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RESEARCH PAPER

Development and validation of an UPLC-MS/MS assay for quantitative analysis of the ghrelin receptor inverse agonist PF-5190457 in human or rat plasma and rat brain

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Abstract PF-5190457 is a ghrelin receptor inverse agonist that is currently undergoing clinical development for the treatment of alcoholism. Our aim was to develop and validate a simple and sensitive assay for quantitative analysis of PF-5190457 in human or rat plasma and rat brain using liquid chromatography-tandem mass spectrometry. The analyte and stable isotope internal standard were extracted from 50 μL plasma or rat brain homogenate by protein precipitation using 0.1 % formic acid in acetonitrile. Chromatography was carried out on an Acquity UPLC BEH C18 (2.1 mm × 50 mm) column with 1.7 μm particle size and 130 Å pore size. The flow rate was 0.5 mL/min and total chromatographic run time was 2.2 min. The mobile phase consisted of a gradient mixture of water: acetonitrile 95:5 % (*v*/*v*) containing 0.1 % formic acid (solvent A) and 100 % acetonitrile containing 0.1 % formic acid (solvent B). Multiple reaction monitoring was carried out in positive electro-spray ionization mode using *m*/*z* 513.35→209.30 for PF-5190457 and *m*/*z* 518.47→214.43 for the internal standard. The recovery ranged from 102 to 118 % with coefficient of variation (CV) less than 6 % for all matrices. The calibration curves for all matrices were linear over the studied concentration range ($R^2 \geq 0.998$, $n=3$). The lower limit of quantification was 1 ng/mL in rat or human plasma and 0.75 ng/g in rat brain. Intra- and inter-run mean percent accuracies were between 85 and 115 % and percent imprecision was ≤15 %. The assays were successfully utilized to measure the concentration of PF-5190457 in pre-clinical and clinical pharmacology studies of the compound.

Keywords Alcoholism · Bioanalytical methods · Ghrelin · LC-MS/MS · PF-5190457 · Pharmacokinetics

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Abbreviations

ACN	Acetonitrile
CV	Coefficient of variation
IS	Internal standard
LLOQ	Lower limit of quantification
ME	Matrix effect
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
Mw	Molecular weight
PLs	Phospholipids
QCs	Quality controls
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry

Introduction

Ghrelin is a 28-amino acid peptide primarily produced by the endocrine X/A-like cells of the fundus mucosa of the stomach and acts as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a). GHS-R1a is a G-protein coupled receptor that induces growth hormone (GH) release from the pituitary [1]. Ghrelin activates hypothalamic orexigenic neurons and inhibits anorectic neurons to induce hunger [2, 3]. In humans, intravenous (IV) acetylated ghrelin administration increases appetite and food intake [4, 5]. Moreover, ghrelin infusion can suppress glucose-dependent insulin secretion in rodents and humans resulting in insulin resistance [6, 2]. Therefore, it is conceivable to believe that pharmacological modulation of ghrelin may be beneficial in regulating appetite and body weight or in treating type 2 diabetes mellitus.

Consistent with converging evidence illustrating that alcohol- and food-seeking behaviors share common neural pathways [7, 8], ghrelin signaling has been proposed as a potential novel pharmacological target for the treatment of alcoholism [9]. In mice, central ghrelin administration to reward nodes of the brain increased alcohol intake while central or peripheral administration of ghrelin receptor antagonists suppressed alcohol intake [10]. Furthermore, clinical studies from our team have shown that plasma concentrations of ghrelin were different in abstinent compared to active drinking alcohol-dependent individuals and correlated with alcohol craving [11]. Additionally, in a human laboratory setting, intravenous administration of 3 μg/kg ghrelin to alcohol-dependent, heavy-drinking individuals resulted in a significant acute increase in cue-induced alcohol craving [12]. Furthermore, there was a positive significant correlation between post-infusion blood ghrelin levels and increased alcohol craving [12].

Generally, it appears that GHS-1Ra antagonism can possibly increase satiety and does not only result in weight loss and improvement in glycemic control but it may also be helpful for treating alcoholism. PF-5190457 is a sensitive and specific ghrelin receptor inverse agonist that is orally bioavailable [13]. It is a member of a spiro-azetidino-piperidine series that was identified through high-throughput screening by Pfizer Pharmaceuticals. PF-5190457 (Mw=512) has a measured logD value of 1.5 at pH 7.4 and a topological polar surface area of 95. Pharmacokinetics studies in rats have shown a high volume of distribution and clearance and an almost 100 % fraction absorbed in portal vein-cannulated rats [10]. Here, we report the development and validation of a sensitive, specific, and robust assay for measurements of PF-5190457 in either human or rat plasma or in rat brain homogenate using an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) technique.

Chemicals and reagents

PF-5190457 and the internal standard (IS) PF-06340740 (stable labeled isotope) were kindly donated by Pfizer. Optima™ LC/MS grade of acetonitrile, ammonium acetate, formic acid, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained using a Milli-Q Synthesis system fitted with a Q-Gard 2 Purification Pack (Millipore, Bedford, MA, USA). Drug-free K_2EDTA rat plasma or brain specimens were from Wistar rats (n=6) aged between 2 and 4 months and weighing between 300 and 500 g (Bioreclamation IVT Inc., Westbury, NY, USA). Similarly, K_2EDTA human plasma from six subjects (three male, three female) were obtained from Bioreclamation IVT Inc.

