# Analytical Methods

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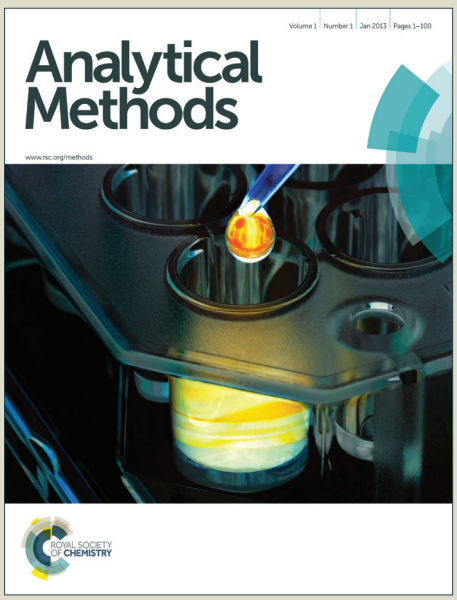





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# Development and validation of an UPLC-MS/MS method for quantitative analysis of OTX015 in human plasma samples

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## ABSTRACT

OTX015 is a novel synthetic thienodiazepine analog, which potently inhibits bromodomains (BRD) 2, 3 and 4 of the BET (bromodomain and extraterminal) protein family. It is currently undergoing phase I evaluation in patients with hematologic malignancies using an oral formulation. We developed and validated an Ultra Performance Liquid Chromatography method with tandem Mass Spectrometry detection (UPLC-MS/MS) for quantification of OTX015 in plasma in order to investigate its pharmacokinetics in humans, using small plasma samples (50 µL), and an internal standard, Y-401. Chromatographic separation was performed on a BEH C18 UPLC column with a mobile phase gradient at a flow rate of 0.5 mL/min for 5 minutes. Quantification was performed using the transition 492-383 (*m/z*) for OTX015 and 506-383 (*m/z*) for Y-401. The lower limit of quantification (LLOQ) was established as 1 ng/mL with 10.80% precision and 94.67% accuracy. The calibration curve was linear up to 250 ng/mL (upper limit of quantification). Intra-assay precision ranged from 7.3% to 11.6% for the three quality control (QC) concentrations evaluated and was 16.0% for the LLOQ, and intra-assay accuracy ranged from 93.7% to 109.8% for the three QC concentrations and was 92.0% for the LLOQ. Inter-assay precision and accuracy ranged from 4.1% to 14.0% and from 92.3% to 104.8%, respectively. These data show that the UPLC-MS/MS procedure is sensitive, accurate, precise and robust, and was validated for determining OTX015 concentrations in plasma. The method was successfully applied to determine the pharmacokinetic profile of OTX015 in patients treated in an ongoing phase I clinical study.

## Introduction

The BET (bromodomain and extraterminal domain) family of proteins, characterized by a tandem bromodomain (BRD) repeat, includes BRD2, BRD3, BRD4 and BRDT. BRD2[1] and BRD4[2] bind to acetylated lysine residues on histone proteins, mobilizing chromatin modifications[3] that alter chromatin accessibility to transcription factors and allow recruitment and activation of RNA polymerases. Several BET proteins, including BRD2, BRD3 and BRD4, have emerged as key epigenetic regulators of proliferation and differentiation. Recent studies have shown that inhibition of the BET family proteins results in antitumor activity in a range of leukemia and lymphoma cell lines.[4-9]

OTX015, 2-[(6*S*)-4-(4-Chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*]-[1,2,4] triazolo [4,3-*a*] [1,4] diazepin-6-yl]-*N*-(4-hydroxyphenyl)-acetamide dehydrate is a novel synthetic thienodiazepine analog. Experimental evidence showed that OTX015 targets BRD2, BRD3 and BRD4 and inhibits binding of these proteins to acetylated histones.[10] OTX015 demonstrates significant antitumor activity in several hematologic malignancy models[10] including acute leukemia (AL),[11] multiple myeloma,[12] and lymphomas.[13,14]

OTX015 is currently under clinical development, including an ongoing phase I study in hematologic malignancies. Appropriate methodology for determining OTX015 concentrations in human plasma is essential for clinical pharmacokinetic studies. Liquid chromatography tandem mass spectrometry (LC/MS/MS) is a reference method for drug quantification in plasma samples.[15] We employed an Ultra Performance Liquid Chromatography (UPLC) system with a mass spectrometer used in a positive electrospray ionization mode for detection, to develop and validate a sensitive, specific, rapid and robust UPLC-MS/MS method for OTX015 quantification in plasma.

