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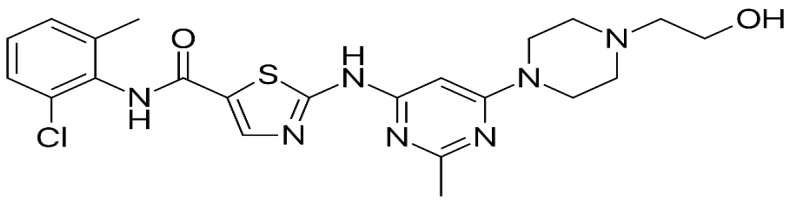
DEVELOPMENT AND VALIDATION OF AN LC–MSMS TECHNIQUE FOR THE QUANTITATION OF TEPOTINIB IN HUMAN PLASMA

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ABSTRACT
The goal of the study was to develop a sensitive, accurate, and linear LC-MSMS technique for quantifying Tepotinib. Chromatographic separation was attained by a Zorbax ODS/C18 column (50 mm ×4.6mm, 2.1µm) with a solvent system consisting of methyl alcohol, 0.1% HCOOH, and ACN in the proportion of 25:15:60 (v/v/v), delivered at 0.5mL/min flow. In MRM mode, detection was performed by monitoring ion transitions between m/z 493.17 and 436.17 for Tepotinib and between m/z 488.2 and 401.1 for the Dasatinib internal standard. The linear equation was y=0.0026x-0.0078, with an r^2 values of 0.9994. The %CV findings for matrix effect of LQC and HQC levels were 3.63% and 3.91%, correspondingly. Mean recovery percentages for Tepotinib at HQC (525µg/ml), MQC (350µg/ml), and LQC (49 µg/ml) were 102.94%, 96.61%, and 95.27%, correspondingly. The accuracy of the analytical method, both interday and intraday, was assessed by estimating %CV values. The quality controls exhibited variability ranging from 2.86% to 4.75% at concentrations of 17.5, 49.0, 350.0, and 525.0 ng/mL. The developed method was validated in compliance with USFDA guiding principles and demonstrated suitability for determining tepotinib in plasma for kinetics and quality control studies.
Keywords: Cancer, Tepotinib, LC-MS/MS, Validation, Accuracy
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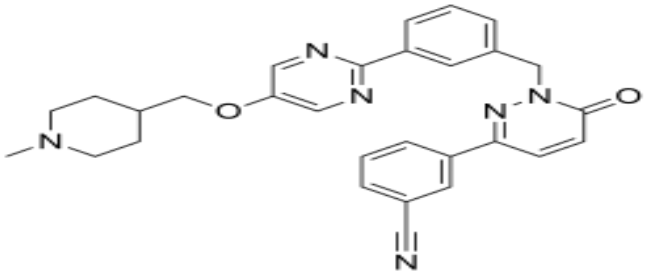
INTRODUCTION

Adults with MET exon 14 skipping mutation-positive metastatic non-small cell lung cancer may be eligible for tepotinib treatment. This medication is designed to prevent and inhibit the functions of MET tyrosine kinases, which are frequently overexpressed in certain solid tumours. Its therapeutic potential has been investigated across multiple malignancies, including gastric cancers, neuroblastoma, non-small cell lung cancers, and hepatocellular carcinomas. Accordingly, MET inhibition has been recognised as a promising strategy in targeted cancer therapy. Tepotinib is identified by its chemical structure as 3-{1-[(3-{5-[(1-methylpiperidin-4-yl)methoxy]pyrimidin-2-yl}phenyl)methyl]} -6-oxo-1, 6-dihydropyridazin3-yl} benzonitrile, characterized by the molecular weight of 492.583 g.mol^{-1} (Fig.-1).[1-3] Tepotinib was granted its first clearance in Japan in March 2020 for the management of NSCLC accompanied by MET mutations. This once-daily oral medication is the first of its type to target the MET tyrosine kinase. This feature is advantageous as it minimises the number of medications patients must take, alleviating a significant burden during chemotherapy treatment.



