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Application Note

Rapid, High Sensitivity Quantification of a Proteolysis Targeting Chimera, PROTAC, in Rat Plasma From a Microsampling DMPK Study Using UPLC-MS/MS

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Abstract

Proteolysis targeting chimeras are bifunctional molecules with two molecular entities connected by a linker, that are highly specific medicines which act by degrading harmful proteins in cells. As these PROTACs molecules are now beginning to enter late-stage development and early human clinical trial, it is necessary to accurately determine the pharmacokinetics of the candidate drug. Hence, there is a need for reliable bioanalytical assays for the quantification of these compounds in plasma and serum. Here we present the application of a rapid, high sensitivity reversed – phase UPLC-MS/MS bioanalytical assay for the quantification of PROTACs 3 gefitinib in plasma from a rat DMPK study using microsampling for blood collection.

Benefits

- Rapid, high sensitivity ACQUITY Premier UPLC and Xevo TQ Absolute UPLC-MS/MS method for the quantification of a proteolysis targeting chimera in plasma
- Improved chromatographic peak shape and reduced analyte binding using ACQUITY Premier UPLC

technology

- Wide dynamic range of Xevo TQ Absolute facilitated the accurate quantification of samples across all the sampling time points
- High sensitivity of the system allowed for the analysis of small sample volumes from a rodent DMPK study

Introduction

PROteolysis TArgeting Chimeras (PROTACs) are an emerging class of therapeutic modalities which operate by hijacking the cell proteasome system to specifically degrade target proteins related to a disease state.[1] PROTACs molecules have attracted significant interest by both academia and the pharmaceutical industry due to their ability to medicinally control specific proteins that been challenging to address with conventional small molecules.[2,3] Over two decades after the initial publication[4] on the potential of protein degraders, protein degrader therapeutics are now beginning to enter the clinical trial stage of drug development.[5,6] Critical to the development of PROTACs molecules as successful medicines is the ability to understand their metabolism and pharmacokinetics.[7] UPLC-MS/(MS) has established itself as the technology of choice for quantification of candidate medicines and identification of drug metabolites in biofluids.[8]

The 3Rs (replacement, reduction and refinement) initiative within the pharmaceutical industry has increased the focus on microsampling, ***e.g.*** dried bloodspots,[9] to not only reduce animal usage but also to improve data quality. Previously we have demonstrated the use of reversed – phase LC-MS/MS using ACQUITY UPLC coupled to tandem quadrupole MS for the quantification of PROTACs 3 gefitinib in rat plasma over the range of 20 pg/mL to 1000 ng/mL. Here we report the further development of this platform method for the quantification of serial bleed plasma samples obtained following the subcutaneous administration of PROTACs 3 gefitinib, Figure 1, to male Wistar rats at 10 mg/kg.

