RESEARCH ARTICLE

QBD Driven Bioanalytical Method Development and Validation of Tepotinib using RP-UPLC

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ABSTRACT: The research aims to establish a new UPLC method for estimating Tepotinib in human plasma. A simple, precise, and accurate RP-UPLC technique has been developed to quantify Tepotinib (TEP) in human plasma by employing a Quality by Design (QbD) approach. The effective separation of TEP was accomplished using the ACQUITY UPLC HSS C18 Column (1.8 µm, 2.1 mm×100 mm) and 0.01N Ammonium formate : Methanol in 70:30 v/v delivered at a flow rate of 0.3ml/min. The eluted TEP and internal standard Linagliptin (LIN) were detected at 258nm wavelength with good resolution. A temperature of 30^0C is maintained in the column throughout the experimental study. Equal volumes of methanol and water are considered as diluent to prepare the standard stock, working standard, and quality control sample solutions. TEP and LIN were eluted at 2.023 min, and 1.668 min, respectively, achieving a resolution of 4.3. The standard curve was linear (r^2 =0.999) across the 8-320 ng/ml concentration range of TEP. The validated method exhibited satisfactory performance aligning with ICH guidelines. The robustness and suitability of the method were further authenticated through QbD studies, making it compatible with therapeutic drug monitoring and determining the pharmacokinetic profile of TEP in *In-Vivo* studies.

KEYWORDS: Tepotinib, Internal Standard, RP-UPLC, Bio-analysis, Human Plasma, QbD

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INTRODUCTION

Tepotinib (TEP) is an anti-cancer agent inhibiting hepatocyte growth factor receptor (HGFR), also known as MET (mesenchymal-epithelial transition factor), which is crucial for cell growth and metastasis [1]. It efficiently blocks gene alterations like MET exon 14 skipping mutations, markedly non-small cell lung cancer (NSCLC), thereby impeding cancer progression and spread. The FDA has approved TEP to treat NSCLC in 2021[1-3]. It selectively inhibits MET by blocking the ATP-binding site, thereby preventing the receptor's activation and subsequent downstream signaling pathways involved in cancer cell proliferation [3-6]. TEP consists of a pyrimidine fraction linked to an indazole ring and a piperazine moiety, contributing to its inhibitory activity against MET. Chemically it is (6-((1-((R)-1-(isopropylamine) ethyl) indazol-6-yl)oxy) pyrimidine-4-yl) (4-methylpiperazin-1-yl) methanone with a molecular weight of 492.5 g/mol (Figure 1) [7].

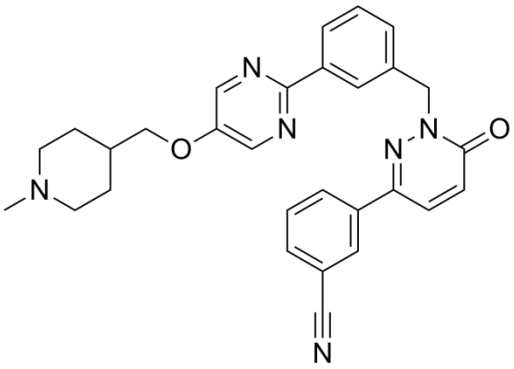

Fig: 1 Chemical structure of TEP

Liquid chromatographic techniques, such as Ultra-Performance Liquid Chromatography (UPLC) and High-Performance Liquid Chromatography (HPLC), are widely utilized for the qualitative and quantitative assessment of pharmaceuticals in both *in vitro* and *in vivo*

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models [8-10]. A comprehensive literature review reveals the development of one ultraviolet (UV) spectroscopic method and four RP-HPLC methods for quantifying TEP in bulk and tablet dosage forms [11-15]. Additionally, one LC-MS/MS method has been reported for the analysis of TEP in human liver microsomes [16].

Despite these advancements, there remains a critical need for a validated HPLC or UPLC-based bioanalytical method for quantifying TEP in human plasma. Such a method is indispensable for accurately characterizing the in vivo pharmacokinetic profile of the drug. To date, no single HPLC or UPLC-based bioanalytical method has been reported for this purpose. UPLC offers several advantages over traditional HPLC, including superior analyte resolution and sensitivity, reduced solvent consumption, and shorter analysis times [10]. These benefits make UPLC an ideal choice for developing robust bioanalytical methods. QbD principles, as outlined in ICH guidelines Q8 to Q11, have been increasingly adopted in the development of analytical procedures [17-19]. Applying QbD to analytical methods provides several benefits, including identifying and mitigating sources of variability that could compromise method robustness [18-20]. This approach ensures that the analytical method consistently performs as intended, enhancing its reliability and resilience under varying conditions [19-21]. Consideration of the advantages of QbD and UPLC, aimed to develop a new robust UPLC-based bioanalytical method by implementation of QbD principles.