(A)

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(B)

Fig.-1: (A) Dasatinib and (B) Tepotinib Chemical Structures

In February 2022, tepotinib was approved in Europe.[4-6] In the current investigation, dasatinib served as an internal standard, exhibiting chromatographic characteristics comparable to those of the analyte, for quantitating tepotinib in biological matrices.[7,8]According to the available literature, only a single analytical approach was documented for the quantitation of tepotinib using LC-MS/MS. Various alternative techniques are referenced in the literature, including HPLC[9,10], UV[11], and LC-MS/MS.[12] So, a specific LC-MSMS method is required to analyse tepotinib levels in plasma samples.

EXPERIMENTAL

Reagents Chemicals

Reference standards of Tepotinib and Dasatinib (internal standard, IS) were kindly supplied by Dr. Reddy's Laboratories Ltd., Hyderabad, India. LC–MS grade methanol and acetonitrile (ACN) were obtained from J.T. Baker (Hyderabad, India). Ultrapure water was produced using a Milli-Q water purification system. Analytical-grade formic acid was purchased from Merck Pvt. Ltd. (Mumbai, India).

LC-MSMS System

A Sciex Applied Biosystems API4000 tandem mass system was coupled with a Shimadzu LC-20ADvp liquid chromatographic system consisting of an autosampler (Shimadzu, Japan). Solvent delivery was achieved using a Shimadzu LC-20AD pump. Data collection and chromatographic integration were carried out using Analyst version 1.4.2 software.

Standards for Quality Controls and Calibration Plot

Tepotinib and Dasatinib at 0.1mg/mL were dissolved in the mobile phase to generate stock solutions. Blank human plasma was used to produce QC and linearity standards. Eight non-zero linearity standards were generated by spiking plasma blank with the Tepotinib stock solution to obtain final concentrations of 17.5, 50.6, 105.0, 181.5, 272.2, 401.8, 537.9, and 700.0 ng/mL. QCs were prepared at 3 levels: 49.0ng/mL (low Q. C), 350.0 ng/Ml (medium Q. C), and 525.0 ng/mL (high Q. C). The Dasatinib stock was further diluted with 70% acetonitrile to obtain an IS working solution at 150 ng/mL. Before analysis, all processed samples were stored at -20 °C.

Chromatographic Parameters

Chromatographic separation was achieved using a Zorbax ODS (50 mm × 4.6 mm, 2.1µm) column with a solvent system consisting of methyl alcohol, 0.1% HCOOH, and ACN in the proportion of 25:15:60, with 0.5mL/min flow. The column oven was maintained at 40 °C, and the autosampler at 5 °C. A volume of 5 µL was used for the sample injection. The analysis was performed quickly with sufficient peak resolution, as the entire chromatographic run time was 2.0 minutes.

Mass System Parameters

API/4000 SCIEX triple quadrupole system with an ESI source running in +ve ion mode was utilised for mass spectrometric detection. Nitrogen served as the heater gas (30psi), CAD gas (set to 6), nebuliser gas (45 psi), and curtain gas (25 psi). A steady 5500V was maintained for the ion spray voltage, while the source temperature was fixed at 500°C. At 10 V and 80 V, respectively, the entry potential and declustering potential were maximised. For both Tepotinib and the internal standard, Dasatinib, the collision energy was fixed at 28 V. For Tepotinib, the collision cell exit potential was −23 V; for the

internal standard, it was −14 V. The transitions m/z 493.17 → 436.17 for Tepotinib and m/z 488.2 → 401.1 for Dasatinib were used for quantification in multiple reaction monitoring (MRM) mode.

Sample Protocol

A 100 µL internal standard solution containing dasatinib at a dose of 1 µg/mL was spiked into 150 µL of plasma samples that had been fortified with Tepotinib at the required concentration. Protein precipitation was carried out by the addition of 4 mL of acetonitrile; next, the mixture was centrifuged at 5 °C for 15-20 minutes at 5000 rpm. The transparent liquid remaining after phase separation was collected and then lyophilised until it was completely dry. To conduct LC-MS/MS analysis, portions of the reconstituted residue were transferred to autosampler vials after being mixed with 250 µL of the mobile phase.[13-16]

Validation

The quantification of Tepotinib in plasma was assessed for accuracy, linearity, recovery, precision, selectivity, and matrix effect as per bioanalytical validation guidelines. The goal of the present research was to validate a quantitative method for the determination of Tepotinib in plasma.[17-19]

RESULTS AND DISCUSSION

The assessment criteria were successfully satisfied by the chromatograms that were created throughout the validation procedure. The blank, blank with dasatinib, and LLOQ figures were shown in Fig.-2 and Fig.-3, respectively.

