chromatograms with lead SRM transition for rat serum sample 72 h after dosing. **a** Full-length C-terminal peptide dC0 [404–414], DSTGRSPSYAS. **b** dC1 peptide [404–413], DSTGRSPSYA. **c** dC2 peptide [404–412], DSTGRSPSY. **d** dC3 peptide [404–411], DSTGRSPS

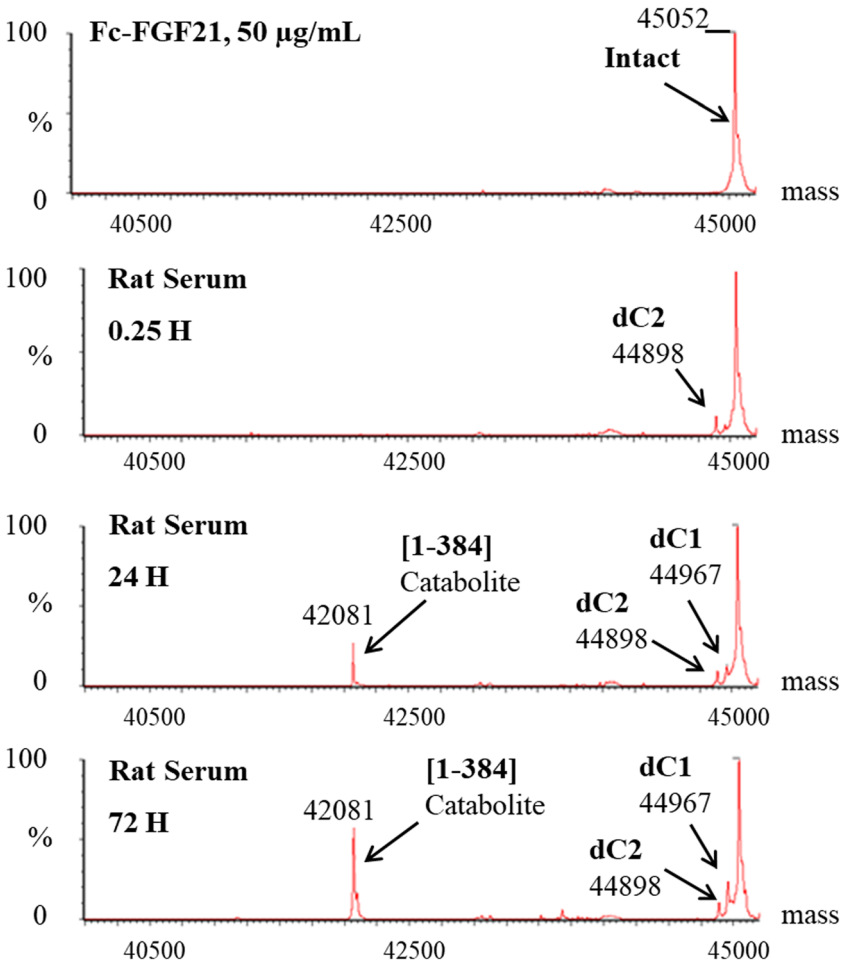

Fig. 4. Fc-FGF21 in control sample and catabolite profiling in rat serum *in vivo* at 0.25, 24, and 72 h post dosage

catabolite is unknown and could be different. Overall, the only pharmacologically active catabolite [1–413] was about 19.6% of the total active moieties including the intact protein [1–414] after 72 h dosage in rat (Fig. 4).