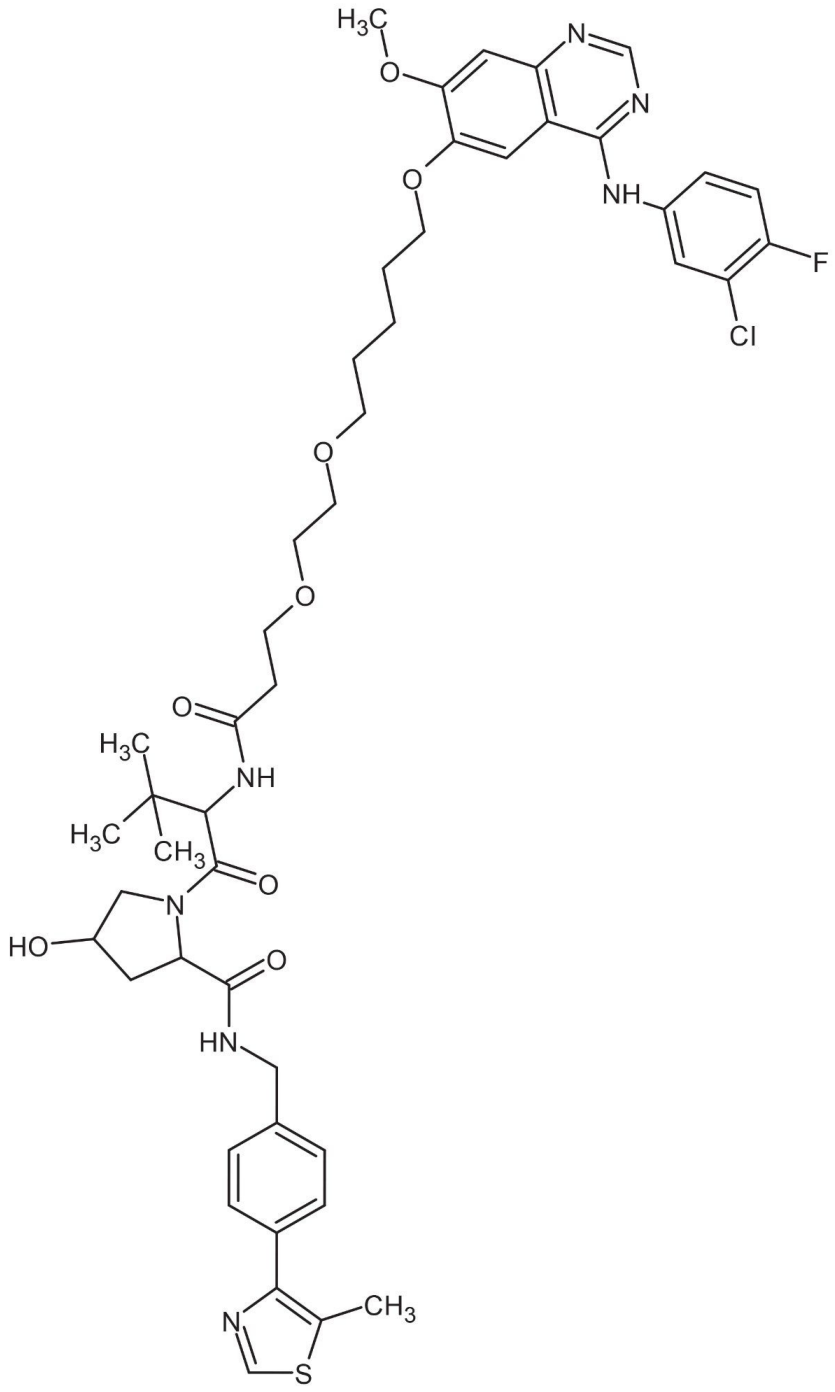

Figure 1. Structure of PROTACs 3 gefitinib.

Experimental

Sample Description

Animal Study

PROTAC 3 gefitinib, Figure 1, was dosed subcutaneously at 10 mg/kg to 4 male Wistar rats. The dose was formulated in 5% DMSO, 40% PEG 400, 5% Cremophor and 50% saline for injection (SFI). The rats were housed individually in a metabowl and had free access to food and water. Blood samples were collected via cannulation of the jugular vein at pre dose, 0.083, 0.25, 0.5, 1, 3, 6, 8, and 24 hr post dose. The samples were centrifuged at 10,000 g for 5 min to separate the plasma. Aliquots were transferred into either a 96 well plate (20 µL) or 1.5 mL Eppendorf tube (50 µL) for analysis.

The study was performed under UK Home Office License PP9552589 Protocol 2 - Pharmacokinetic Study, with local ethical committee clearance. The study was performed by technicians who have completed parts A and B of the UK Home Office Personal License course and who hold current personal licenses.

A calibration line was prepared over the range 0.25–500 ng/mL by spiking PROTAC 3 gefitinib authentic standard solutions into control blank rat plasma. Quality control (QC) standards were also prepared in the same manner at concentrations of 0.5, 5, and 250 ng/mL.

Method Conditions

Calibration standards, QC and study plasma samples were prepared by solid phase extraction using an OASIS HLB SPE plate (p/n 186001828BA). A 10 µL aliquot of plasma was diluted with 10 µL of 4% phosphoric acid containing gefitinib d6 (50 ng/mL) as the internal standard and added to a SPE plate which had previously been conditioned with 200 µL methanol, and 200 µL water, the plate was then washed with 50 µL of 5% aqueous methanol and eluted with (2x) 25 µL acetonitrile.