MATERIALS AND METHODS

The TEP and LIN are provided as complimentary samples from Spectrum Laboratories, Hyderabad. The HPLC and analytical grade solvents used in the current study were procured from Merck India Limited, India. Waters Alliance UPLC, equipped with a photodiode array (PDA) detector and integrated with Empower 2 software, was used to develop and validate the method.

METHOD DEVELOPMENT

The method was optimized using a quadratic model based on the Central Composite Design (CCD) within a randomized response surface methodology study. Preliminary trials were conducted to refine the method for optimal performance. Three critical qualities attributes (CQAs), including the percentage of organic solvent in the mobile phase, column temperature, and flow rate, were identified as key factors influencing the dependent responses or the Quality Target Product Profile (QTPP) of the method. The key QTPP parameters, such as retention time (RT) and USP plate count were evaluated to ensure the method's quality. The CCD approach generated 20 experimental runs, including six center points, six axial points, and eight factorial points derived from the three CQAs (Table 1). A desirability function was applied to the optimized conditions to anticipate and accomplish the specified QTPP parameters.

Chroma*tographic conditions*

The successful separation of TEP was accomplished using the ACQUITY UPLC HSS C18 Column (1.8 µm, 2.1 mm×100 mm) and a mobile phase of 0.01N Ammonium formate: Methanol in 70:30 v/v delivered at a flow rate of 0.3ml/min. The eluted TEP and internal standard LIN were detected at 258nm wavelength with good resolution. A temperature of 30^0C is maintained in the column throughout the experimental study. Equal volumes of methanol and water portions are considered as diluent to prepare the standard stock, working standard, and quality control sample solutions.

Preparation of standard stock solution (16 µg/mL)

Accurately weigh 4 mg of TEP and transfer it into a volumetric flask of 250 mL clean and add 200mL of diluent (1:1 of water and methanol), and sonicate for 5 minutes. The remaining free volume was filled with the same diluent to attain a concentration of 16 µg/mL of TEP.

Preparation of working standard solutions

A volume of 0.05ml, 0.1ml, 0.15ml, 0.4ml, 1.0ml, 1.2ml, 1.6ml & 2.0 ml of standard stock solution was pipette and transferred to 8 individuals of 10 ml volumetric flask and fill the remaining volume with diluent to obtain 0.08 µg/mL, 0.016µg/mL, 0.024 µg/mL, 0.064 µg/mL, 0.160 µg/mL, 0.192 µg/mL, 0.256µg/mL, &0.320 µg/mL of TEP respectively.

Preparation of internal standard stock Solution (50 µg/mL)

Accurately weigh 4 mg of LIN and transfer it into a 100 mL volumetric flask. Add 80mL of

diluent and sonicate for 5 minutes. The remaining free volume was filled with the same diluent to attain a concentration of 50 µg/mL of LIN.

Table 1: CCD design of TEP with independent and dependent variables

		Factor			Response				
		1	2	3	1	2	3	4	5
Std	Run	A:Flow rate	B: Methanol	C:Temp^{0}C	RT of LIN	RT of TEP	R	NTP	TF
		ml/min	%	^{0}C	min	min	num	num	num
1	17	0.27	25	27	1.78	2.038	3	9325.2	1.1
2	7	0.33	25	27	1.466	1.671	2.7	8439.6	1.19
3	3	0.27	35	27	1.839	2.381	5.9	9535	1.05
4	11	0.33	35	27	1.499	1.933	5.3	9038.1	1.12
5	20	0.27	25	33	1.653	1.896	3.1	9097.1	1.04
6	14	0.33	25	33	1.373	1.57	2.8	7843.4	1.21
7	15	0.27	35	33	1.712	2.184	5.6	8715.2	1.1
8	10	0.33	35	33	1.418	1.82	4.9	7852.6	1.28
9	4	0.249546	30	30	1.868	2.271	4.5	9407.4	0.97
10	12	0.350454	30	30	1.348	1.63	3.8	7821.2	1.21
11	19	0.3	21.591	30	1.566	1.746	2.3	8806.7	1.04
12	8	0.3	38.409	30	1.634	2.2	6.3	8893.2	1.07
13	5	0.3	30	24.9546	1.664	2.023	4.1	9324.6	1.21
14	18	0.3	30	35.0454	1.473	1.784	4.1	8220.5	1.3
15	2	0.3	30	30	1.65	2.02	4.3	9023	1.09
16	13	0.3	30	30	1.653	2.028	4.3	9005	1.08
17	9	0.3	30	30	1.662	2.038	4.3	9050	1.09
18	6	0.3	30	30	1.654	2.031	4.4	9090	1.1
19	1	0.3	30	30	1.655	2.032	4.4	9003	1.08
20	16	0.3	30	30	1.658	2.026	4.4	9041	1.09

Preparation of Linearity standards and quality control (QC) samples

A volume of 0.05ml, 0.1ml, 0.15ml, 0.4ml, 1.0ml, 1.2ml, 1.6ml, and 2.0 ml of standard stock solution was pipette and transferred to 8 individuals of 10 ml volumetric flask and 2.5 mL of blank plasma. The remaining volume is filled with diluent to achieve a final concentration of 8 ng/mL (LLOQ), 16/mL, 24 ng/mL (LQC), 64 ng/mL, 160 ng/mL (MQC), 192 ng/mL, 256ng/mL (HQC), &320 ng/mL (ULOQ) of TEP, respectively.