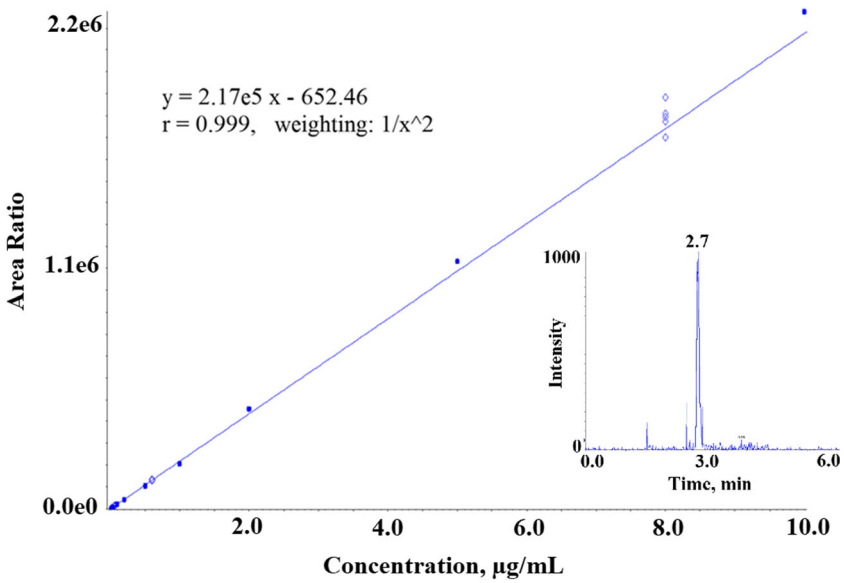

Fig. 5. Calibration curve using digestion LC-MS/MS method in rat serum. The insert is S1 (20 ng/mL) chromatogram

Table I. Inaccuracy and Imprecision of Three Runs of the Digestion LC-MS/MS Method

Fc-FGF21 concentration (μg/mL)	QC1 0.02 Batch 1	QC1 0.02 Batch 2	QC1 0.02 Batch 3	QC2 0.06 Batch 1	QC2 0.06 Batch 2	QC2 0.06 Batch 3	QC3 0.6 Batch 1	QC3 0.6 Batch 2	QC3 0.6 Batch 3	QC4 8 Batch 1	QC4 8 Batch 2	QC4 8 Batch 3
Replicate 1	0.0232	0.0224	0.0212	0.0634	0.0558	0.0656	0.633	0.633	0.564	8.77	8.72	7.23
Replicate 2	0.0214	0.0191	0.0219	0.0587	0.0560	0.0542	0.606	0.595	0.583	8.27	8.26	7.88
Replicate 3	0.0240	0.0225	0.0199	0.0679	0.0613	0.0594	0.601	0.591	0.558	8.46	8.57	7.44
Replicate 4	0.0226	0.0214	0.0179	0.0620	0.0592	0.0618	0.625	0.613	0.472	8.26	8.43	7.57
Replicate 5	0.0223	0.0210	0.0191	0.0614	0.0611	0.0626	0.595	0.591	0.495	8.34	8.38	7.99
Replicate 6	0.0231	0.0212	0.0249	0.0567	0.0574	0.0583	0.587	0.594	0.488	7.92	8.02	7.75
Intra-assay data												
Mean	0.0227	0.0213	0.0208	0.0617	0.0585	0.0603	0.608	0.603	0.527	8.34	8.40	7.64
SD	0.000887	0.00123	0.00246	0.00389	0.00244	0.00394	0.0178	0.0169	0.047	0.278	0.244	0.285
CV (%)	3.90	5.79	11.8	6.30	4.18	6.53	2.92	2.81	8.92	3.34	2.91	3.72
RE (%)	13.5	6.50	4.00	2.83	−2.50	0.500	1.33	0.50	−12.2	4.25	5.00	−4.50
Inter-assay data												
Mean	0.0216			0.0602			0.579			8.13		
SD	0.00179			0.00355			0.0478			0.433		
CV (%)	8.28			5.90			8.25			5.33		
RE (%)	8.00			0.278			−3.44			1.58		

SD standard deviation, *CV* coefficient of variance, *RE* reduction of error, *RE* stands for relative error

Quantification of Intact PF-06645849 by LC-TOF and LC-MS/MS

For the digestion LC-MS/MS method, the linearity range was from 20 to 10 μg/mL in 50 μL of 50-fold diluted rat serum based on the protein level (Fig. 5). The first calibrant (S1) could be lowered further but 20 ng/mL was sufficient for the preclinical samples. The inaccuracy and imprecision for QCs were mostly within 10% except for QC1 at 20 ng/mL (Table I).