The plasma extracts (1 µL) were analysed using an ACQUITY™ Premier UPLC™ System, comprising a binary solvent manager, sample manager and column manager connected to a Xevo™ TQ Absolute Tandem quadrupole mass spectrometer. Chromatographic separations were performed using a 2.1 x 100 mm ACQUITY Premier HSS T3 1.7 µm column maintained at 60 °C at a flow rate of 0.6 mL/min. The data was collected using multiple reaction monitoring (MRM) mode using the transitions, cone voltages and collision energies given below. Quantification was performed using gefitinib d6 stable labelled isotope as the internal standard, and 1/X weighting.

LC Conditions

LC system:	ACQUITY Premier UPLC
Vials:	LCGC Certified Clear Glass 12 x 32 mm Screw Neck Vial, Total Recovery. p/n:186000384C
Column(s):	ACQUITY UPLC HSS T3 Column, 100 Å, 1.8 µm, 2.1 mm X 100 mm. p/n:186003539
Sample preparation:	OASIS HLB SPE plate (p/n:186001828BA)
Column temp.:	60 °C
Sample temp.:	20 °C
Injection volume:	2 µL
Flow rate:	0.6 mL/min
Mobile phase A:	Aqueous 0.1% formic acid, 1 mM ammonium formate
Mobile phase B:	95% acetonitrile 5% water, 0.1% formic acid, 1 mM ammonium formate

Gradient Table

Time (min)	Flow rate (mL/min)	%A	%B
0	0.6	95	5
0.5	0.6	95	5
2	0.6	5	95
4	0.6	5	95
4.1	0.6	95	5
5	0.6	95	5

MS Conditions

MS system:	Xevo TQ Absolute
Ionization mode:	Positive ion ElectroSpray Ionization (ESI)
Capillary voltage:	1 kV

PROTAC 3 gefitinib and Internal Standard MRM Transitions

Compound	Precursor ion	Product ion	Dwell time (ms)	Cone voltage	Collision energy
PROTACs	934.4	617.3	0.024	60	34
Gefitinib d6	453.16	134.2	0.024	50	48

Data Management

Acquisition Software:	MassLynx™ 4.2 Software
Processing Software:	TargetLynx™ XS Software

Results and Discussion

Study

PROTACs 3 gefitinib, (2S,4R)-1-((S)-2-(3-(2-((5-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)pentyl)oxy)ethoxy)propanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide, is a PROTAC version of the tyrosine kinase inhibitor (TKI) gefitinib, which has demonstrated an anti-tumour immune response in non-small cell lung cancer models (NSCLC) via epidermal growth factor receptor (EGFR) inhibition.[10] It has a molecular formula of $C_{47}H_{57}ClFN_7O_8S$ and mono isotopic mass of 933.3662 Da. The aim of this study was to determine the pharmacokinetic of this model PROTACs compound following a single subcutaneous administration to male Wistar rats at 10 mg/Kg.

Chromatography

Critical to the success of any LC-MS/MS bioanalytical assay is the ability to separate the target analyte(s) from the endogenous components in the sample and any drug related metabolites and confidently quantify them at low concentrations. The presence of sub structures in analytes which can act as Lewis Acids, able to donate lone pairs of electrons (***e.g.*** uncharged amines, phosphates, and deprotonated carboxylic acids), can interact with the transition metals in the chromatography system and column. These unwanted chemical interactions can result in poor peak shape, analyte absorption onto the LC system and reduction in signal response, which can adversely affect the analysis of low concentrations of drugs and their metabolites in biological samples. ACQUITY Premier UPLC chromatography system and columns employed for this analysis are constructed with high performance surfaces (HPS)[11] which provides an inert chemical barrier preventing the non-specific binding of analytes to the metal surfaces in the chromatography system and columns. The data shown in Figure 2 illustrates the benefits of HPS technology for the chromatographic analysis of PROTACs 3 gefitinib, with the ACQUITY Premier UPLC chromatography system and column delivering a sharper, more symmetrical peak.