Instruments

An Eppendorf 5810 refrigerated centrifuge from Micro and Nanotechnology (Urbana, IL, USA) was used to obtain supernatants. Acquity UPLC from Waters Corp (Milford, MA, USA) connected to Xevo TQ MS mass spectrometry (Waters Corp) was used to quantify PF-5190457 concentrations. The Acquity UPLC system had a binary pump and was equipped with a built-in column heater. A 20 μL sample loop was used to deliver samples in partial loop injection mode. The system was controlled with MassLynx™ software (V 4.1) and data was processed using TargetLynx™ tool.

Chromatographic conditions

Chromatographic separation was carried out in an Acquity UPLC BEH C18 (2.1 mm×50 mm) with 1.7 μm particle size and 130 Å pore size analytical column (Waters Corp, Milford, MA). An Acquity UPLC BEH C18 pre-column was used to preserve the performance of the analytical column. The column was maintained at 55 °C and an auto-sampler temperature was kept at 20 °C. A gradient elution method was utilized with a mobile phase consisting of water: acetonitrile 95:5 % (*v*/*v*) containing 0.1 % formic acid (solvent A) and 100 % acetonitrile containing 0.1 % formic acid (solvent B). The mobile phase was delivered at 0.5 mL/min flow rate. Each chromatographic cycle started and maintained at 2 % solvent (B) for 0.3 min and increased gradually to 98 % over 0.7 min and maintained at this level until 1.8 min. To re-equilibrate the column for the next run, the proportion of solvent (B) was decreased within 0.1 min to 2 % and kept constant until the end of the run at 2.2 min. To minimize detector contamination, a diversion valve was

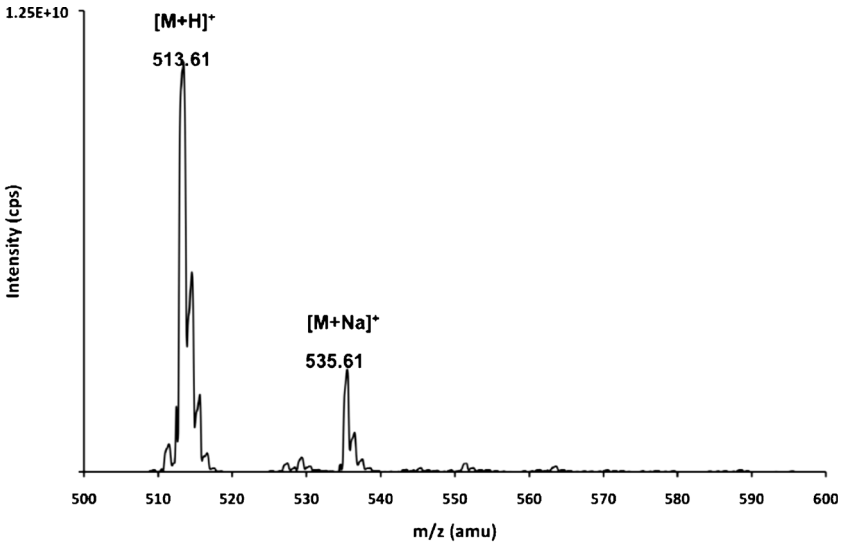

Fig. 1 Q1 scan of PF-5190457 shows the abundant adducts, $[M+H]^+$ and $[M+H_4]^+$

set to deliver the first 0.60 min and from 1.10 min until the end of each run to waste. The elution time for both analyte and IS was 0.83 min.

Mass spectrometry conditions

Multiple reaction monitoring (MRM) in positive electrospray ionization (ESI) mode was used for detection and quantification of analytes and IS. The MS scan of infused PF-5190457 detected protonated molecules $[M+H]^+$ (m/z=513.61) with highest intensity, followed by sodium adduct (m/z=535.61) $[M+Na]^+$ as seen in Fig. 1. Therefore, the protonated form was selected. Protonated precursors were fragmented into two compounds of similar intensity with m/z values of 127.21 and 209.30; a third compound (m/z=335.35) was fragmented with 50 % intensity compared to the first two fragments (Fig. 2). The two fragments with the highest m/z values were selected. MRM transitions were monitored (m/z Q1→Q3); m/z 513.35→209.30 transition was used for quantification while m/z 513.35→335.35 transition was used as a qualifier. m/z