## Results and discussion

OTX015 and Y-401, the internal standard (IS), were detected and quantified over a total run time of 5 minutes. For the selected transitions of OTX015 (m/z 492 → 383) and the IS (m/z 506 → 383), retention times were 2.10 and 2.02 minutes, respectively.

*Validation of the analytical method*

*Linearity and sensitivity*

The assay was linear over the range 1-250 ng/mL OTX015, and linear regression yielded the following equation: y = 0.0718 (0.0694-0.0742) x + 0.0018 (-0.0219-0.0255) ($r^2$=0.990). A reproducible linear relationship between concentration and response was found over the measured concentration range. Deviation from -14% to 12.7% was observed across the six calibration levels (six replicates).

The lower limit of quantification (LLOQ) of OTX015 was established (using six replicates) to be 1 ng/mL with a precision of 10.80% and an accuracy of 94.67%, with a signal surface more than five times that of blank plasma based on a signal-to-noise ratio of 89 (RMS signal-to-noise calculation method). The LLOQ of M1 (an OTX015 metabolite) was established as 5 ng/mL with a precision of 5.52% and an accuracy of 98.10%, with a peak area more than five times that of blank plasma based on a signal-to-noise ratio of 110. Responses at the LLOQ were identifiable, discrete and reproducible, and precision was within 20% and accuracy within 80%-120%, in accordance with the FDA guidelines.[16]

*Precision and accuracy*

The results of the assay performance at four quality control (QC) concentrations (five replicates) are summarized in Table 1. Intra-assay precision ranged from 7.3% to 11.6% for the three QC concentration levels (3, 80 and 220 ng) and was 16.0% for the LLOQ. Intra-assay accuracy ranged from 93.7% to 109.8% for the three QC concentration levels and was 92.0% for the LLOQ.

Inter-assay precision and accuracy ranged from 4.1% to 14.0% and from 92.3% to 104.8%, respectively. All observed data for the intra- and inter-assay were less than 15% for the three QC concentrations and less than 20% for the LLOQ concentration. These data demonstrate that the method provides adequate accuracy and precision for OTX015 determination in plasma samples.

**Table 1: Intra-day and inter-day precision and accuracy for OTX015 (n=5) in human plasma**

| OTX015 | Intra-assay | | | | Inter-assay | | | |
|---|---|---|---|---|---|---|---|---|
| (ng/mL) | 1 | 3 | 80 | 220 | 1 | 3 | 80 | 220 |
| Mean (ng/mL) | 0.9 | 3.2 | 87.8 | 206.1 | 1.1 | 2.9 | 82.5 | 203.1 |
| SD (ng/mL) | 0.2 | 0.2 | 10.2 | 15.5 | 0.2 | 0.2 | 5.3 | 8.4 |
| Precision (CV%) | 16.0 | 7.3 | 11.6 | 7.5 | 14.0 | 5.2 | 6.4 | 4.1 |
| Accuracy (%) | 92.0 | 106.0 | 109.8 | 93.7 | 104.8 | 98.0 | 103.1 | 92.3 |

*Extraction recovery*

Mean extraction recovery ranged from 44.4% to 68.9% with a precision less than 15% at the three QC concentrations for OTX015. For Y-401, mean extraction recovery was 59.7% with a precision less than 15% at a concentration of 500 ng/mL (Table 2).

**Table 2: Recovery of OTX015 and Y-401 following liquid/liquid extraction in human plasma**

| OTX015 (ng/mL) | Mean extraction recovery (%) | CV (%) |
|---|---|---|
| 3 | 44.4 | 14.8 |
| 80 | 68.9 | 3.6 |
| 220 | 60.3 | 0.9 |
| **Y-401 (ng/mL)** | **Mean extraction recovery (%)** | **CV (%)** |
| 500 | 59.7 | 9.6 |

*Selectivity and specificity*

No interference between any of the compounds and blank plasma was observed after injection of six plasma samples spiked with OTX015 and M1 at their respective LLOQs, and Y-401 at the concentration used (500 ng/mL). Each compound had a specific transition and a different retention time (Figure 1).