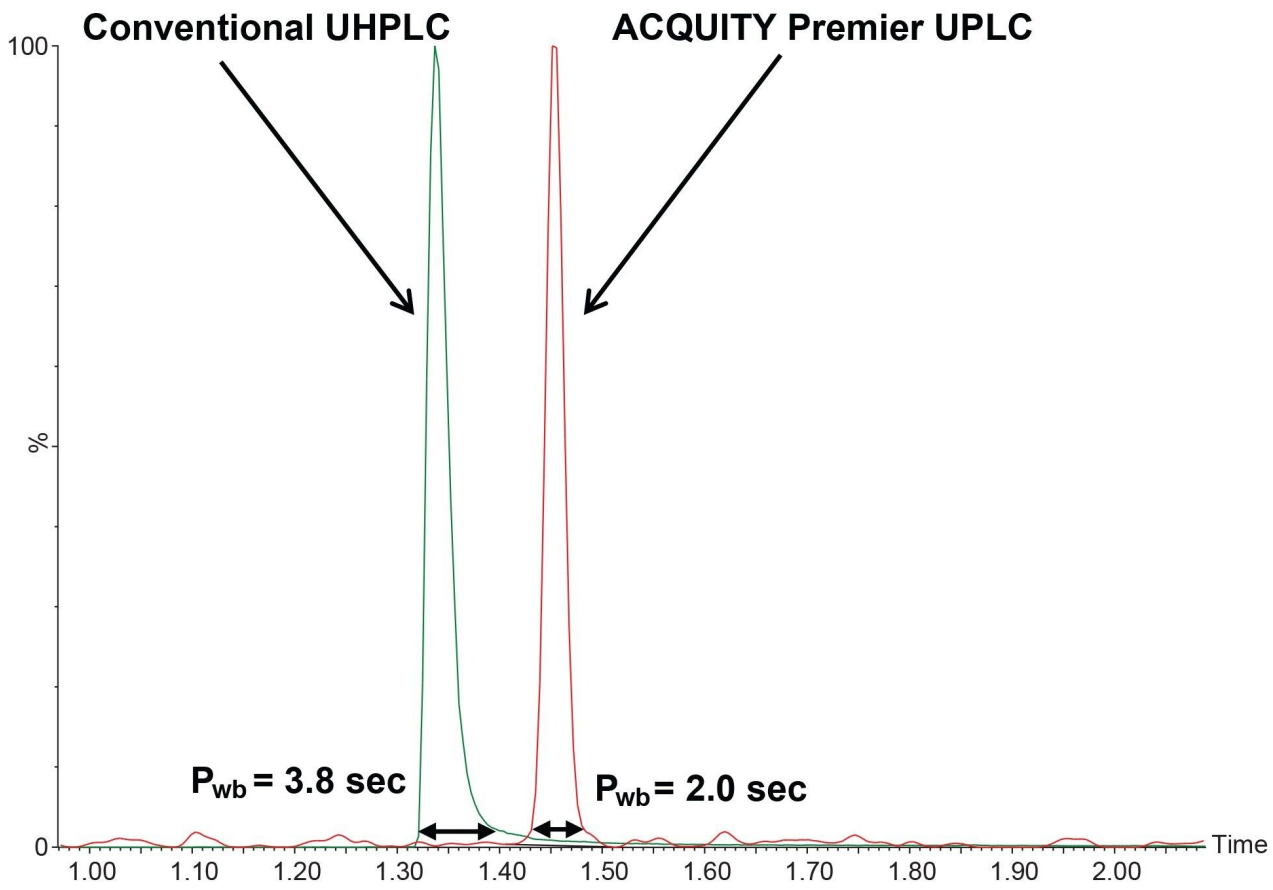

Figure 2. Comparison of chromatographic peak shape for PROTACs 3 gefitinib using ACQUITY Premier chromatography system/column and Conventional UPLC system/column.

LC MS/MS Results

The plasma samples, derived from the PROTACs 3 gefitinib rat study were analysed by reversed – phase UPLC-MS/MS operated in positive ion ESI MRM mode following solid phase extraction using Oasis HLB. The analyte extraction procedure was optimized by comparing the analyte recovery, matrix effects and residual phospholipid concentration for five different sample preparation procedures i) protein precipitation with organic solvent, ii) Sirocco Oasis filter plates, iii) Ostro phospholipid removal plates, iv) Oasis HLB and v) Oasis HLB. The Oasis HLB was selected for this study as it gave the highest analyte recovery. Oasis PRIME HLB produced the lowest matrix effect and residual phospholipids.