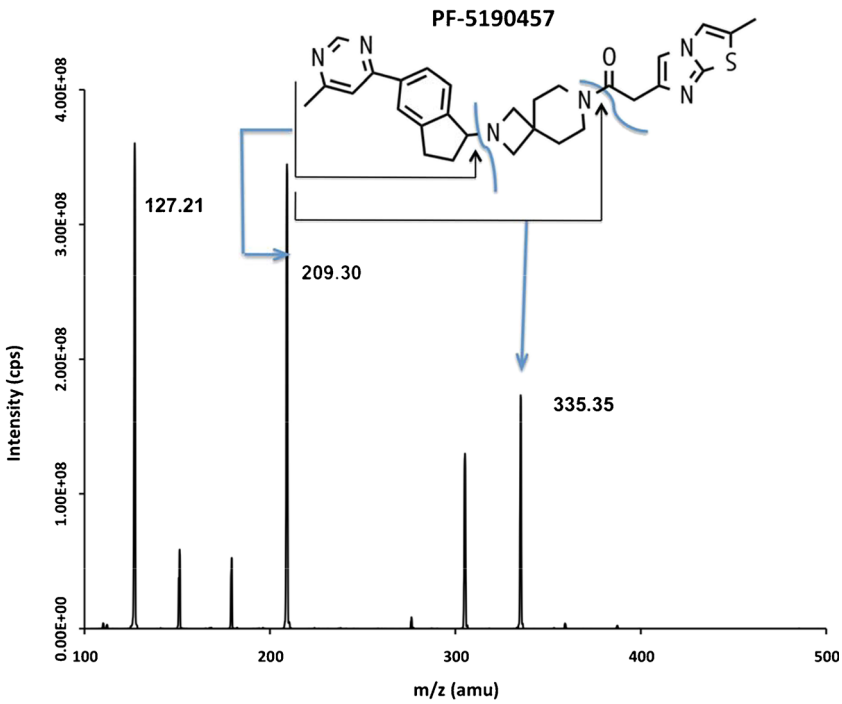

Fig. 2 Q3 scan shows fragmentation pattern of PF-5190457 $[M+H]^+$ and intensity of daughter ions

518.47→214.43 transition was selected for the internal standard. The proposed fragment formation of PF-5190457 is illustrated in Fig. 3. All chemical structures were produced using ChemDraw version 14.0.0.117 from PerkinElmer Inc (Waltham, MA, USA). After automatically obtaining initial mass spectrometry parameters with the IntelliStart tool, manual tuning of final parameters was performed to achieve the highest possible signal. Final mass spectrometry parameters were: capillary voltage=0.30 kV, extractor voltage=3 V, source temperature=150 °C, desolvation temperature=650 °C, desolvation gas flow=400 L/h, and collision gas flow 0.15 mL/min. Cone voltages and collision energy were 32 and 38 for analytes with *m/z* 513.35→209.30 transition and 32 and 18 for analytes with *m/z* 513.35→335.35 transition, respectively, and 38 and 44 for the internal standard.

Preparation of standards, quality controls, and IS solutions

Sub-stock and working stock solutions of PF-5190457 and IS were prepared using 50 % acetonitrile (ACN) and were stored at 4 °C. Standards and quality control (QC) samples were prepared by spiking rat or human