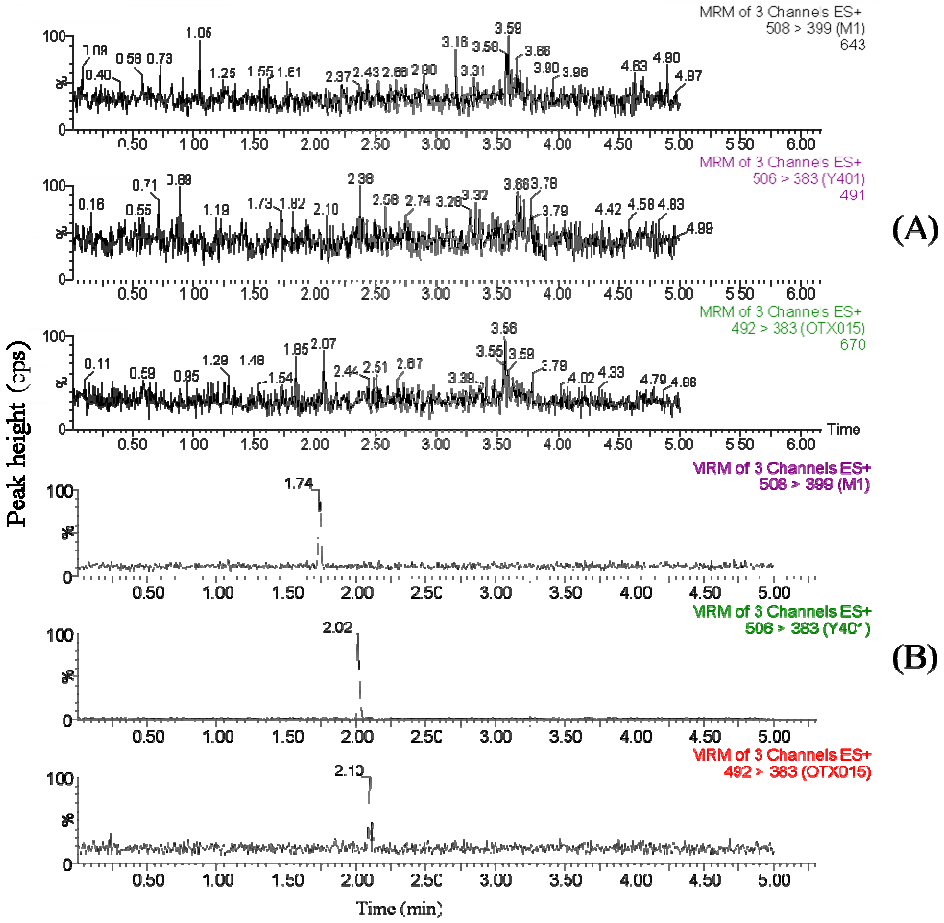


**Figure 1: Representative chromatograms of blank plasma at each mass transition for M1, Y-401 and OTX015 (A), and representative chromatograms for plasma spiked with M1 at 5 ng/mL (LLOQ), Y-401 at 500 ng/mL (concentration used), and OTX015 at 1 ng/mL (LLOQ) (B).**

*Matrix effect*

The matrix effect was evaluated by comparing the area obtained from six blank plasma samples spiked with OTX015 at 3 ng/mL (three times the LLOQ) and from a direct injection of diluted stock solution (three replicates) at the same concentration. This evaluation was repeated with OTX015 at 200 ng/mL (close to the upper limit of quantification), and matrix ratios were calculated for both conditions (Table 3). A typical matrix effect was observed, with a mean ratio of 0.93 ± 0.05 for OTX015 at 3 ng/mL and 0.83 ± 0.03 for OTX015 at 200 ng/mL (mean ± standard deviation [SD]).

