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Quantitation of the Next-Generation Imipridone ONC206 in Human Plasma by a Simple and Sensitive UPLC-MS/MS Assay for Clinical Pharmacokinetic Application

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Keywords

ONC206; Ultra-HPLC; Mass spectrometry; Clinical Pharmacology

1. Introduction

In the context of recombinant tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) as a potential anticancer therapeutic, Allen *et al.* suggests that use of small molecules to upregulate endogenous apoptotic proteins can avoid efficacy limitations faced by protein-based strategies [1]. I*n vitro* and *in vivo* studies of TIC10 (ONC201), a small molecule that transcriptionally induces TRAIL expression, demonstrated monotherapy efficacy and broad-spectrum activity in preclinical models against a variety of cancer types

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CRediT authorship contribution statement

JCG, SMZ, WJR performed research. CJP, VP, TY, JAD, MM, designed experiments and analyzed data. JCG, SMZ, CJP, VP, AA, JAD, MM wrote the manuscript. BJT, MG, WDF designed clinical trial.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

[1-3]. Further mechanistic studies revealed that ONC201 is a selective antagonist of G-protein coupled receptors (GPCR) dopamine receptor D2 (DRD2) and D3 as well as an agonist of mitochondrial protease ClpP. Downstream of target engagement, ONC201 induces the integrated stress response pathway and inhibits Akt/ERK signaling that ultimately results in selective inhibition of tumor cell viability and apoptosis [4]. Additionally, ONC201 has demonstrated clinical efficacy based on radiographic response rates and disease-free survival outcomes in H3 K27M mutant Diffuse Midline Gliomas [5].

Further investigation revealed the integral role of its unique pharmacophore structure, a triheterocyclic scaffold called imidazopyridopyrimidone, in determining therapeutic potency and range of efficacy [6-9]. Additional analogues were synthesized as a potential means to modulate the target engagement and phenotypic efficacy. Select derivative structures that maintained the inner imipridone structure with altered peripheral moieties exhibited distinct target engagement and increased efficacy and sensitivity in biochemical assays compared to ONC201 [10].

ONC206, the second imipridone to enter clinical development, is a DRD2/3 antagonist and ClpP agonist that exhibits differentiated receptor pharmacology and gene expression profiles in tumors relative to ONC201. The compound is orally bioavailable, penetrates the blood-brain barrier, and exhibits anti-cancer efficacy and safety in several preclinical cancer models with pronounced efficacy in myc-overexpressing CNS tumors [11, 7, 12, 12, 13]. This agent also activates the same downstream signaling pathways including integrated stress response to elicit TRAIL-induced apoptosis [14, 15]. As such, ONC206 is under investigation in an ongoing, first-in-human, Phase I clinical trial for adults with recurrent primary central nervous system (CNS) neoplasms (NCT04541082).

To characterize the clinical pharmacokinetic profile of ONC206, a simple and sensitive, fully validated method for quantifying ONC206 in human plasma at clinically relevant concentrations is crucial. This report demonstrates that this assay is sensitive, selective, accurate, precise, and robust.

2. Experimental

2.1. Materials

Both ONC206 (HPLC purity 99%) and the internal standard, $[^2H]_7$-ONC206 (HPLC purity 98.54%), were provided by Chimerix Inc. (Durham, NC). Analyte and internal standard chemical structures are shown in Figure 1. Drug-free human plasma (EDTA-treated, pooled mixed gender) and drug-free human whole blood (EDTA-treated, individual donor) were provided by the National Institutes of Health Clinical Center Blood Bank (Bethesda, MD). Optima® HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium formate (≥99.0%) was purchased from Honeywell Fluka™ (Charlotte, NC). Ammonium hydroxide solution (NH_4OH; ACS reagent grade) was purchased from Sigma-Aldrich (St. Louis, MO). Milli-Q water used in the preparation of stock solutions and mobile phases was deionized and ultra-filtered (18.2 MΩ·cm) on a MilliPore system (EMD MilliPore, Milford, MA). Human hemoglobin and trigycleride mix were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Preparation of stock solutions