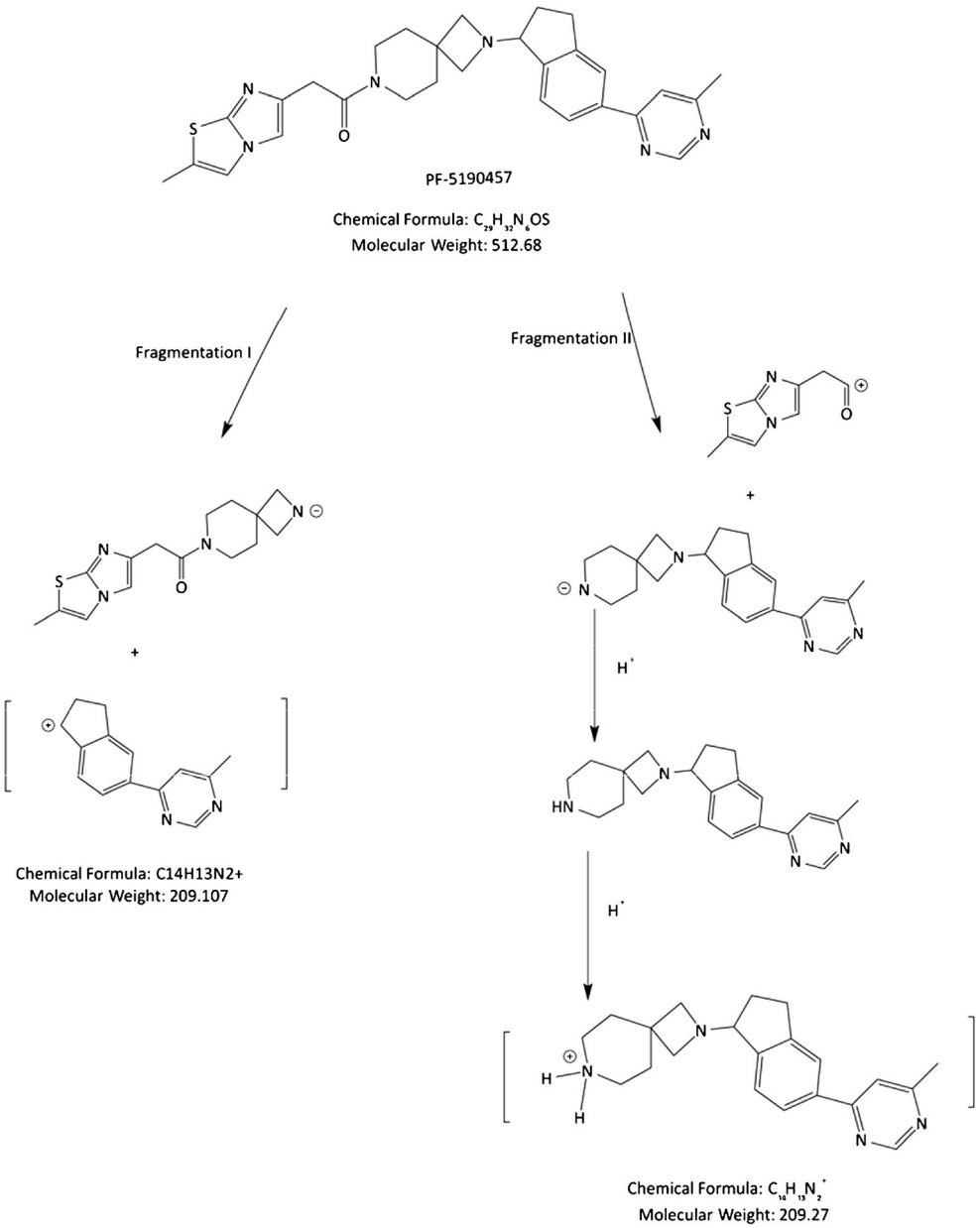

Fig. 3 Proposed fragmentation pathway of PF-5190457 $[M+H]^+$ into daughter ions with *m/z* of 209 and 335

Table 1 Summary of standards and calibration curve parameters from three individual runs

		STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	R^2
Matrix	Nominal conc. (μg/L)	0.75	1.50	4.50	12.00	24.00	48.00	96.00	120.00	
Rat brain	Mean	0.83	1.47	4.04	11.86	23.47	44.59	97.84	117.85	0.9989
	SD	0.06	0.12	0.63	0.60	0.96	2.91	7.99	7.53	
	%Bias	10.20	−1.92	−10.33	−1.14	−2.20	−7.10	1.91	−1.79	
	CV	7.60	7.87	15.73	5.03	4.08	6.53	8.17	6.39	
Rat plasma	Nominal conc. (ng/mL)	1.00	2.00	10.00	100.00	250.00	500.00	800.00	1000.00	
	Mean	1.00	2.06	9.95	103.18	258.39	500.11	795.00	999.22	0.9999
	SD	0.10	0.27	1.11	12.63	33.70	23.88	35.87	33.04	
	%Bias	0.13	3.23	−0.47	3.18	3.36	0.02	−0.63	−0.08	
Human plasma	Nominal conc. (ng/mL)	1.00	2.00	10.00	100.00	250.00	500.00	800.00	1000.00	
	Mean	0.98	1.89	9.42	99.79	251.93	476.21	831.43	993.69	0.9989
	SD	0.09	0.25	3.89	11.29	14.59	30.72	38.45	28.31	
	%Bias	−2.12	−5.51	−5.81	−0.21	0.77	−4.76	3.93	−0.63	
	CV	9.05	12.99	41.25	11.32	5.79	6.45	4.62	2.85	

n=3 (one replicate for each of the three validation runs); %bias calculated as 100×(mean−nominal)/nominal; CV calculated as 100×SD/mean

plasma or rat brain homogenate to achieve desired PF-5190457 concentrations while keeping the organic solvent ≤5 % of the total volume. Standard concentrations of PF-5190457 in rat brain homogenates before extraction were 0.15, 0.30, 0.68, 2.40, 4.80, 9.60, 19.20, and 24.00 μg/L; QC concentrations were 0.45, 3.00, and 18.00 μg/L for low QC (LQC), medium QC (MQC), and high (HQC), respectively. The final standard and QC concentrations in brain samples are shown in Tables 1 and 2.

Plasma standard concentrations before extraction were 1, 2, 10, 100, 250, 500, 800, and 1000 μg/L; QC concentrations were 3, 200, and 750 μg/L for LQC, MQC, and HQC, respectively. Working internal standard solutions (WIS) composed of 0.1 % formic acid in ACN at concentrations of 5 and 10 μg/L were used as precipitating solvents for brain and plasma samples, respectively. The final standard and QC concentrations in plasma samples are shown in Tables 1 and 2.

Table 2 Summary of quality control samples from three individual runs

		LLOQ	QC1	QC2	QC3
Matrix	Nominal conc. (μg/L)	0.75	2.25	15.00	90.00
Rat brain	Inter-run mean	0.77	2.28	14.51	96.95
	Inter-run SD	0.10	0.24	1.14	5.47
	Inter-run %bias	2.86	1.46	−3.25	7.72
	Inter-run CV	12.64	10.40	7.84	5.65
Rat plasma	Nominal conc. (ng/mL)	1.00	3.00	200.00	750.00
	Inter-run mean	1.00	3.12	241.9	786.99
	Inter-run SD	0.17	0.35	18.30	57.02
	Inter-run %bias	0.21	4.12	7.40	4.93
	Inter-run CV	16.99	11.17	8.50	7.24
Human plasma	Nominal conc. (ng/mL)	1.00	3.00	200.00	750.00
	Inter-run mean	0.94	2.96	205.45	758.24
	Inter-run SD	0.15	0.40	13.99	83.22
	Inter-run %bias	−5.67	−1.17	2.73	1.10
	Inter-run CV	15.55	13.63	6.81	10.97

n=18 (six replicates for each validation run); %bias calculated as 100×(mean−nominal)/normal; CV calculated as 100×SD/mean

Protein precipitation and sample extraction

Rat brain samples

Brain segments from each rat were weighed individually and homogenized manually on ice using a glass tissue homogenizer with a fourfold volume of deionized water (*w*:*v*) until a homogenous mixture was formed. One part brain homogenate of control blank, standards, QCs, and samples was extracted with two parts of 5 μg/L WIS in 1.5-mL Eppendorf tubes. Double blank samples were extracted with 100 % ACN. After vortex mixing for 10 s, samples were centrifuged at 5000×*g* for 5 min and 10 μL of supernatant was injected onto LC-MS/MS.