For the intact protein method, six replicates of 5 QC levels (0.2, 0.6, 1.5, 8, and 40 μg/mL) were evaluated in 3 accuracy and precision runs (Table II). Deconvolution spectra of representative QC samples were shown in Fig. 6. The calibration curve was from 0.2 to 40 μg/mL in 50 μL of 10-fold diluted rat serum in Fig. 6. The intra- and inter-assay %CV and %RE were all within 20%.

Quantification of PF-06645849 using the intact protein LC-TOF assay was accomplished in serum samples from the rat PK study. In rat serum at 2 h, 24 h, 48 h, or 72 h post dosing, the concentrations of Fc-FGF21 were 120, 93.3, 68.7, or 56.3 μg/mL, about 13.2% higher, 5.5% higher, 4.7% lower, or 10.4% lower than the corresponding concentration measured by the digestion method at each time point, respectively. Thus, the quantification results of intact Fc-FGF21 were comparable for *in vivo* rat samples between the two LC-MS methods (Fig. 7).

DISCUSSION

Digestion LC-MS/MS Method

The engineered FGF21 molecule has a glycan linked to an asparagine (N) near the C-terminus. After the PNGase F deglycosylation, asparagine converts to aspartic acid (D) which is cleavable by Asp-N enzyme. Considering there are only few arginines and lysines in the molecule, if trypsin were used, a C-terminal peptide with 26 amino acids would be generated which is not ideal for high-sensitivity measurement based on its length. Therefore, Asp-N was chosen for digestion, which selectively cleaves peptide bonds N-terminal to aspartic acid.

On-bead digestion proved to be time efficient and yielded better results compared with acid elution prior to in-solution digestion (Supplemental Fig. 8). Sample preparation time of the method was within a day, which was suitable for preclinical sample analysis requiring increased throughput and fast data turnaround.

Potential impurities in the SIL peptide preparation, if any, whether isotopic or otherwise, do not interfere with the detection of the non-labeled peptide (Supplemental Fig. 10). Quantification of the intact PF-06645849 was evaluated in 3 accuracy and precision runs. The digestion LC-MS/MS method has 10-fold better sensitivity and wider calibration range than the intact protein LC-MS method. For the preclinical rat sample bioanalysis, both of the methods were sensitive enough to measure the full PK profile and yielded comparable quantification results. For clinical samples, however, the digestion method would be a better choice in order to detect the much lower concentration that would be predicted at a clinically relevant dose. Digestion methods have been applied to analyze biological therapeutics in clinical samples (14,15). In this case, to enable clinical sample analysis, a more specific antibody would likely be required to enrich PF-06645849 while not binding to endogenous IgG. Furthermore, low-flow LC-MS and incorporation of anti-peptide antibodies could be considered as components of a clinical assay to reduce matrix effect and to enhance bioanalytical sensitivities.

Intact LC-MS Method

The intact protein quantification method requires less sample processing and method development and measures ions over a large m/z range, thus providing a broader view of

Table II. Inaccuracy and Imprecision of Three Runs of the Intact Protein Fc-FGF21 Quantification Assay