For preparation of the master stock solutions, ONC206 and $[^2H]_7$-ONC206 were individually dissolved in 50/50 (v/v) water/ACN at concentrations of 1 mg/mL. Master stocks were vortexed and sonicated, then transferred to amber glass vials for storage at −80 °C. Working stock solutions were prepared by serial dilution of an ONC206 master stock in 50/50 (v/v) water/ACN at concentrations of 10000, 5000, 2000, 1000, 500, 100, 40 and 20 ng/mL. Working stock solutions were stored in amber glass vials at −80 °C and thawed in a water bath to prepare calibration and quality control (QC) standards. To prepare the internal standard solution, an $[^2H]_7$-ONC206 master stock (1 mg/mL) was diluted in 50/50 (v/v) water/ACN for a final concentration of 10 ng/mL in a glass solvent bottle and stored at 4 °C.

2.3. Sample preparation

For each analytical set, calibration and QC standards were freshly prepared in duplicate and quintuplet, respectively, by diluting the required amounts of ONC206 working stocks in drug-free human plasma (EDTA-treated, pooled mixed gender). Concentrations for calibration standards were 500, 250, 100, 50, 25, 5, 2 and 1 ng/mL. QC standards had concentrations of 1 (lower limit of quantitation; LLOQ), 3 (low-range, LQC), 40 (mid-range, MQC), 400 (high-range, HQC), and 4000 ng/mL (10-fold dilution; DQC).

Calibration standards were spiked with 5 uL working stock into 95 uL of blank plasma, then aliquoted to 25 uL. QCs and study samples were aliquoted at 25 μL of plasma. All were mixed with 25 μL of the internal standard solution and 500 μL of additional pure ACN. This mixture was vortexed vigorously, then centrifuged at 17,000 x g for 10 min at 4 °C. Approximately 450 μL of supernatant was transferred to a 96-well, 2 mL collection plate. Samples were dried under nitrogen gas at 40 °C, before reconstitution with 300 μL of (60/40, v/v) water/methanol. Reconstituted samples in the plate were briefly vortexed, then centrifuged at 4 °C for 5 min at 8720 x g before injection.

2.4. Instrument conditions

Ultra-high performance liquid chromatography–tandem mass spectrometry (uHPLC-MS/MS) was used to develop and optimize this assay. Each sample (10 μL) was injected onto a Phenomenex Kinetex C18, 50 x 2.1 mm, 1.3 μm, 100 Å analytical column kept at ambient temperature. Mobile phases A and B were composed of 0.1% ammonium formate (w/v, aqueous), pH 8.5 and 1/1 (v/v) ACN/methanol, respectively. Isocratic conditions of 60% B at a 0.4 mL/min flow rate were maintained for the entirety of the 2 min run using a Waters ACQUITY UPLC® system (Waters, Milford, MA) with a binary solvent pump and refrigerated autosampler set to 4 °C.

An AB SCIEX QTRAP® 5500 mass spectrometer (Sciex, Foster City, CA) was used to detect the product ion of ONC206 (*m/z* 408.8→127.2) and $[^2H]_7$-ONC206 (*m/z* 416.0→127.1) using multiple reaction monitoring (MRM). The electrospray ionization (ESI) source and MRM parameters were optimized using SCIEX Analyst®, v1.7. The Turbo Ionspray source was set to operate in ESI positive mode a source temperature of 500 °C, ion spray voltage of 5500 V, desolvation gas 1 and 2 (N_2) of 30, curtain gas (N_2) of 30

and medium collision gas. The optimized MRM parameters for the analyte and IS were set, respectively, to the following: collision energies of 45 and 70 V, declustering potentials of 59 and 50 V, entrance potentials of 12 and 14 V, collision cell exit potentials of 19 and 14 V, and dwell times of 100 msec for both transitions. MRM peak integration, quantitation, and other data analyses were obtained with the Sciex OS-MQ® software.

2.5. Validation

2.5.1. Linearity—Assay calibration for ONC206 was established with least-squares linear regression analysis of an eight-point calibration curve (1–500 ng/mL) by plotting the analyte-to-internal standard peak area ratio versus the analyte to internal standard concentration ratio. A weighting factor of $1/x^2$, where x is the ratio of nominal analyte to internal standard concentration, was applied to each calibration curve. Calibrator response functions and choice of regression analysis were evaluated by percent deviation (% DEV) and determination coefficients (r^2) for all calibrators.