Rat and human plasma samples

One part of rat or human plasma as control blank, standards, QCs, and plasma samples was mixed with four parts of 10 ng/mL WIS in a 1.5-mL microfuge tube. Double blank

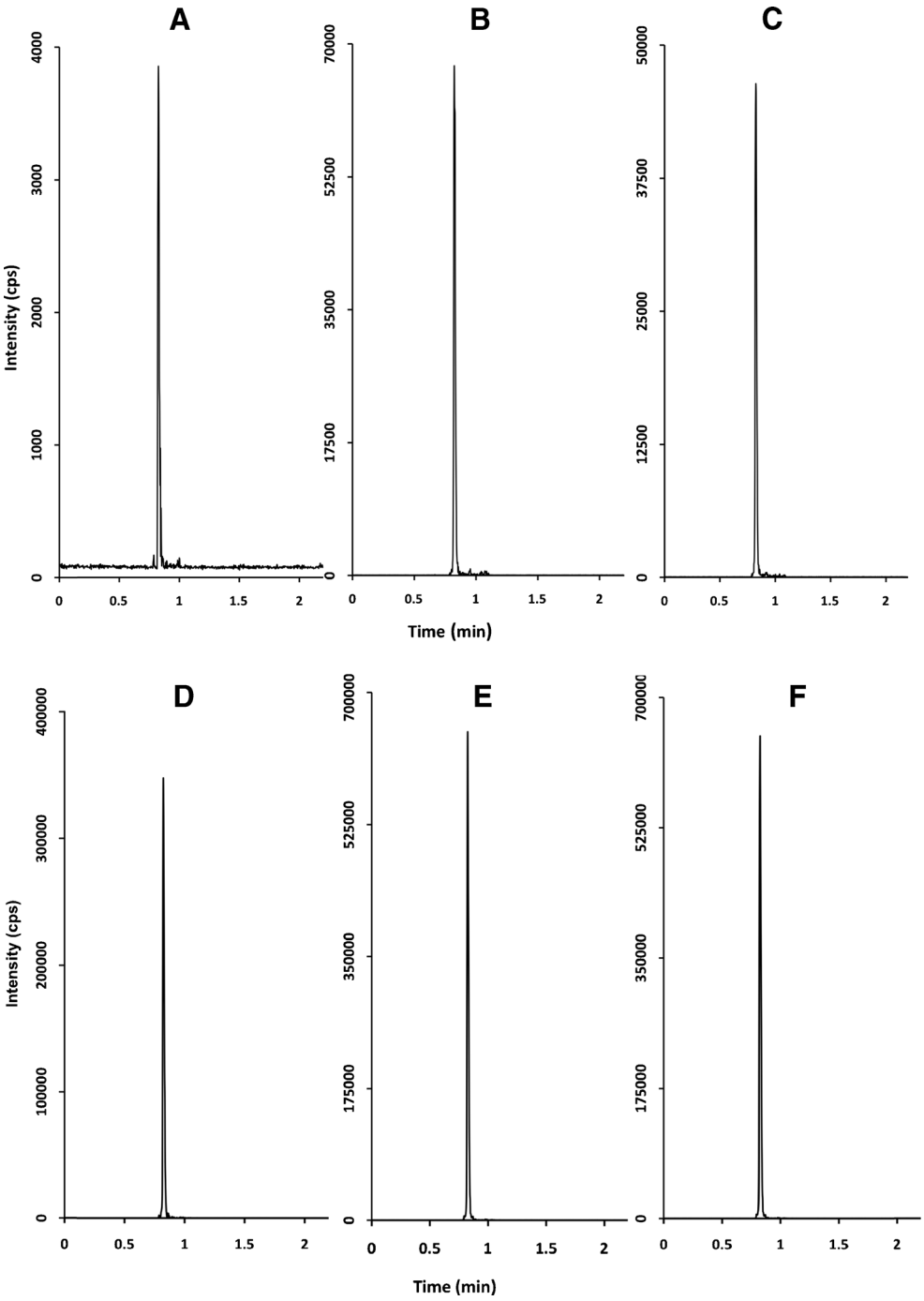

Fig. 4 Chromatograms of ghrelin antagonist (PF-5190457) at LLOQ (**A**, **B**, and **C**) and the internal standard (**D**, **E**, and **F**) in rat brain, rat plasma, and human plasma samples, respectively

samples were extracted with 100 % ACN. After vortex mixing for 10 s, samples were centrifuged at 5000×*g* for 5 min and 5 μL of the supernatant was injected onto LC-MS/MS.

Assay validation

Standards and QCs

The method was validated in accordance with the current version of the Food and Drug Administration (FDA) guidance for industry on bioanalytical method validation [14]. Calibration curves were constructed by plotting the analyte/IS peak area ratio against the nominal concentration of analytes and fitted using a $(1/x)$ weighting method. Accuracy and precision of the assay were determined using three different batches of brain or plasma that were spiked with working stock solutions to achieve standard and QC concentrations (six replicates) and extracted as described in the sample extraction section.

Sensitivity and selectivity

The lower limit of quantification (LLOQ) was determined by concentrations that had % bias ≤ ± 20, coefficient of variation (CV)≤±20 %, and signal to noise ratio (*S/N*) ≥ 10. Acceptance criteria for QCs (LQC, MQC, and HQC) were %bias≤±15 % and CV≥±15 %. Selectivity assessed by inspecting the presence of noise or peaks at analyte and IS elution time on chromatograms represented blank brain or plasma samples (from six subjects).