PROTACs 3 gefitinib eluted with a retention time of t_R = 1.45 min and the gefitinib d6 internal standard eluted a retention time of t_R = 0.88 min, Figure 3. This data shows that the chromatography system produced a narrow, symmetrical LC peak for both the PROTACs 3 gefitinib and the gefitinib d6 internal standard. There was no evidence of any interference in the blank or control samples. In this study, the plasma sample was diluted 1:5 by the SPE sample extraction process and 2 µL of the sample extract was loaded onto the column giving an overall sensitivity of 100 fg on column.

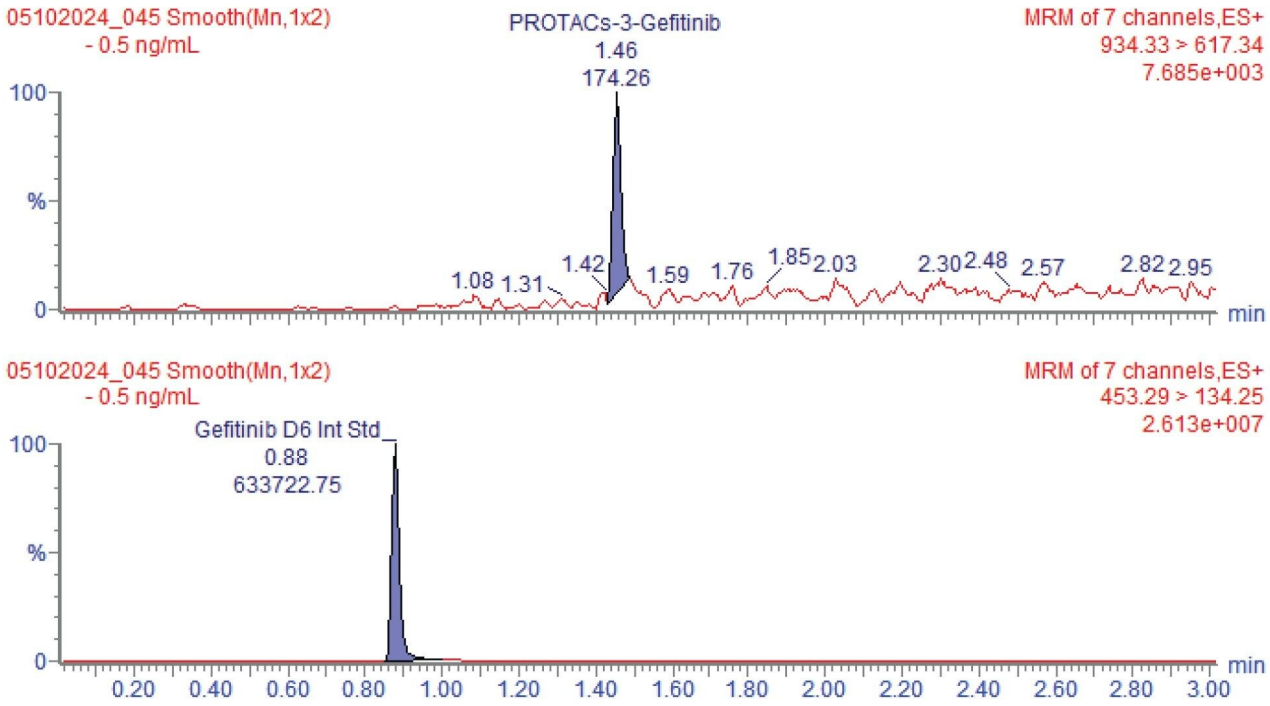

Figure 3. Extracted ion chromatogram of PROTACs 3 gefitinib, 934.33 > 617.34 (upper) and gefitinib d6 453.29 > 134.25 (lower) from the 0.5 ng/mL plasma calibration standard.