2.5.2. Accuracy and precision—Accuracy and precision were investigated by quantifying ONC206 at five different concentrations of QC samples over four separate days. Each analytical set was freshly prepared and consisted of a blank plasma control in duplicate, an internal standard-only plasma control in duplicate, calibration standards in duplicate ($n = 8$), and all QC and LLOQ-QC standards in quintuplet ($n = 20$). Accuracy (% DEV) was calculated as the percent difference between the mean observed analyte concentration and the nominal concentration. Precision was evaluated using within-run precision (WRP) and between-run precision (BRP), which were calculated according to the following equations:

$$\mathrm{WRP} = 100 \times [(\mathrm{MS_{WIT}})^{0.5} / \mathrm{GM}]$$

$$\mathrm{BRP} = 100 \times [((\mathrm{MS_{BET}} - \mathrm{MS_{WIT}}) / \mathrm{n})^{0.5} / \mathrm{GM}],$$

where GM represents grand mean, defined as the mean of all observed concentrations for each concentration; $\mathrm{MS_{WIT}}$ represents within-group mean squared; $\mathrm{MS_{BET}}$ represents between-group mean squared; and n represents the number of repetitions. The parameters GM, $\mathrm{MS_{WIT}}$, and MSBET were calculated from the 1-way ANOVA using GraphPad Prism 8 (v.8.3.0). In accordance with FDA Bioanalytical Guidelines [21], accuracy (less than ±15% bias) and precision (less than 15% CV) was acceptable, except for LLOQ standards, which were allowed less than ± 20% bias (accuracy) and <20% variability (precision).

2.5.3. Plasma stability—The stability of ONC206 in plasma was evaluated after 2 hr at both room temperature and 4 °C to assess optimal preparation temperature of thawed plasma samples. Two sets of low- and high-range concentrations of 2 ng/mL and 250 ng/mL, respectively, were prepared in matrix and aliquoted in triplicate ($n = 6$, per set). The first set of low- and high-range samples were left to sit for 2 hr at room temperature while the second was kept at 4 °C for the same duration before protein precipitation. The

mean calculated analyte concentrations of the low-range samples (n = 3) and high-range samples (n = 3) from each set, room temperature and 4 °C, were compared to that of the corresponding standard samples freshly prepared in duplicate for the calibration curve of the same analytical run.

2.5.4. Whole blood stability—The stability of ONC206 in individual EDTA-treated whole blood was assessed after 2 hr at both room temperature and 4 °C. Two sets of low- and high-range concentrations of 2 ng/mL and 250 ng/mL, respectively, were prepared in matrix and sat at room temperature for 15 min to achieve plasma-RBC equilibrium. The first set of low- and high-range samples were left to sit for 2 hr at room temperature while the second was kept at 4 °C for the same duration. Following the resting period, all whole blood samples were placed in a microcentrifuge (4 °C) and centrifuged for 15 min at 1500 x g. The separated plasma of each sample was aliquoted in triplicate, then processed with the same sample preparation procedure as all other calibrator and QC standards. The mean calculated analyte concentrations of the low-range samples (n = 3) and high-range samples (n = 3) from each set, room temperature and 4 °C, were compared to that of corresponding samples freshly prepared, following the same whole blood extraction procedure, at the same time as the calibration and QC standards.

2.5.5. Autosampler re-injectability—The re-injectability of ONC206 in the 96-well, 2 mL collection plate for 24 hr in a refrigerated autosampler set at 4 °C was performed. A full validation set—double blanks (no analyte, no IS), IS-only blanks, calibration standards, and QC standards—was prepared in plasma, injected, and kept in refrigerated autosampler. All samples were re-injected and re-analyzed 24 hr after the initial injection. The mean calculated concentrations of each QC sample set of replicates (n = 5, per QC level) were compared to that of the initial values obtained from those same samples.