Fc-FGF21 concentration (μg/mL)	QC1 0.2 Batch 1	QC1 0.2 Batch 2	QC1 0.2 Batch 3	QC2 0.6 Batch 1	QC2 0.6 Batch 2	QC2 0.6 Batch 3	QC3 1.5 Batch 1	QC3 1.5 Batch 2	QC3 1.5 Batch 3	QC4 8 Batch 1	QC4 8 Batch 2	QC4 8 Batch 3	QC5 40 Batch 1	QC5 40 Batch 2	QC5 40 Batch 3
Replicate 1	0.219	0.203	0.220	0.584	0.673	0.630	1.35	1.66	1.44	7.53	9.15	7.74	38.2	36.2	36.6
Replicate 2	0.213	0.195	0.213	0.557	0.568	0.636	1.52	1.49	1.54	7.95	8.52	8.12	42.1	34.8	41.2
Replicate 3	0.219	0.173	0.213	0.546	0.617	0.603	1.37	1.50	1.37	8.13	8.42	7.94	39.6	36.5	37.2
Replicate 4	0.274	0.150	0.248	0.606	0.594	0.588	1.29	1.51	0.837	7.73	8.13	7.68	42.9	37.7	39.6
Replicate 5	0.229	0.196	0.234	0.580	0.636	0.576	1.41	1.46	1.43	7.53	8.02	7.59	41.9	35.6	41.3
Replicate 6	0.238	0.145	0.221	0.542	0.604	0.611	1.45	1.47	1.52	7.86	8.26	7.94	47.6	35.3	39.0
Intra-assay															
Mean	0.232	0.177	0.225	0.569	0.615	0.607	1.40	1.51	1.35	7.79	8.42	7.83	42.0	36.0	39.2
SD	0.0223	0.0250	0.0135	0.0247	0.0363	0.0233	0.0814	0.0752	0.260	0.239	0.405	0.198	3.25	1.03	1.97
CV (%)	9.61	14.1	6.01	4.34	5.89	3.83	5.82	4.97	19.2	3.07	4.81	2.53	7.74	2.86	5.03
RE (%)	16.0	− 11.4	12.4	− 5.11	2.58	1.24	− 6.81	0.935	− 9.71	− 2.64	5.22	− 2.08	5.12	− 9.96	− 2.10
Inter-assay															
Mean	0.211			0.597			1.42			8.01			39.1		
SD	0.0318			0.0340			0.168			0.405			3.32		
CV (%)	15.1			5.69			11.8			5.05			8.48		
RE (%)	5.67			− 0.432			− 5.20			0.166			− 2.31		

SD standard deviation, *CV* coefficient of variance, *RE* reduction of error, *RE* stands for relative error

protein catabolism. As an internal standard, the molecular weight of anti-Her2 mAb heavy chain is 49,530 Da, different from that of the reduced Fc-FGF21 which is 45,052 Da. No peaks derived from the intact Fc-FGF21, PF-06645849 with MW of 90,104 Da, or dimer of the Her2 Ab heavy chain with MW of 99,061 Da were detected (Supplemental Fig. 11), suggesting the reduction was complete and the intact protein quantification can be reliably performed under the described reduction conditions. The mAb heavy chain and the reduced Fc-FGF21 coeluted from the capillary C4 column, making it easy to select and analyze them in the same deconvolution spectrum window (Fig. 5). This internal standard can compensate for variations from sample preparation and MS detection. It can be applied as an IS for preclinical samples using anti-human mAb as a capture reagent. The assay sensitivity was 0.2 μg/mL, sufficient for the current toxicokinetic studies.

An extracted ion chromatogram (XIC) method was tried initially for quantification as an alternative approach to deconvolution. Three to four high-intensity ions were selected to retrieve chromatographic peaks and the peak areas were summed for quantification. While this approach has been demonstrated for some small proteins, e.g., 10 kDa (11), the chromatographic peaks of the reduced 45 kDa Fc-FGF21were broad even at an elevated column temperature of 85°C, compromising the quantitative performance. An example is shown in Supplemental Fig. 12. In comparison, deconvolution spectra gave a higher signal to noise ratio with a cleaner background, which may be related to background subtraction in the deconvolution process. It does not have a high requirement for chromatographic separation. Kang *et al.* took the same approach and used deconvolution data for quantification (13). Although deconvolution brings an additional mathematic conversion from *m*/*z* ratios to molecular weights, the results from the 3 accuracy and precision runs suggest that this conversion can be performed reliably for quantification when all the parameters are predefined, such as selected HPLC retention time window and deconvolution parameters. Overall, for the current preclinical study, the intact protein LC-TOF method was sufficient for the characterization and quantification of the fusion protein PF-06645849.