A calibration line was constructed over the range 0.25–500 ng/mL. Samples were analysed in the following order; solvent blank; plasma blank; calibration curve (0.25, 0.75, 1, 2.5, 7.5, 10, 25, 50, 75, 100, 500 ng/mL); extracted samples (n = 6) with two QCs at each concentration spaced evenly throughout the analysis (0.5, 5, 250 ng/mL); blank; calibration curve. To be accepted, the batch QC samples had to be within ±15% of the nominal value with a minimum of 2/3 achieving this, and with no more than one failure at each concentration. In the absence of a stable isotope labelled authentic standard for PROTACs 3 gefitinib, gefitinib d6 was employed as an

internal standard. The bioanalytical assay was shown to be linear over the concentration range 0.25–500 ng/mL with a correlation coefficient of r^2 = 0.987 and an intercept of 0.0002, Figure 4.

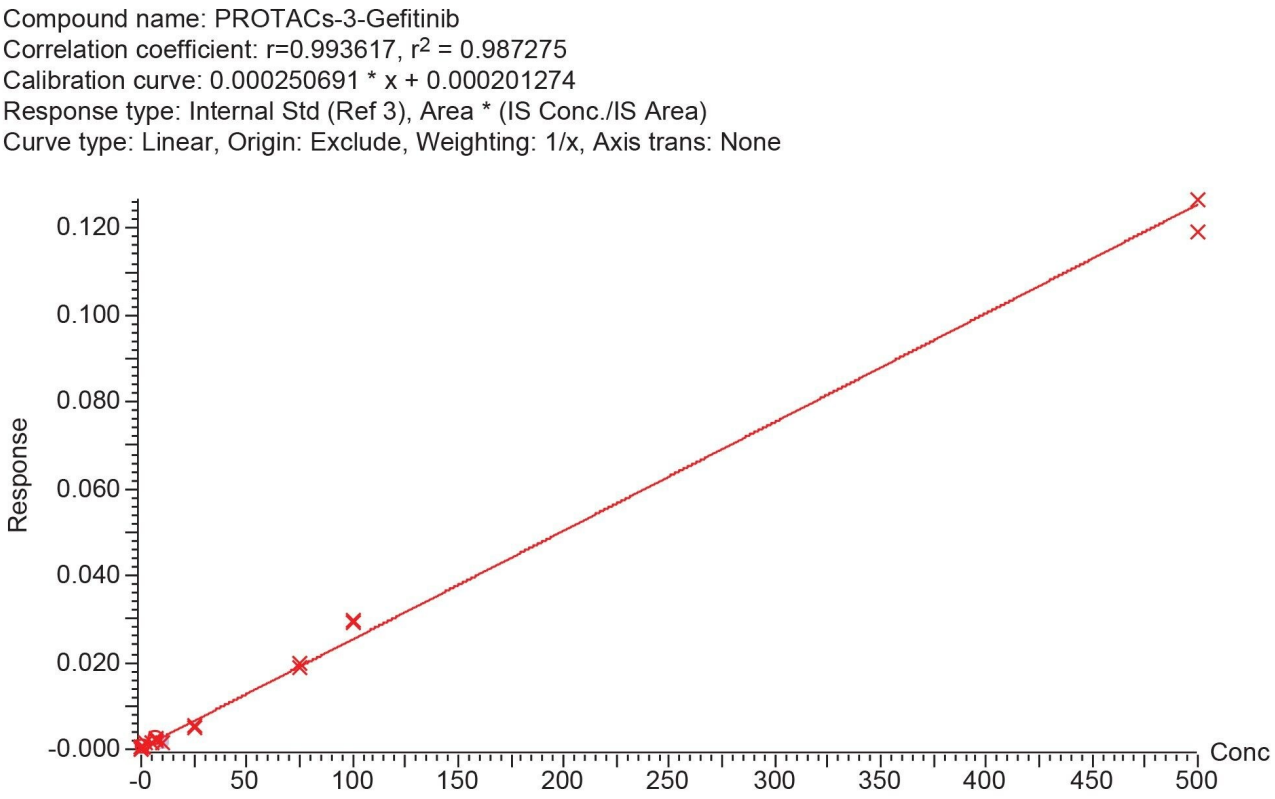

Figure 4. Example calibration line for the quantification of PROTACs 3 gefitinib in rat plasma over the concentration range 0.25–500 ng/mL

Following subcutaneous administration of PROTACs 3 gefitinib at 10 mg/kg, mean plasma concentrations gradually increased following dosing with a peak value of 71.8 ng/mL (62.8 nM) obtained at the 6 h time point. The plasma concentration then declined gradually to a value of 11.3 ng/mL (12.1 nM) at the 24 h time point, Figure 5. The pharmacokinetic half–life ($T_{1/2}$) was determined to be 7.2 h with an area under the curve (AUC _0-t) = 898 nM*hr, Table 1.