2.5.6. Long-term working stock stability—The long-term freezer storage stability of ONC206 in 50/50 (v/v) ACN/water was tested. Two sets of low- and high-range calibration standards at concentrations of 2 ng/mL and 250 ng/mL, respectively, were prepared in reconstitution solution (60/40 (v/v) water/methanol) using previously prepared working stocks and aliquoted in triplicate (n = 6, per set). Working stocks prepared on the following dates were used: 10/29/20 (8.5 months-old); 04/29/21 (2.5 months-old); and 06/16/21 (1 month-old). All working stocks were stored at −80 °C when not in use. The mean calculated analyte concentrations of the low-range samples (n = 2) and high-range samples (n = 2) from each set, 8.5- and 2.5-month-old stocks, were compared to that of the corresponding standard samples freshly prepared in duplicate for the calibration curve of the same analytical run.

2.5.7. Freeze-thaw stability—Frequently thawing and refreezing samples for assay preparation and analysis can increase the risk of compound degradation over time. Potential degradation of ONC206 after various freeze/thaw cycles was assessed at low- and high-range concentrations of 2 ng/mL and 250 ng/mL, respectively. Four sample sets, consisting of one low and one high-range standard, were prepared in plasma and aliquoted in triplicate (n = 6, per set). In one freeze/thaw cycle, samples were thawed at room temperature

then refrozen at −80 °C for a storage interval of at least 12 hr. The first set of samples underwent one freeze/thaw cycle; the second set, two; the third, three; and the fourth, four. The mean calculated analyte concentrations of the low-range samples (n = 3) and high-range samples (n = 3) from each set, 1-4 freeze/thaw cycles, were compared to that of the corresponding standard samples freshly prepared in duplicate for the calibration curve of the same analytical run.

2.5.8. Matrix effects—Matrix effects of EDTA human plasma on ONC206 signal was assessed. Individual lots of blank plasma were extracted with ACN, then spiked with ONC206 at low (2 ng/mL) and high concentrations (250 ng/mL) in quintuplet, representing 100% recovery of ONC206. These samples were run alongside neat reconstitution solution spiked with ONC206 at 2 ng/mL and 250 ng/mL in quintuplet, representing 100% recovery of ONC206 and no matrix. The ratios of average peak areas were calculated to determine matrix factor.

Hemolysis effect was assessed in low (160 mg/dL) and high (800 mg/dL) concentrations of hemoglobin in EDTA plasma. These represent mild and severe hemolysis, respectively.[16] EDTA plasma was spiked at these concentrations with human hemoglobin, vortexed, then spiked with ONC206 at LQC and HQC levels, in triplicate, and processed alongside LQC and HQCs in non-hemolyzed EDTA plasma. Average peak area ratios of ONC206/$[^2H]_7$-ONC206 were compared to assess hemolysis effect.

Lipemic effect was also assessed in low (100 mg/dL) and high (1000 mg/dL) concentrations of triglyceride in EDTA plasma. These represent minimal and moderate to severe lipemia, respectively.[17] EDTA plasma was spiked at these concentrations with triglyceride mix, vortexed, then spiked with ONC206 at LQC and HQC levels, in triplicate, and processed alongside LQC and HQCs in blank human EDTA plasma. Average peak area ratios of ONC206/$[^2H]_7$-ONC206 were compared to assess lipemic effect.

2.5.9. Extraction and processing efficiency—Extraction efficiency, or extraction recovery, was assessed by extracting individual lots of blank EDTA plasma with ACN, then spiking ONC206 at low (2 ng/mL) and high concentrations (250 ng/mL) in quintuplet, representing 100% extraction. These samples were run alongside samples prepared as usual; individual plasma lots were spiked at the same low and high concentrations, then extracted with ACN. The ratio of the average signal areas represents the extraction efficiency.

Process efficiency, or overall recovery throughout sample processing, was assessed by spiking neat reconstitution solution at low (2 ng/mL) and high (250 ng/mL) concentrations. The average signal areas were compared against plasma lots spiked at the same low and high concentrations, then extracted and prepared as described in section 2.3.

2.5.10. Selectivity—Selectivity was assessed using a set of double blank plasma controls in duplicate (n = 12) and LLOQ samples in quintuplet (n = 30), individually prepared using six unique lots of plasma. Peak areas from blank matrix samples and spiked matrix samples were compared.