Stability

Stability of PF-5190457 was investigated by quantifying QC1 and QC3 concentrations in three replicates. Freeze and thaw (three freeze and thaw cycles), bench-top, and short-term stability for up to 1 month were investigated. Auto-sampler stability was assessed by re-injecting one of the validation batches kept in the auto-sampler for 72 h.

Matrix effect and recovery

Possible interference of matrix effect (ME) in brain and plasma samples was inspected visually by two methods. First, possible interference of matrix components was visually inspected on chromatograms generated using post-column infusion [15]. The test was performed by continuously infusing, after the column via a Tee connection, 98 % ACN solution (represents the composition of the mobile phase at elution time) containing PF-5190457 and IS at the highest standard concentrations at a flow rate of 10 μL/min. Simultaneously, extracted blank brain or plasma samples, and neat solution (%50 ACN) were injected using the pre-established LC method. Chromatograms

Table 3 Results of stability studies

Matrix	QCs		Bench top 12 h	Freeze and thaw	Auto-sampler 75 h	Short term 1 week	Recovery %
Rat brain	QC1 (2.25) (ng/g)	Mean	2.3	2.2	2.4	2.5	102.0
		%Bias	2.2	−2.2	6.6	11.1	–
		CV	4.3	9.5	6.4	6.1	−1.2
	QC3 (90.0) (ng/g)	Mean	77.2	82.1	83.6	79.4	115.0
		%Bias	−14.2	−8.8	−7.1	−11.8	–
		CV	6.2	4.5	11.6	5.8	−1.4
Rat plasma	QC1 (3.0) (ng/mL)	Mean	3.4	3.1	3.0	3.1	117.0
		%Bias	14.4	3.3	1.2	3.3	–
		CV	11.0	8.5	11.5	5.6	−1.6
	QC3 (750) (ng/mL)	Mean	709.5	826.2	757.5	765.5	104.0
		%Bias	−5.4	10.2	1.0	2.1	−1.3
		CV	4.0	4.4	10.6	6.5	
Human plasma	QC1 (3.0) (ng/g)	Mean	3.4	3.3	2.9	3.4	114.0
		%Bias	13.3	10.0	−3.2	13.3	–
		CV	6.1	6.1	9.4	7.8	−5.5
	QC3 (90.0) (ng/g)	Mean	797.3	826.5	801.2	800.2	118.0
		%Bias	6.3	10.2	6.8	6.7	–
		CV	2.5	2.8	11.1	1.8	−1.4

n=3; %bias calculated as 100×(mean−nominal)/nominal; CV calculated as 100×SD/mean

obtained from injecting blank brain or plasma samples were compared with a chromatogram that represented injection of neat for any signs of suppression and/or enhancement at the analyte and IS elution region. Second, possible co-elution of analytes and IS with phospholipids (PLs) was also checked [16, 17]. By including MRM transitions of abundant PLs in the MS method, we were able to visually locate the PL elution region at early stages of method development. Co-elution was avoided by manipulating liquid chromatography conditions and mobile phase gradients.