Specificity

To determine selectivity, endogenous plasma components were separated from Tepotinib using optimal chromatographic conditions, and plasma samples spiked at LLOQ (17.5 ng/mL) were injected under these circumstances. By keeping an eye on the internal standard and Tepotinib retention periods, we were able to assess plasma interference.[20,21] If the interference peak's reaction at the analyte retention time was ≤ 20% of the mean LLOQ response and less than or equal to 5% of the mean Dasatinib area, the technique was deemed selective. Furthermore, it was necessary for the obtained concentration at the limit of quantification to fall within ±20% of the theoretical value.

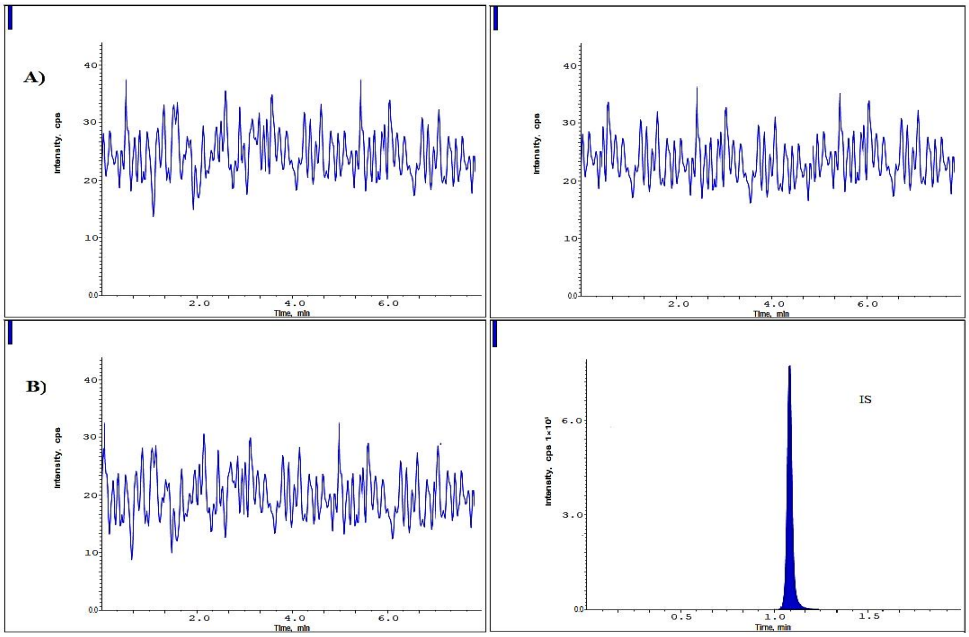


Fig.-2: Chromatogram of Plasma Blank(A), Blank with Dasatinib (B)

Calibration Graph

The construction of the calibration curve included graphing the peak response ratio of Tepotinib to the internal standard vs nominal concentration levels that corresponded to those ratios. The approach demonstrated linearity in concentration levels of 17.5–700ng/mL (Fig.-4), as shown in references.[22,23] The

equation y = 0.0026x − 0.0078 was obtained by the use of linear regression analysis, which resulted in an r^2 value of 0.9994. The calibration curve parameters and linearity findings were summarised in Table-1.