2.5.11. Clinical application—Patient samples of unknown ONC206 concentrations were prepared and analyzed to determine the reliability of this assay for accurate patient data and applicability to clinical pharmacokinetics. In the associated Phase I dose-escalation trial, blood samples were collected in lavender top (K2 EDTA) tubes pre-dose and following an oral dose at 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 hr post-dose. After blood samples were centrifuged for 15 min at 1500 x g, an aliquot of the separated plasma was removed and processed with the same sample preparation procedure as all other calibrator and QC standards. The National Cancer Institute Institutional Review Board approved this clinical protocol (NCT04541082) and participating patients provided informed consent.

3. Results

The optimized uHPLC-MS/MS assay produced reliable chromatograms with a sharp, symmetrical peak eluting at 0.99 min of the 2 min run (k'= 2.3). There were no interfering peaks present in plasma (Figure 2A) nor cross-inference between the deuterated IS and the unlabeled analyte (Figure 2B). Further, there was a strong signal at the LLOQ of 1 ng/mL (Figure 2C) and no carryover, given that ONC206 signal area in a blank injection following the ULOQ was <5% of the LLOQ signal area.

3.1. Validation

Each standard included in the 8-point calibration curve, ranging from 1 – 500 ng/mL, was run in duplicate on 4 different days ($n = 8$). The standards demonstrated good accuracy (range - 3.5 to 8.1% deviation) and precision (range 3.2 - 10.9 %CV), with excellent correlation ($r^2 = 0.99732 \pm 0.0010$; $n = 4$) using linear regression and $1/x^2$ weighting (Table 1). QC standards also demonstrated high accuracy (range −4.1 to 2.4% deviation) and precision (BRP range 1.5 to 5.3 %CV; WRP range 2.8 to 6.7 %CV) (Table 2). This high between run precision across 4 separate days demonstrates the ruggedness of this assay. Calibration standards and QC standards easily fall within FDA Bioanalytical Guidance acceptance criteria for method validation [18].

3.2. Stability

Short-term 2-hr benchtop stability at room temperature and at 4 °C demonstrated nonsignificant deviation of ONC206 in human plasma from nominal values (range 0.3 to 2.8% deviation relative to fresh controls; Table 3), indicating reliable stability over the duration of sample preparation. ONC206 was stable in whole blood for 2 hr at room temperature and 4 °C (range −2.4 to 7.0% deviation relative to fresh controls), which is relevant for processing to plasma upon sampling from subjects. Working stocks of ONC206 dissolved in 50/50 (v/v) water/ACN stored at −80 °C for 8.5 months showed nonsignificant deviation (range −3.8 to 8.6%) relative to freshly prepared stocks. Extracted plasma samples were injected, then kept at 4 °C in the autosampler for 24 hr and re-injected. Nonsignificant deviation (range −3.3 to −1.4%) of calculated concentrations demonstrated accurate re-injectability. Plasma low- and high-range concentration samples showed nonsignificant deviation (range −7.0 to 2.8%) over 4 freeze-thaw cycles. These data allow working stocks to be used over multiple days, samples to be frozen and thawed up to 4 cycles, and re-analysis of extracted samples within 24 hr in the instance of instrument hardware failure.

3.3. Matrix effects

The matrix effect of EDTA plasma at low (2 ng/mL) and high (250 ng/mL) concentrations was 89.9% and 85.3%, respectively. The hemolysis effect of EDTA plasma with low (160 mg/dL) hemoglobin at low (3 ng/mL) and high (400 ng/mL) ONC206 concentrations were 103 and 98%, respectively, and in high (800 mg/dL) hemoglobin samples, 106 and 105%, respectively. The lipemic effect of EDTA plasma with low (100 mg/dL) triglyceride at low (3 ng/mL) and high (400 ng/mL) ONC206 concentrations were 111 and 109%, and in high (1000 mg/dL) triglyceride samples, 103 and 108%, respectively. Matrix effects were nominal.

3.4. Extraction and processing efficiency

The extraction efficiency at low (2 ng/mL) and high (250 ng/mL) concentrations was 95.4 and 93.6%, respectively (Table 4). The process efficiency at low (2 ng/mL) and high (250 ng/mL) concentrations was 85.8 and 80.0%, respectively (Table 4).

3.5. Selectivity

The uHPLC-MS/MS method selectively detected and quantified ONC206 at 1 ng/mL (LLQC), as the calculated concentration of LLOQ spiked into six different lots of plasma had a bias (percent deviation from the expected 1 ng/mL) of < 5% (range −3.9 to 4.9% from nominal 1.0 ng/mL). These same six lots of blank plasma (no ONC206, no IS) were prepared and analyzed with this uHPLC-MS/MS assay, and there was no quantifiable peak present in any lot.