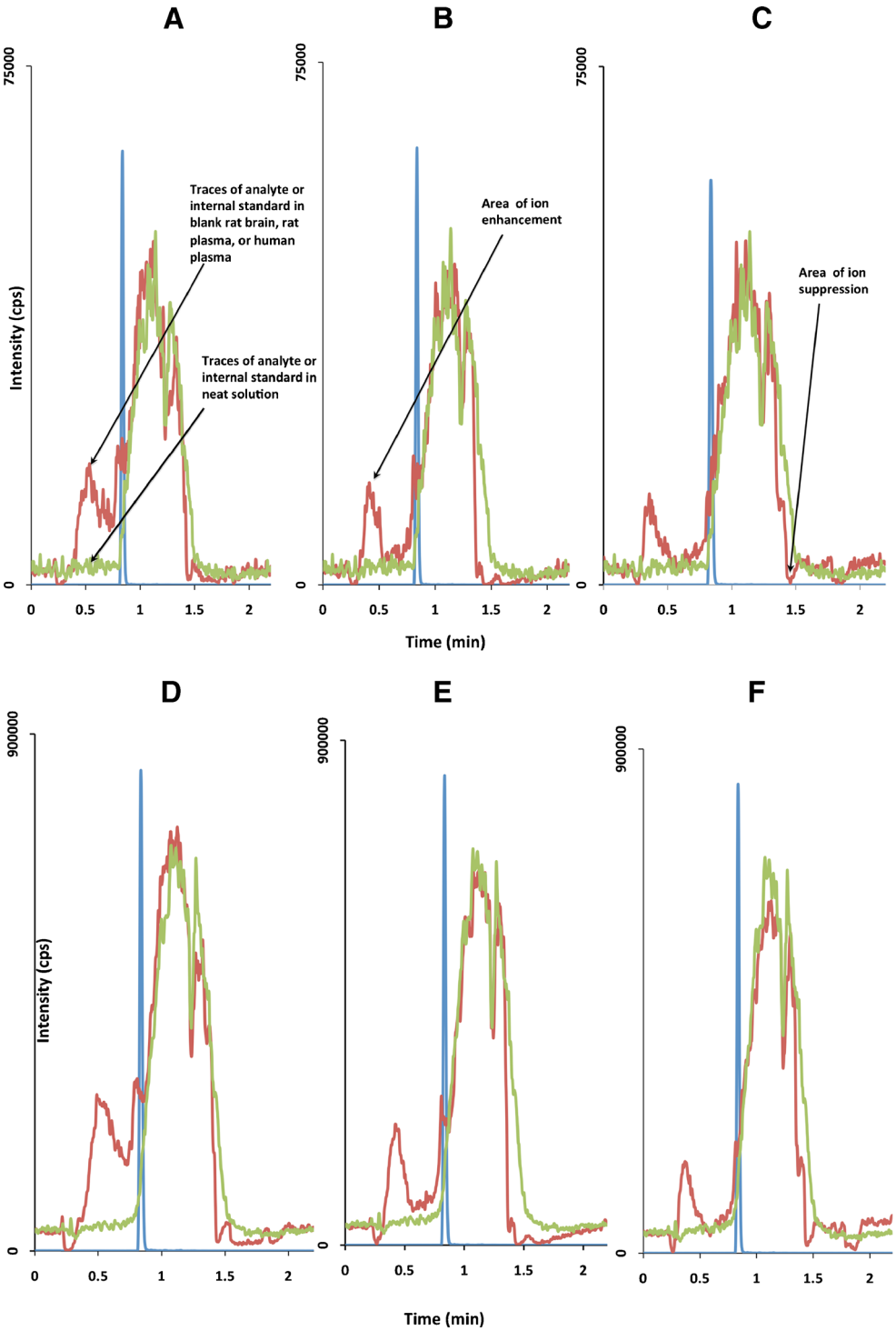

Fig. 5 A composite chromatogram of traces obtained from continuous post-column infusion chromatograms of PF-5190457 (**A**, **B**, and **C**) and the internal standard (**D**, **E**, and **F**) overlaid on chromatograms of injections of rat brain (*left column*), rat plasma (*middle column*), and human plasma (*right column*)

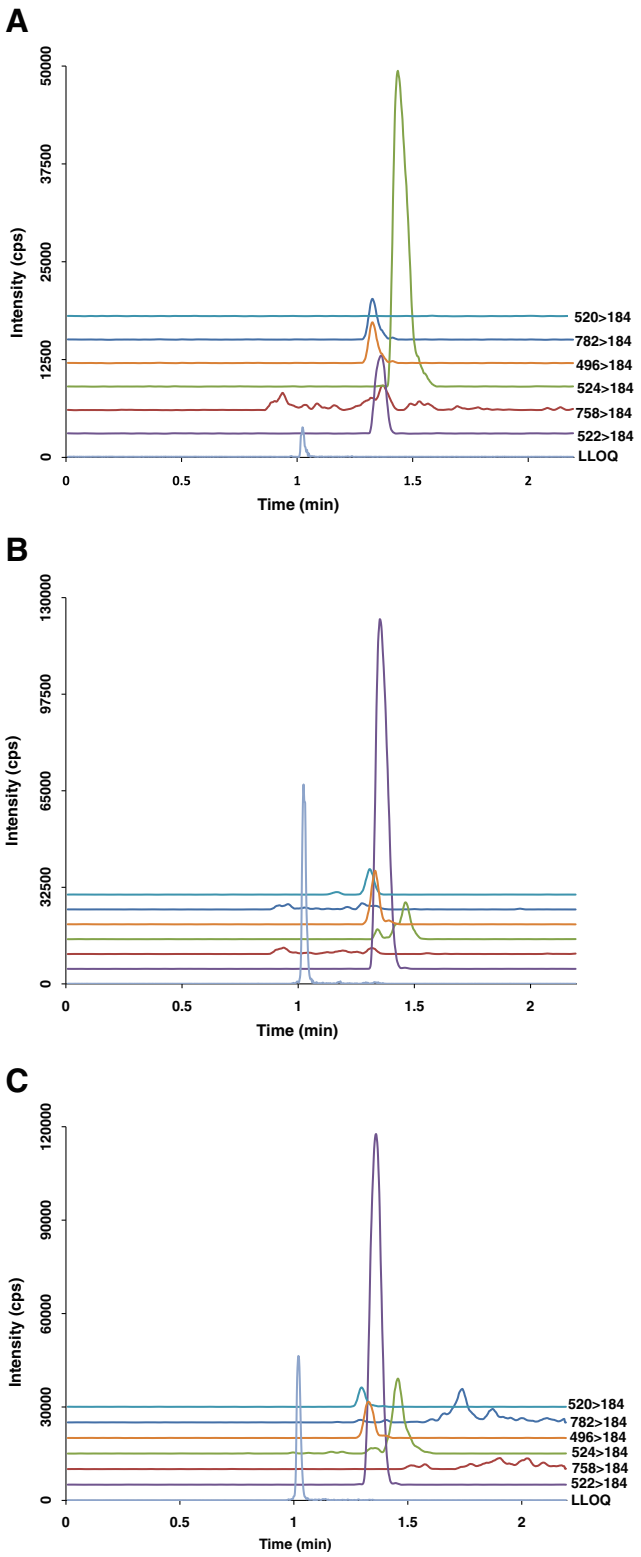

Fig. 6 Chromatograms depicting traces of phospholipids obtained from injecting pooled blank samples of rat brain (**A**), rat plasma (**B**), and human plasma (**C**). MRM transition of each individual phospholipid species is shown on the *right side* of the graph. The figures show the relative amount of PF-5190457 to PLs in each matrix

To determine recovery, two sets of QCs (from six subjects) were prepared. The first set of QCs was prepared in either brain or plasma and was extracted as prescribed in the sample extraction section (pre-extracted matrix QCs). The second set was prepared by spiking extracted blank matrices with standard working solutions to achieve the same final concentration as the concentration in the first set. The percentage ratio of mean peak areas of pre-extracted samples to mean post-extracted spiked samples was used to calculate recovery.