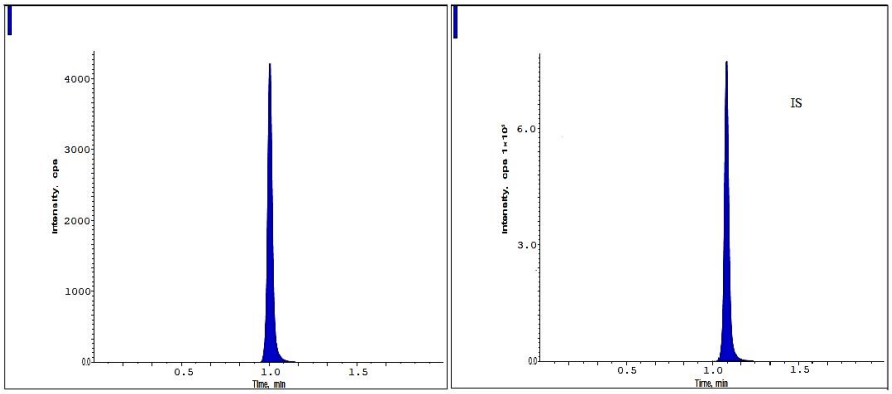


Fig.-3: Tepotinib Chromatogram at LLOQ with IS

Table-1: Tepotinib linearity data

LS-ID	Conc. (ng/ml)	Analyte response^N	IS responses	Analyte/IS responses
LS -1	17.5	4658	95114	0.0489728
LS -2	50.6	11877	95235	0.1247126
LS -3	105	24795	95003	0.2609918
LS -4	181.5	43117	95194	0.4529382
LS -5	272.2	65129	95347	0.6830734
LS -6	401.8	98662	95364	1.0345833
LS -7	537.9	129972	95985	1.3540866
LS -8	700	173867	95147	1.8273514

LS: Linearity standard; N=3 replicates

Precision

The inter-day and intra-day precision and accuracy of the method were evaluated by analysing QC samples at four concentration levels: LLOQ QC (17.5 ng/mL), LQC (49 ng/mL), MQC (350 ng/mL), and HQC (525 ng/mL). Within the scope of a single analytical run, five duplicate analyses of each quality control level were carried out for intra-day evaluation. Analysing the same quality control levels over the course of five different days allowed for the determination of interday precision and accuracy. To meet the acceptance standards, the %CV had to be below 15% for high, medium, and low QC samples, and it had to be below 20% for the low-level observation quality control samples. In all of the QC samples, inter- and intra-day percentage CV findings that were found ranged from 2.86 per cent to 4.75 per cent, indicating that the accuracy and precision were satisfactory, and there was no substantial variability. Table-2 provides a summary of the findings that were carried out.

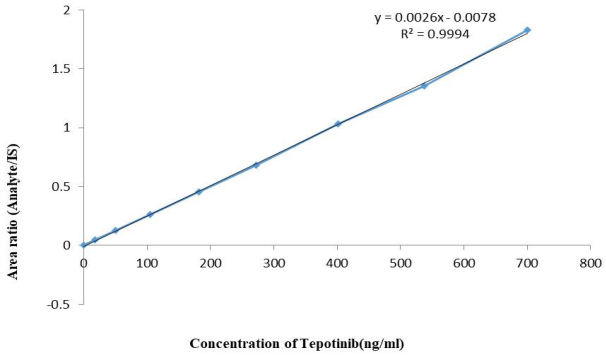


Fig.-4: Tepotinib Linearity

Table-2: Tepotinib intra- and Intraday Precision and Accuracy Findings

Intra batch	Concentration (ng/ml)	Average	SD	%CV
LLOQ	17.5	16.47	0.61	3.70
Low-QC	49	47.37	2.25	4.75
Median-QC	350	333.52	15.36	4.60
High-QC	525	538.28	20.36	3.78
LLOQ	17.5	16.73	0.71	4.24
Low-QC	49	47.32	1.98	4.18
Median-QC	350	338.95	11.95	3.52
High-QC	525	512.18	19.37	3.78
LLOQ	17.5	16.75	0.48	2.86
LLOQ	49	47.70	2.06	4.32
Low-QC	350	329.01	12.86	3.91
Median-QC	525	532.51	21.94	4.12
Inter batch		Average	SD	%CV
LLOQ	17.5	16.95	0.58	3.42
Low-QC	49	47.03	2.01	4.27
Median-QC	350	335.08	14.27	4.26
High-QC	525	504.62	21.07	4.18

Average=6 replicates

Extraction Recovery

Extraction recovery was assessed by comparing peak responses of Tepotinib isolated from plasma at quality control (QC) levels with those obtained from post-extraction spiking samples at similar doses.[24,2] This was done in order to determine recovery from the extraction process. According to Table-3, the mean recovery of Tepotinib was 102.94% at the high-quality control level (525 ng/mL), 96.61% at the mid-quality control level (350 ng/mL), and 95.27% at the low-quality control level (49 ng/mL). These results indicate that the recovery is consistent and repeatable throughout the concentration range.