3.6. Clinical Application

To demonstrate the reliability of this assay and application to clinical pharmacokinetic analysis, samples were analyzed from 3 clinical trial participants collected pre-dose and 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 hr following administration of either a 50, 100, or 150 mg dose. Figure 3 depicts the plasma concentration versus time curves of 3 individual patients. Preliminary results suggest rapid absorption of ONC206 following an oral dose, an average maximal concentration occurring around 2 hr for these three patients, and that this assay adequately measures drug concentrations across clinical dose escalation.

4. Conclusions

Herein, we present a novel, sensitive, and fully validated uHPLC-MS/MS assay for the quantification of ONC206 in human plasma. This assay meets FDA Bioanalytical Guidelines for accuracy, precision, and selectivity. ONC206 is stable during working conditions and multiple freeze-thaw cycles. Further, we demonstrate the clinical pharmacokinetic application and ability to quantify therapeutically relevant, first-in-human plasma concentrations of oral ONC206. This assay offers a simple and robust method for ongoing (NCT04541082) and future ONC206 clinical trials.

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Abbreviations

HPLC	high performance liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
QC	Quality control
LLOQ	lower limit of quantification
ULOQ	upper limit of quantification

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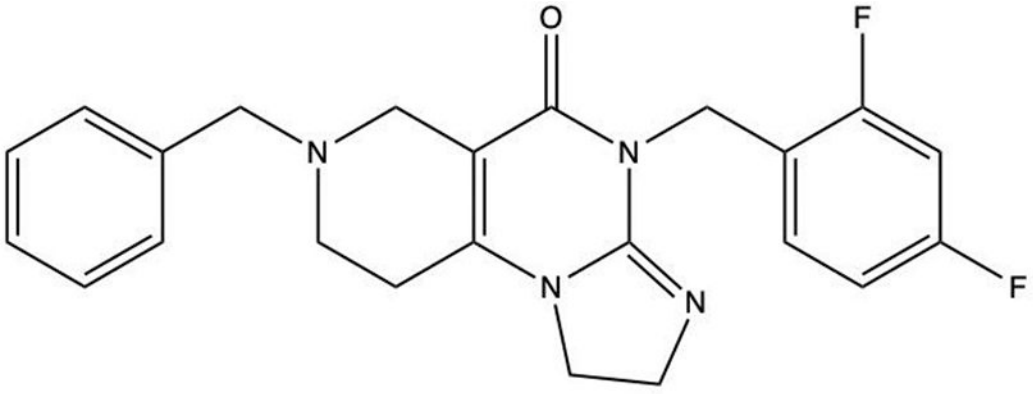
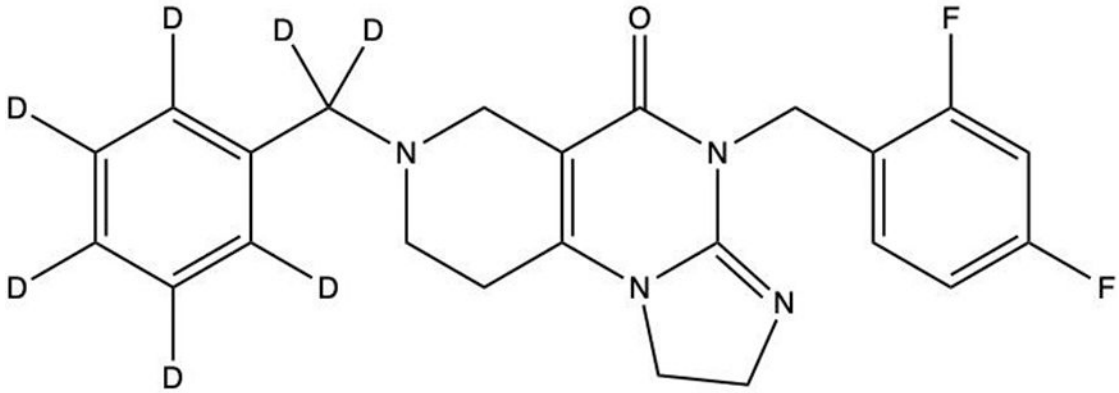
Highlights