Results and discussion

Sensitivity and selectivity

The brain concentration of the analyte was expected to be very low compared to that of plasma. Therefore, mass spectrometry and chromatographic conditions were optimized using extracted brain samples to improve the lower limit of quantification. Adequate sensitivity and selectivity were obtained using an Acquity UPLC BEH C18 column. The final UPLC and mass spectrometry parameters were appropriate to set LLOQs at 0.75 and 1 μg/L for brain and plasma, respectively (Fig. 4). Chromatograms obtained from pooled blank samples from six subjects and blank neat solutions (50 % ACN) were visually inspected and compared for any peaks or noises at elution regions. No sign of interference was noticed. No carryover was detected when double blank samples were injected following the highest calibration concentration.

Curve fitting of the standard curve was comprised of $1/x$ weighted least squares linear regression. The average correlation coefficient (R^2) of the three validation batches was 0.999. The inter-run %bias and coefficient of variation (CV) were in the recommended limit of ±20 for LLOQ and ±15 for QCs (Table 2).

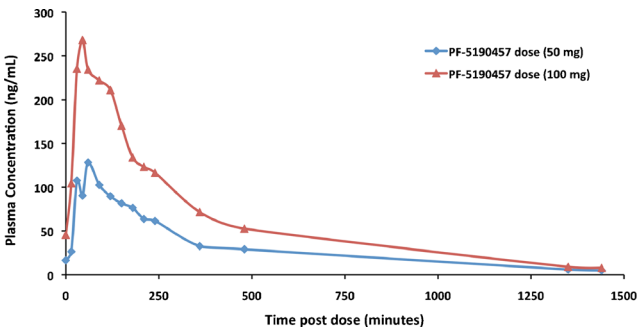

Fig. 7 Concentration-time profiles of PF-5190457 in a representative study volunteer after ingestion of 50- and 100-mg doses of PF-5190457 by oral route

Stability

Bench top, freeze and thaw, auto-sampler, and short-term storage at −80 °C for up to 4 weeks were studied (Table 3). No stability problems were noticed and analytes were stable in extracted matrices for up to 72 h.

Recovery and matrix effect

Sample processing and extraction procedures showed excellent recovery. The recovery ranged from 102 to 118 % with CV less than 6 % for all matrices (Table 3). Endogenous components in biological fluids may interfere and compete for ionization with the analytes of interest [15]. The ME could be either ionization suppression or enhancement, both of which can potentially compromise the integrity of the data [16]. A post-column infusion technique was utilized to examine possible interference of components present in matrices of interest. Figure 5 shows a representative composite of PF-5190457 and IS traces obtained from post-column infusion at a concentration of 1 μg/mL overlaid on chromatograms obtained from injecting samples. An area of ionization suppression was seen at around 0.25 min in the chromatogram from all matrices; slight ionization enhancement was also seen at around 0.5 min in all matrices (Fig. 5). There was no sign of ionization suppression or enhancement at the retention time of analyte or IS.

The ME was also investigated visually first by detecting elution regions of PLs components of rat brain, rat plasma, and human plasma. The MRM of transitions of the most common PLs [17, 16] was added to the mass spectrometry method. Mass transitions of PLs include *m/z* 496→184, 520→184, 522→184, 524→184, 758→184, and 782→184. As shown in Fig. 6, the investigated PLs eluted far enough after analytes of interest in rat brain (A), rat plasma (B), and human plasma (C). It must be noted that PLs that have an *m/z* of 524 are more abundant in the brain when compared to those of rat and human plasma. In contrast, PLs with an *m/z* of 522 seem to be more abundant in rat and human plasma than in rat brain. Since the dilution factors (15 and 5 times for brain and plasma, respectively) and final water proportion in each final matrix extract was different, direct quantitative comparison was not possible.

Assay application

The assay was successfully utilized to measure compound concentrations in rat brains and plasma after administration of PF-5190457 as well as preliminary pharmacokinetic studies in human plasma conducted in the context of the phase 1b study. Appropriate approvals were granted by the appropriate NIH Institutional Animal Care and Use Committee (IACUC) and the Institutional Review Board (IRB). Figure 7 depicts a concentration-time profile of PF-5190457 in a representative human subject at steady state after administration of 50- and 100-mg oral doses of PF-5190457.

Conclusion

This is the first reported analytical method for quantification of PF-5190457 in rat brain, rat plasma, and human plasma. This LC-MS/MS method was developed and validated in accordance with the current FDA guideline and showed high sensitivity, selectivity, and robustness. Simple extraction processes with excellent recovery and sufficient sample cleanness were used. The method allowed us to examine the presence and describe relative components and elution behaviors of the investigated PL species. The assays were successfully applied for quantification of PF-5190457 in both pre-clinical and clinical studies.

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