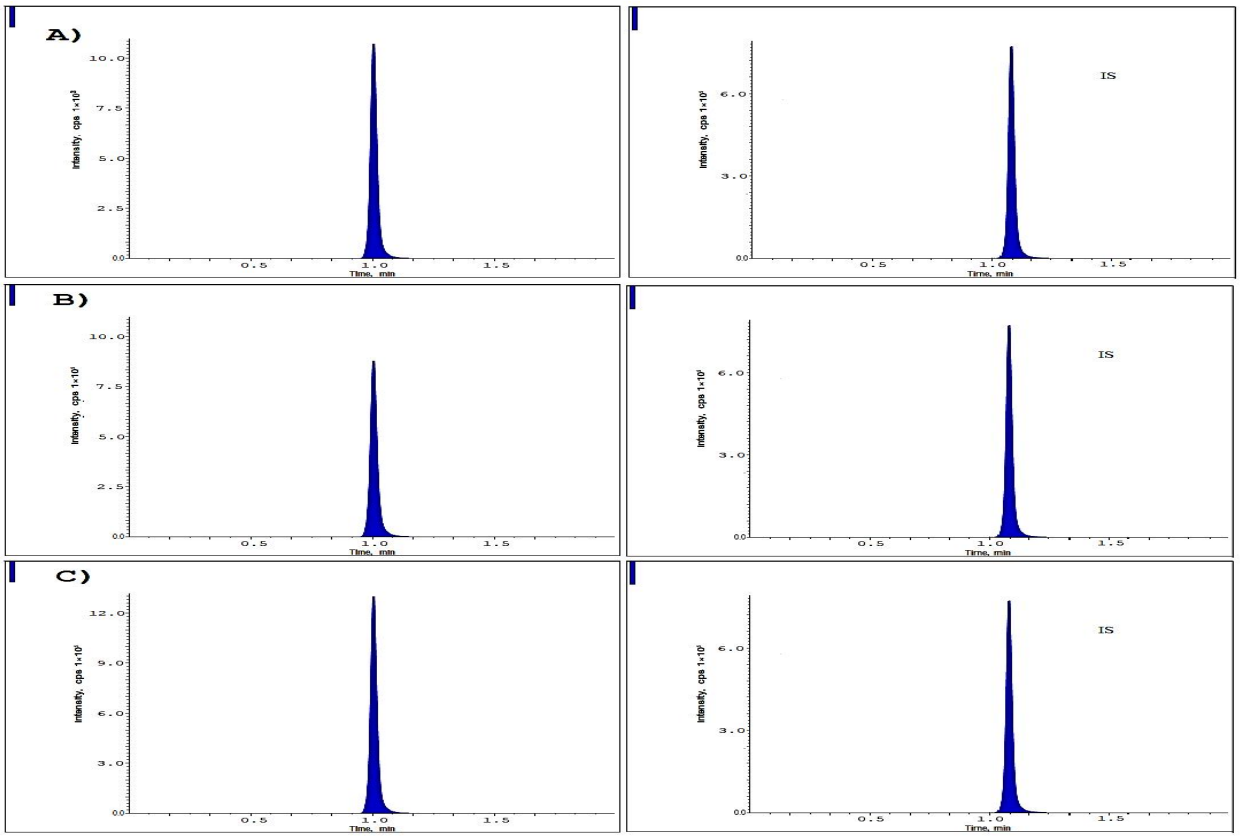


Fig.-5: Analyte LQC(A), MQC(B) and HQC(C) Level Chromatograms

Table-3: Recoveries of Tepotinib after Extraction

QC levels	X	Y	%Recoveries	%Average recovery	%RSD
LQC	12196	11619	95.27	98.27	4.09
MQC	87126	84172	96.61		
HQC	130687	134529	102.94		
IS	95114	94229	99.07		