**Table 3: Results of matrix effect assay for a concentration of OTX015 at 3 ng/mL and at 200 ng/mL**

| Peak area of OTX015 at 3 ng/mL in plasma (mean ± SD) (n=6) | Peak area of OTX015 at 3 ng/mL pure (n=3) | Ratio (mean peak area OTX015 at 3 ng/mL in matrix)/ (OTX015 at 3 ng/mL pure) |
|---|---|---|
| 97.28 ± 6.73 | 107.70 | 0.90 |
| | 108.90 | 0.89 |
| | 97.00 | 1.00 |
| | **Mean of 3 ratios ± SD** | 0.93 ± 0.05 |
| **Peak area of OTX015 at 200 ng/mL in plasma (mean ± SD) (n=6)** | **Peak area of OTX015 at 200 ng/mL pure (n=3)** | **Ratio (mean peak area OTX015 at 200 ng/mL in matrix)/ (OTX015 at 200 ng/mL pure)** |
| 3866 ± 111 | 4650 | 0.79 |
| | 4653 | 0.85 |
| | 4571 | 0.86 |
| | **Mean of 3 ratios ± SD** | 0.83 ± 0.03 |

Assessment of matrix suppression after post-column infusion of OTX015 into the MS detector was also performed. Several suppression bands were observed across the entire run however none were reported around the OTX015 retention time (2.10 minutes) (Figure 2).

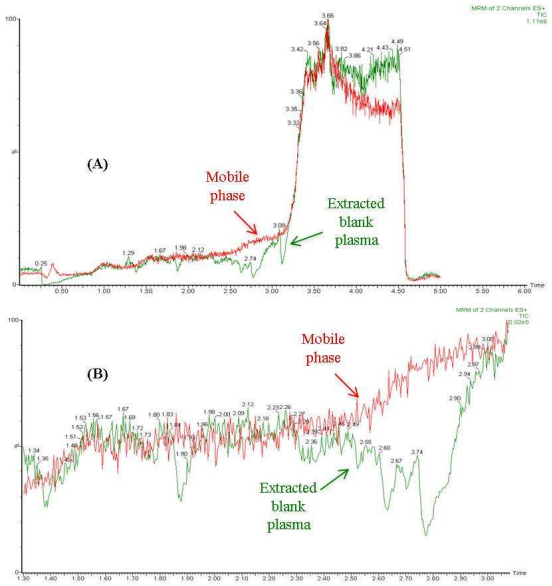


**Figure 2: Post-column infusion chromatogram of OTX015 UPLC-ESI-MS/MS analysis of blank mobile phase injection and blank extracted plasma by acetonitrile protein precipitation. Matrix suppression profile across the entire chromatographic run (A) and across an interval around the OTX015 retention time (B).**

*Stability*

The stability of OTX015 in plasma maintained at room temperature for 4 h and 24 h was demonstrated for both low and high concentrations (Table 4). Two QC concentrations (3 and 220 ng/mL) were analyzed in triplicate with a standard curve obtained from freshly prepared standard solutions. Deviation of the triplicate concentrations was less than ±15% for both concentrations.

A freeze/thaw stability study demonstrated that OTX015 in plasma was stable after one freeze/thaw cycle, at both QC concentrations. After three freeze/thaw cycles, OTX015 was stable at the high concentration (220 ng/mL) but not at 3 ng/mL, for which accuracy was greater than 15% (Table 4).

Evaluation of long-term stability of OTX015 in plasma at -30 °C for 1 month showed OTX015 was stable at both concentrations (Table 4).

**Table 4: Data for stability studies**

| | Nominal concentration (ng/mL) | Mean concentration identified (ng/mL) | Precision (%) | Accuracy (%) |
|---|---|---|---|---|
| Short term stability, 4 h, n=3 | 3 | 3.27 | 3.82 | 108.89 |
| | 220 | 214.03 | 4.87 | 97.29 |
| Short term stability, 24 h, n=3 | 3 | 3.07 | 4.07 | 102.22 |
| | 220 | 220.63 | 2.79 | 100.29 |
| Freeze/thaw stability at -30 °C, 1 cycle, n=3 | 3 | 3.03 | 12.72 | 101.11 |
| | 220 | 236.23 | 1.21 | 107.38 |
| Freeze/thaw stability at -30 °C, 3 cycles, n=3 | 3 | 2.47 | 3.82 | 82.22 |
| | 220 | 200.8 | 5.05 | 91.27 |
| Long term stability at -30 °C 1 month, n=3 | 3 | 3.02 | 2.00 | 100.56 |
| | 220 | 200.43 | 0.45 | 91.10 |

*Dilution test*

The mean of the calculated concentrations (n=5) for the dilution test was 121.70 ± 9.82 ng/mL with a precision of 8.07% and an accuracy of 110.64%.