- A uHPLC-MS/MS assay was developed and validated for the quantification of a novel anticancer drug, ONC206
- Rapid, one-step sample preparation was implemented
- Accuracy, precision, and matrix effects of human plasma on analyte met FDA Bioanalytical Guidelines
- Lower limit of quantitation was 1 ng/mL
- Pharmacokinetic application for first-in-human clinical trial of oral ONC206 for adult patients with recurrent primary central nervous system neoplasms



ONC206



ONC206-D_7

Figure 1.
Structures of ONC206 and the internal standard, ONC206-D_7.

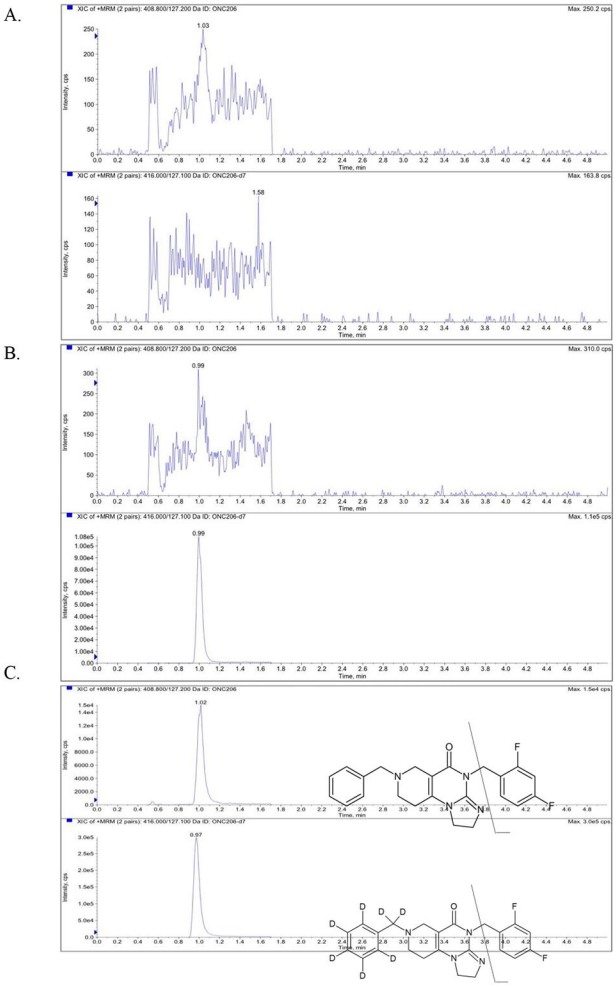


Figure 2.

LC-MS/MS chromatograms of (A) a blank plasma extract, (B) internal standard only extract, and (C) the lower limit of quantification (LLOQ) with proposed fragmentation patterns.

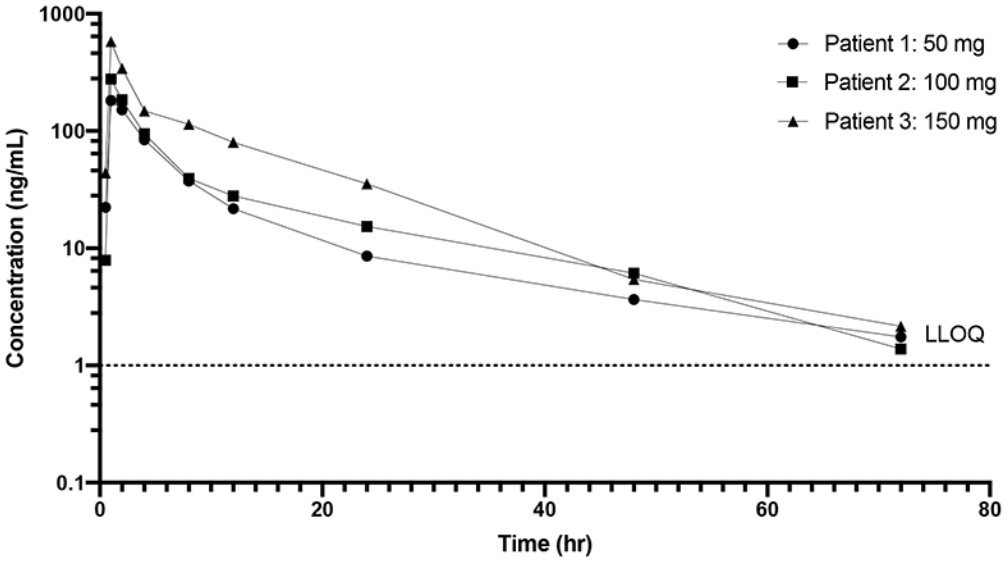


Figure 3.