X, unextracted sample recoveries; Y, extracted sample recoveries; n=3 replicates

Effects of Matrix Components

In order to investigate the effects of the matrix, 6 variable lots of plasma blanks were used, and three duplicates were generated at both low and high QC levels. It was determined whether or not the peak responses found from post-extracted spike samples were comparable to those obtained from equivalent clean reference solutions. The %CV for matrix factor was 3.63% at the low-QC level and 3.91% at the high-QC level, both within the acceptable limit of ≤15%, indicating minimal matrix interference. Table-4 provides a summary of the computed matrix factors and their associated findings.

Table-4: Tepotinib Matrix Effect Outcomes

	LQC			HQC		
S. No	Peak areas without matrices	Peak areas with matrices	Matrix factors	Peak areas without matrices	Peak areas with matrices	Matrix factors
1	12141	11811	97.28	130687	127223	97.35
2	12296	11726	95.37	130687	134699	103.07
3	12391	12668	102.24	130687	124636	95.37
4	12283	12694	103.35	130687	123525	94.52
5	12174	11721	96.28	130687	124531	95.29
6	12108	11540	95.31	130687	134045	102.57
Mean			98.30			98.03
± SD			3.57			3.83
% CV			3.63			3.91

n=6 replicates

Stability Study

Following the recommendations for FDA bioanalytical technique validation, stability tests were carried out under different storage and handling settings. Stability of Tepotinib in human plasma was assessed under different environments, including three freezing and thawing sequences, short (25 °C for 19h) term, long (−70 °C for 36 days) term, post-preparative (10 °C for 24 h), and stock solution stabilities.[23-25] Tepotinib was shown to be stable throughout sample processing, chromatographic analysis, and storage under all examined conditions. Table-5 presents stability statistics.

Table-5: Tepotinib Stability Studies.

	LQC		MQC		HQC	
Storage condition	%Accuracy	Precision (%RSD)	%Accuracy	Precision (%RSD)	%Accuracy	Precision (%RSD)
Short-term (25°C for 19 h)	97.37	4.34	96.64	3.29	95.61	3.84
Post-preparative (10℃ for 24 h)	95.42	3.24	94.28	4.09	103.34	4.28
Three freeze-thaw cycles (3 cycles)	94.54	3.67	94.66	4.61	96.79	3.17
Stock solution (20 h at 25℃)	96.61	2.84	95.91	3.68	97.52	4.06
Long-term (-70°C for 36 days)	96.18	3.61	98.37	3.44	103.32	3.29
Stock solution (17days at 2 to 8℃)	95.37	4.26	93.83	2.57	93.17	4.08

Effect of Carryover

To test carryover, six identical blank plasma samples were injected just after the ULOQ. Responses from blanks did not exceed 20% of analyte responses at LLOQ or 5% of Dasatinib, thereby confirming that carryover was negligible and within acceptable validation limits.

CONCLUSION

To quantify Tepotinib in biological matrices, a reliable, accurate, and repeatable LC-MSMS approach was designed and validated. Chromatographic separation was achieved by a Zorbax ODS (50 mm × 4.6 mm, 2.1µm) column with a solvent system of methyl alcohol, 0.1% HCOOH, and ACN in the proportion of 25:15:60, provided at 0.5mL/min flow. In MRM mode, detection was done by keeping an eye on the ion shifts between m/z 493.17 and 436.17 for Tepotinib and between m/z 488.2 and 401.1 for the Dasatinib internal standard. The linear equation was y=0.0026x-0.0078, with an r^2 values of 0.9994. The %RSD findings for ME of LQC and HQC levels were 3.63% and 3.91%, correspondingly. Mean recovery percentages for Tepotinib at HQC (525µg/ml), MQC (350µg/ml), and LQC (49 µg/ml) were 102.94%, 96.61%, and 95.27%, correspondingly. The method complied with FDA bioanalytical validation guidelines and is suitable for the quantitative determination of Tepotinib in biological matrices for quality control, bioavailability, and forensic applications.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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