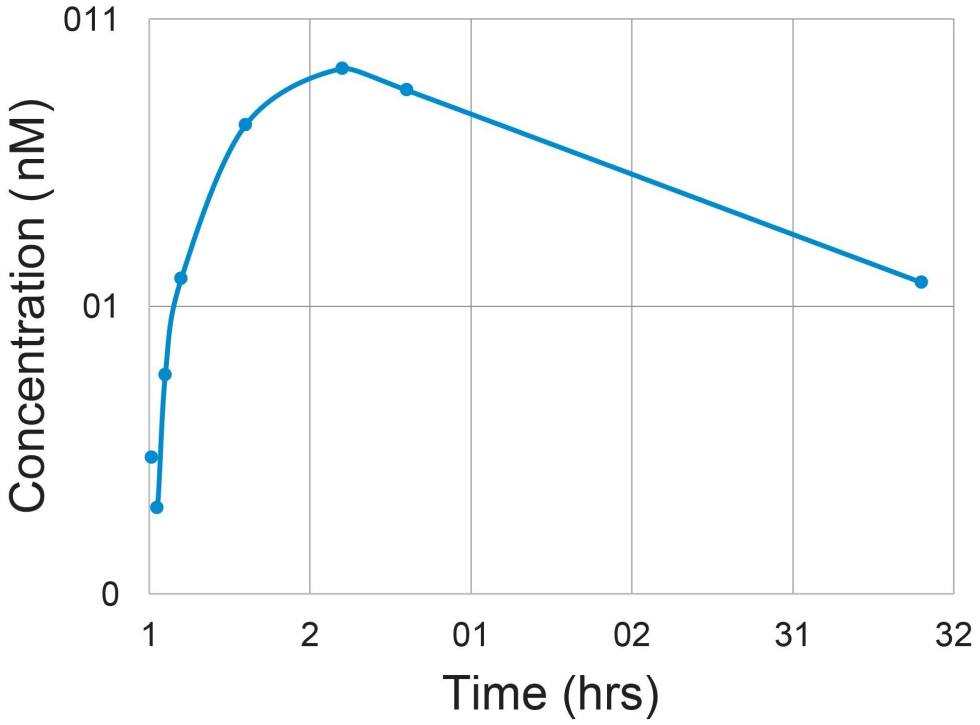

Figure 5. Pharmacokinetics elimination curve of PROTACS 3 gefitinib following subcutaneous administration to the male Wistar rat at 10 mg/kg.

Parameter	PROTACs – 3 gefitinib mean value
$T_{1/2}$ (hr)	7.2
AUC _0-t (nM*hr)	898
AUC _0-inf (nM*hr)	1025
% Extrap	12.3
C_{max} (nM)	67.2
T_{max} (hr)	6

Table 1. Summary of Pharmacokinetics PROTACS 3 gefitinib following subcutaneous administration to the male Wistar rat at 10 mg/kg.

Conclusion

PROTACs molecules are a new class of synthetic therapeutic which hold the potential to improve both efficacy and potency. Unlike small molecules they do not rely on accessing drug-binding cavities deep in a protein structure, instead they degrade the target protein and thus potentially have a long-term pharmacological effect. In this study, we have employed a high sensitivity reversed – phase UPLC-MS/MS bioanalytical assay for the quantification of a PROTACs tyrosine kinase inhibitor in plasma from a rat microsampling study. The UPLC-MS/MS assay showed excellent sensitivity and was linear over the concentration range 0.25–500 ng/mL. The use of the ACQUITY Premier UPLC system and column reduced analyte absorption to the chromatography system and column resulting in a sharper, more symmetrical LC peak. The derived pharmacokinetics showed that, following a subcutaneous administration, a maximum peak plasma concentration of 72 ng/mL was observed 6 h post dose. The compound was eliminated with a half-life ($T_{1/2}$) of 7 h and was still detectable at the 24 h time point.

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