Phase I clinical trial data. ONC206 Cycle 1 plasma concentration vs. time curve for individual patients dosed at 50 mg, 100 mg, and 150 mg.

Table 1

Calibration linearity.

Nominal (ng/mL)	GM (ng/mL)	SD (ng/mL)	DEV (%)	R.S.D (%)	n
1	0.987	0.0343	−1.28	3.47	8
2	2.16	0.235	8.07	10.9	8
5	5.12	0.163	2.30	3.18	8
25	26.6	2.24	6.53	8.41	8
50	50.7	1.61	1.46	3.17	8
100	101	5.52	0.947	5.46	8
250	243	9.19	−2.94	3.79	8
500	483	26.6	−3.48	5.50	8

Table 2

Accuracy and precision.

Nominal (ng/mL)	GM (ng/mL)	SD (ng/mL)	DEV (%)	WRP (%)	BRP (%)	n
1	1.01	0.0823	0.951	6.70	5.27	20
3	2.99	0.137	−0.314	3.77	2.87	20
40	40.9	1.26	2.43	2.80	1.51	20
400	386	21.8	−3.46	5.22	2.43	20
4000	3840	201	−4.11	5.39	-	19

*A hyphen indicates the within-run mean square > between-run mean square, thus producing a negative number (cannot take square root of a negative number). Thus, it is safely assumed that no additional variability was observed as a result of performing the assay in different runs.

**One DQC replicate did not inject.

Table 3

Analyte stability.

	Matrix/ Conditions	Nominal (ng/mL)	GM (ng/mL)	DEV from Nominal (%)	R.S.D (%)	DEV from (%)	n
2 hr Plasma Stability	Plasma, Fresh	2.0	2.0	0.1	2.8	-	2
		250.0	238.9	−4.4	3.5	-	2
	Plasma/2hr, RT	2.0	2.0	1.9	4.2	1.9	3
		250.0	243.0	−2.8	1.0	1.7	3
	Plasma/2hr, 4°C	2.0	2.1	2.9	3.8	2.8	3
		250.0	239.7	−4.1	1.9	0.3	3
Autosampler Re-injectability	Fresh	1.0	1.1	12	2.7	-	5
		3.0	3.1	3.8	1.3	-	5
		40.0	43.2	8.1	1.4	-	5
		400.0	401	0.2	0.6	-	5
	24 hr, 4°C	1.0	1.1	8.4	3.1	−2.9	5
		3.0	3.0	1.5	1.0	−2.2	5
		40.0	41.8	4.6	1.7	−3.3	5
		400.0	395	−1.2	2.0	−1.4	5
Freeze-Thaw Stability	Plasma, Fresh	2.0	2.2	8.5	2.6	-	2
		250.0	261.8	4.7	0.25	-	2
	Plasma/1x FT cycle	2.0	2.1	6.7	2.6	−1.7	3
		250.0	251.8	0.7	9.0	−3.8	3
	Plasma/2x FT cycle	2.0	2.2	11.5	1.2	2.8	3
		250.0	255.3	2.1	1.3	−2.5	3
	Plasma/3x FT cycle	2.0	2.1	3.8	2.9	−4.3	3
		250.0	243.4	−2.6	4.1	−7.0	3
	Plasma/4x FT cycle	2.0	2.0	1.8	1.9	−6.1	3
		250.0	252.0	0.8	6.8	−3.7	3

Table 4

Assay Efficiency.

	Matrix	Nominal (ng/mL)	%
Matrix Effect	Plasma	2.0	89.9
		250.0	85.3
Extraction Efficiency	Plasma	2.0	95.4
		250.0	93.7
Processing Efficiency	Plasma	2.0	85.8
		250.0	80.0