*Method application in a clinical pharmacokinetic study*

The validated method was applied to quantify OTX015 in 38 plasma samples from the first six patients treated with 10 mg oral OTX015 in an ongoing phase I clinical study, three with AL and three with other hematological malignancies (OHM). Representative MRM chromatograms of OTX015 and Y-401 specific mass transitions, and of OTX015 and the IS Y-401 for an extracted plasma sample from a patient at 1 h post-intake is shown in Figure 3.

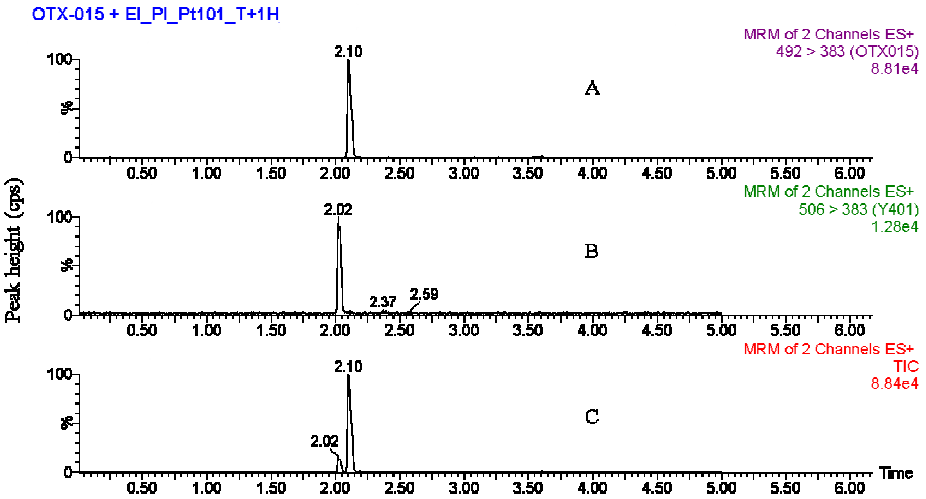


**Figure 3: Representative chromatograms for OTX015 (A) and Y-401 mass transitions (B), and a representative MRM chromatogram of OTX015 and the IS (Y-401) obtained from a patient plasma sample 1 hour after administration of 10 mg OTX015 (C).**

OTX015 concentrations up to 24 h were always above the LLOQ and the lowest OTX015 concentration identified was 4.2 ng/mL (24 h post-administration). The OTX015 plasma concentration-time profile for the first six patients treated at 10 mg OTX015 is shown in Figure 4.

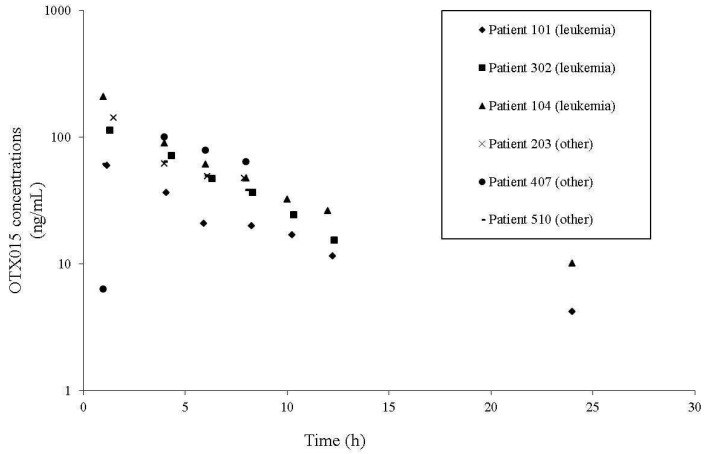


**Figure 4: Semi-logarithmic OTX015 concentration versus time profile in six patients receiving 10 mg OTX015.**

Estimated pharmacokinetic parameters for the six treated patients are shown in Table 5.