Intact protein quantification is appealing as it needs less sample preparation and keeps a protein analyte "intact." The feasibility of the method for quantifying intact PF-06645849 was successfully demonstrated in the preclinical rat samples. There are some key considerations when choosing this approach to quantify proteins with MW of several ten thousand Daltons. This includes current limitations in assay sensitivity, and the need for high-resolution mass spectrometry instrumentation and deconvolution software. Although the intact protein LC-TOF method showed promising quantification results, further technology development is expected to make this approach a more common practice.

Pivotal prior research done by Hager *et al.* used differential ELISAs for the quantification of Fc-FGF21 forms, as well as LC-TOF and LC-MS/MS for catabolite characterization (4). Similarly, Stanislaus used an IA-MALDI method for intact protein characterization and IA-LC-MS/MS method for digested peptide characterization (6). In the current study, both the intact LC-TOF method and

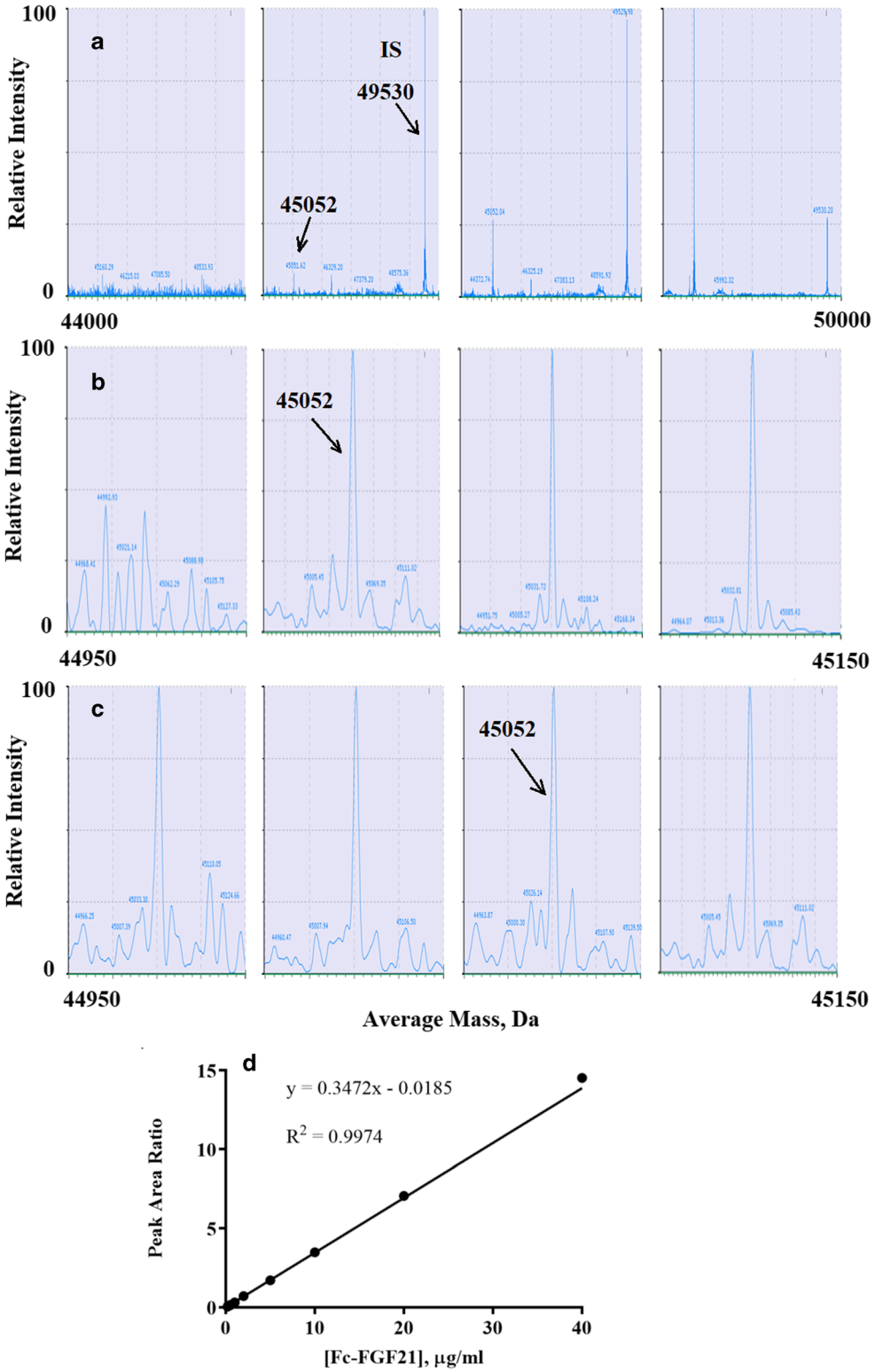

Fig. 6. **a** Deconvoluted spectra of rat serum blank, reduced PF-06645849 at 0.2, 0.6, and 8 μg/mL, from left to right. IS was a heavy chain of reduced anti-Her2 mAb with MW 49,530 Da. **b** Zoomed-in deconvoluted spectra of rat serum blank, reduced PF-06645849 at 0.2, 0.6, and 8 μg/mL, from left to right, from mass 44,950 to 45,150 Da. **c** Deconvoluted spectra of four replicates of reduced PF-06645849 at 0.2 μg/mL in rat serum. **d** Intact protein assay calibration curve ranged from 0.2 to 40 μg/mL