**Table 5: Pharmacokinetic parameters for six patients treated with 10 mg OTX015.**

| Parameters | AL patients (n=3) | OHM patients (n=3) | All patients (n=6) |
|---|---|---|---|
| $C_{max}$ (ng/mL) | 141.5 ± 69.4 | 107.7 ± 43.4 | 124.6 ± 60.3 |
| $T_{max}$ (h) | 0.8 ± 0.6 | 2.5 ± 1.9 | 1.6 ± 1.6 |
| $AUC_{0-\infty}$ (ng.h/mL) | 815.2 ± 242.7 | 908.3 ± 179.6 | 861.8 ± 218.5 |
| $t_{1/2}$ (h) | 4.6 ± 1.5 | 5.6 ± 2.3 | 5.1 ± 2.0 |
| Cl (L/h) | 13.6 ± 4.4 | 9.8 ± 4.3 | 11.7 ± 4.8 |

*General discussion*

We have developed a method for quantification of OTX015 in plasma using an UPLC-MS/MS system with an electrospray ionization source. This methodology is highly specific and sensitive, as well as being rapid and widely used to quantify drugs, metabolites, and endogenous compounds in biological matrices such as plasma.[15,17]

LC-MS/MS methodology occasionally encounters problems. Some of these problems may be caused by matrix effects however no significant matrix effect was observed for the quantified analyte with the UPLC-MS/MS method used here. The degree of enhancement or suppression of ionization is dependent on ionization type, sample preparation, bio-fluid type and the analyte of interest.[18] Protein precipitation was used for sample preparation, considered the simplest and fasted method for preparing samples relative to other extraction techniques such

as solid phase extraction. On the other hand, solid phase extraction may minimize the matrix effect and allow for the preparation of clean samples for injection. Nonetheless this extraction method is more difficult to implement.[17,18]

The UPLC-MS/MS method developed uses a minimal plasma sample (50 µL) and achieves good sensitivity (OTX015 LLOQ of 1 ng/mL). It was established to be suitable for implementation in the clinical context, allowing rapid, sensitive, accurate, and precise quantification of OTX015 in human plasma samples.

## Experimental

*Chemicals and Reagents*

OTX015, Y-401 (IS), and M1 (an OTX015 metabolite) were provided by Oncoethix (Lausanne, Switzerland). Structures of the three compounds are presented in Figure 5. Acetonitrile LC-MS grade, water LC-MS grade, ammonium hydroxide and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasma from healthy donors was purchased from the French Blood Bank ("Etablissement Français du Sang", EFS, France).

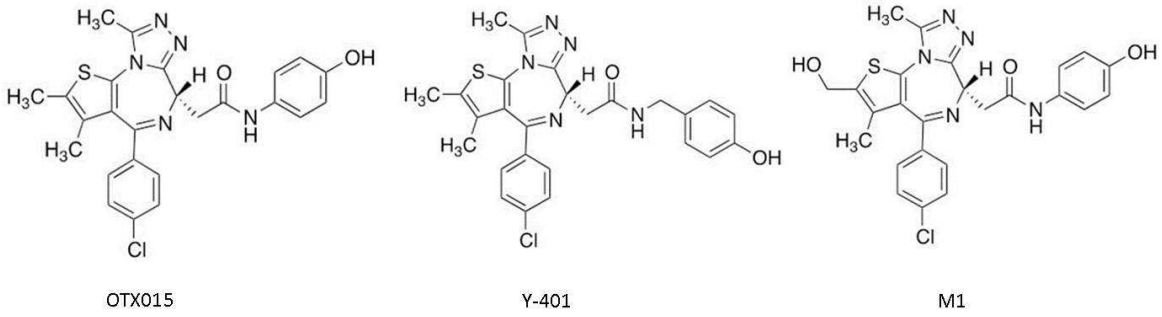


**Figure 5: Chemical structures of OTX015, Y-401 (IS) and M1 (OTX015 metabolite).**

*Preparation of Stock Solutions, Calibration Standards and Quality Control Samples*

OTX015 and IS stock solutions were prepared in DMSO to obtain a final concentration of 10 mg/mL. Aliquots of stock solutions were stored at -30 °C. OTX015 and IS stock solutions were further diluted in a water/acetonitrile mixture (1:1, v/v) on the day of the extraction to obtain working solutions of 1.0, 0.1 and 0.01 µg/mL for OTX015 and 0.05 µg/mL for Y-401. Calibration standards were prepared by diluting the working solution in human plasma, at 1, 5, 25, 50, 100 and 250 ng/mL. QC samples containing OTX015 were obtained from a different OTX015 stock solution and prepared in human plasma at 1, 3, 80 and 220 ng/mL.

*Chromatography Conditions*

An Acquity Ultra Performance LC System (Sample Manager and Binary Solvent Manager) (Waters, Milford, MA, USA) was used. Chromatographic separation in reverse-phase was performed on a BEH C18 UPLC 1.7 µm 2.1 x 50 mm column (Waters) maintained at +40 °C. Mobile phases consisted of water with 5 mM ammonium acetate (mobile phase A) and acetonitrile (mobile phase B). The gradient elution was performed at a flow rate of 0.5 mL/min (Table 6).

**Table 6: Gradient elution for chromatographic conditions**

| Time (min) | Flow (mL/min) | % Phase A | % Phase B | Curve |
|---|---|---|---|---|
| 0 | 0.5 | 98 | 2 | |
| 0.25 | 0.5 | 98 | 2 | 6 |
| 3 | 0.5 | 2 | 98 | 6 |
| 4 | 0.5 | 2 | 98 | 6 |
| 4.5 | 0.5 | 98 | 2 | 6 |
| 5 | 0.5 | 98 | 2 | 6 |

*Mass Spectrometry Conditions*

A Micromass Quattro *micro* API mass spectrometer (Waters) was used with a positive electrospray ionization (ESI) mode for detection and MassLynx 4.1 software for data acquisition and processing. MS/MS detection was conducted by monitoring fragmentation of 492→383 (*m/z*) for OTX015, 506→383 (*m/z*) for Y-401, and 508→399 (*m/z*) for M1. Source parameters were: capillary voltage, 3.20 kV; cone voltage, 45 V; extractor voltage, source temperature, 150 °C; desolvation temperature, 400 °C.

*Sample Processing*

A 5-µL volume of the IS working solution (500 ng/mL) in water/acetonitrile mix (1:1, v/v) was added to 50 µL of plasma and vortexed for 1 minute, 1 mL of acetonitrile was added, followed by centrifugation at 2000 g for 30 minutes. The supernatant was transferred to a clean vial and evaporated at 40 °C under a nitrogen stream. Extracted samples were reconstituted in 100 µL of water/acetonitrile (1:1, v/v) and transferred into 96-well plates (Waters). A 5 µL sample was injected into the LC/MS/MS system.

*Validation Procedures*

The assay was validated in accordance with the FDA Guidelines for Bioanalytical Methods Validation for Human Studies[16] and the EMA Guidelines on Bioanalytical Validation.[19] The validation procedure was evaluated in terms of linearity and sensitivity, precision and accuracy at four levels for intra- and inter-assays, recovery tests, matrix effects, selectivity and specificity, stability tests, and dilution tests.

*Linearity and sensitivity*

Calibration standards were prepared and analyzed in six replicates in six independent runs. Calibration curves were fitted using linear regression. For acceptable linearity, deviation of the mean calculated concentrations over six runs had to be within ±15% of nominal concentrations for the non-zero calibration standards, expect for the LLOQ level where a deviation of ±20% was allowed.[16] For sensitivity, the LLOQ was determined by analyzing six replicates in the same run. A precision of ±20% and an accuracy of 80%-120% were allowed.[16]

*Precision and accuracy*

Intra-assay precision and accuracy were determined by analyzing five replicates of each spiked QC sample in a single assay. Inter-assay precision and accuracy were determined by analyzing one QC sample per day at each concentration on five different days. Intra- and inter-assay precisions were expressed as the coefficient of variation (CV) at each of the three QC concentration levels, low (3 ng/mL), mid (80 ng/mL), high (220 ng/mL), and could not exceed 15% except for the LLOQ (1 ng/mL), which could not exceed 20%. Accuracy was calculated as the percent deviation from the nominal concentration and had to be within ±15% for each concentration level, expect for LLOQ, where ±20% was permitted.[16]

*Extraction recovery*

Five blank plasma aliquots were extracted and spiked with pure standard solutions at each QC concentration, low (3 ng/mL), medium (80 ng/mL) and high (220 ng/mL). Recovery was determined by comparison of the mean peak area of these unextracted standards accounting for 100% recovery, with the three extracted plasma samples at the QC concentrations.