digestion LC-MS/MS method were used not only to characterize the biotransformation of Fc-FGF21 but importantly also to quantify the intact Fc-FGF21 in a preclinical *in vivo* study. A new catabolite [1–384] with MW 42,081 Da was identified by the intact protein LC-TOF method in rat serum *in vivo* after 24 h and 72 h dosage. Three accuracy and

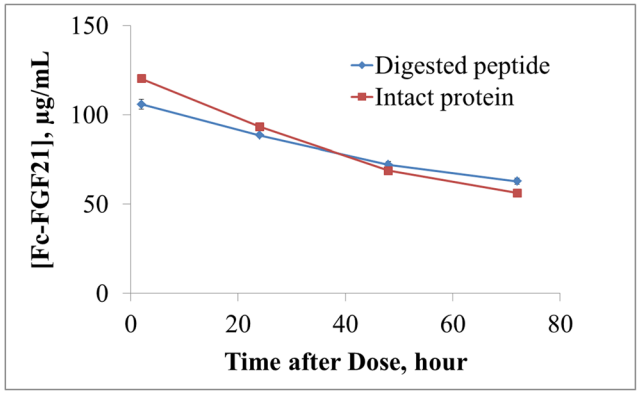

Fig. 7. Comparison of intact protein and digestion peptide LC-MS results of *in vivo* rat serum samples, $n = 2$

precision runs were performed by the two methods separately, and inaccuracy and imprecision were within 20%.

CONCLUSION

In conclusion, in the current study, the intact protein LC-TOF method and digestion LC-MS/MS method were both used to profile the catabolism of fusion protein PF-06645849 and to quantify the intact molecule in rat serum samples. The two approaches were complementary in the study as the intact protein method detected a truncated fragment with a mass of 42,072 Da, and the digestion method provided better sensitivities by monitoring the catabolized peptides. These approaches can be applied to other therapeutic fusion proteins with catabolism or stability concerns.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflicts of interest.

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