For the IS, recovery was evaluated by comparison of the peak area of extracts of blank plasma samples spiked at 500 ng/mL Y-401 with the peak area of extracted plasma samples at the same concentration.

*Matrix effect*

The matrix effect was evaluated in six different extracts of blank plasma samples spiked at 3 ng/mL and six different extracts of blank plasma samples spiked at 200 ng/mL. Peak area obtained from the six extracts was compared with that obtained by direct injection of the diluted stock solution at the same concentration in three replicates prepared with a water/acetonitrile mixture (1:1, v/v). Assessment of matrix suppression was performed using the post-column infusion method. A blank extracted plasma sample was injected during continuous post-column infusion of 10 µg/mL OTX015 at a flow rate of 5 µL/min. Matrix suppression was evaluated by comparing the OTX015 chromatogram profiles obtained from an injection of the blank extracted plasma, with an equivalent injection of the mobile phase. Matrix suppression is shown as a negative peak.

*Selectivity and specificity*

Selectivity and specificity were evaluated in six different plasma samples spiked with OTX015 and M1 at their respective LLOQ concentrations and Y-401 at the concentration used in the method application.

*Stability*

Short-term temperature stability was determined with three aliquots of each of the low (3 ng/mL) and high (220 ng/mL) concentrations kept at room temperature from 4 to 24 hours. Freeze/thaw stability was determined after one and three freeze/thaw cycles (-30 °C to room temperature) in three plasma samples at the low and high concentrations; samples were refrozen for 24 h for each cycle. Long-term temperature stability was determined with three aliquots of each of the low and high concentrations at -30 °C for 1 month.

*Dilution*

A within-assay reproducibility test was performed with a diluted sample to assess the reliability of the method at concentration levels outside the calibration range. Five replicates of a validation standard sample spiked with OTX015 at a concentration of 2200 ng/mL were diluted at 1/20 with blank human plasma to fall within the calibration range. The mean concentration and standard deviation were calculated.

*Clinical Protocol*

The OTX015 analysis method was applied in the context of an ongoing clinical protocol to analyze patient plasma samples. This is an open-label phase I dose-finding and pharmacokinetic study in patients with advanced hematologic malignancies.[20] Dose escalation is performed using a conventional 3+3 design and cohorts of AL or OHM patients are evaluated independently. OTX015 is administered orally, daily, once a day. AL patients receive a discontinuous schedule (14 days on, 7 days off), and OHM patients receive a continuous schedule. The study protocol was approved by the local ethics committee and all patients provided signed informed consent. Blood samples (3 mL) were collected according to complete and limited assessment schedules; at 0 (before dose), 1, 4, 8 and 10, 12 and 24 h post-dose (complete) on days 1 and 2 in AL patients, and at 0, 1, 4, 6 and 8 h (limited) in OHM patients. Samples were centrifuged, and plasma was removed and stored at -30 °C until analysis.

*Pharmacokinetic Analysis*

Pharmacokinetic parameters from clinical trial plasma samples were calculated using the MicroPharm-K (MP-K) program.[21] Area under the OTX015 time-concentration curves (AUC) were calculated using a one-compartment model. The maximum observed plasma concentration ($C_{max}$) and the time taken to achieve this maximum level ($t_{max}$) were observed directly from the curves. The most representative model of the observed concentrations was determined and the pharmacokinetic parameters were assessed.

## Conclusions

We have developed and validated a sensitive, accurate, precise and robust procedure using UPLC-MS/MS for evaluating OTX015 concentrations in human plasma. Good linearity was obtained over the concentration range of 1-250 ng/mL. Moreover, this assay demonstrated high sensitivity with an LLOQ at 1 ng/mL using a small plasma volume for analysis (50 µL), which is appropriate for quantifying OTX015 concentrations in human plasma. The method was successfully applied to determine the pharmacokinetic profile of OTX015 in six patients who received an oral dose of 10 mg OTX015 in the context of an ongoing clinical phase I dose escalation study in patients with hematologic malignancies. This validated UPLC-MS/MS method will be used for complete characterization of OTX015 pharmacokinetics in this and future studies.

## Acknowledgements

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