Extraction procedure

A total volume of 750µL of plasma was combined with 500µL of internal standard and an additional 250µl µl of diluent and mixed well. Further, add 1mL of Acetonitrile to precipitate all the proteins and mix in a vortex cyclo mixture. Centrifuge at 3200 RPM for 5 min. After centrifugation, collect the supernatant liquid and filter through a 0.45µ filter before injecting it into the UPLC system.

METHOD VALIDATION

The validation procedure has concluded in accordance with USFDA guidelines [22].

System suitability test

It was accomplished by injecting replicate injections of the MQC sample of TEP consisting of internal standard (LIN) (50µg/mL) six times. The percentage (%) CV (coefficient of variation) was determined for peak areas of both TEP and Linagliptin. The %CV for peak area ratio for TEP and Linagliptin was calculated & assessed.

Linearity

The linearity of the specified method was evaluated by determining the r^2 value for a set of QC samples ranging from 8 ng/mL - 320 ng/mL, by plotting a curve between concentrations and peak area ratio of TEP and LIN.

Recovery

The recovery of TEP was assessed by analyzing the peak area response from TEP added to and extracted from the biological matrix with the peak area response from a pure standard solution of TEP. This method involved spiking a known quantity of TEP into a plasma matrix at three quality control levels: low (LQC), medium (MQC), and high (HQC). The recovery was calculated by comparing the responses of six replicates of the extracted samples to those of the un-extracted standard solutions. The % CV for the spiked TEP amount was also determined.

Precision & Accuracy

The system precision (Intra-day) was assessed by injecting 6 successive sets of four different levels TEP QC samples [(HQC-256ng/mL), (MQC-160ng/mL) (LQC-24ng/ml and LLOQ-8 ng/mL). The inter-day precision or reproducibility was ascertained by injecting six successive replicates of the same levels of TEP QC samples for three continuous days. The %CV & % mean accuracy of each sample level were measured to confirm the various precision and accuracy.

Specificity

5 µL of blank plasma, TEP standard solution, internal standard solution, and TEP standard solution spiked in plasma were separately injected in a subsequent manner. The recorded chromatograms were interpreted to detect any interference with the RT of TEP and LIN from impurities and matrix components.

Sensitivity

Sensitivity was evaluated using the LLOQ samples which are considered as the lower limit of drug concentration in a sample that can be accurately and precisely determined. Six QC samples at the LLOQ (8ng/mL) level, along with one set of linearity curve standards, were prepared by spiking with appropriate aqueous dilutions in a blank matrix with acceptable interference. The % mean accuracy & coefficient of variation (%CV) were calculated based on the observed peak responses.

Ruggedness

The ruggedness of the method was validated by assessing the precision and accuracy at three different concentrations of TEP standard solutions: high-quality control (HQC, 256 ng/mL), mid-quality control (MQC, 160 ng/mL), and low-quality control (LQC, 24 ng/mL). Each concentration was analyzed in six replicates. The mean accuracy was calculated as a percentage to confirm the reliability of the reported results.

Matrix Effect

The potential ionization effects caused by plasma components were evaluated by comparing the peak responses of post-extracted MQC samples (160 ng/mL of TEP, n=6) with those of freshly prepared drug solutions at the same concentration. The method's assessment for matrix effects was conducted using chromatographically screened human plasma.

Stability studies

Stability testing involves assessing analyte stability across various stages: during sample collection, handling, short-term and long-term storage, freeze-thaw cycles &the analysis process itself. Test conditions should closely mimic real-world scenarios to ensure accuracy. Additionally, stability evaluations should encompass the analyte's stability in the stock solution, using freshly prepared samples in a biologically relevant matrix devoid of any interfering substances.

Bench top stability

In the current method, LQC, MQC, & HQC samples were stored in the freezer for 12 hr. Subsequently, the samples kept at room temperature on the working bench for 6 hours. The resultant QC level samples were analyzed by the UPLC system in six replicates and the % mean accuracy and %CV were calculated from the obtained responses [23].

Auto-sampler injection stability

To prevent bias in results prior to validation, it is more important to check the reinjection or reproducibility of auto sampler stability. In the current method was established by determining the %mean accuracy of LQC, MQC & HQC samples for 24 hr in the auto sampler.

Freeze-thaw, short and long-term stability

Freeze-thaw stability was tested by storing LQC, MQC, HQC samples at -20 ±5°C for 24 hours, then thawing them at room temperature for 6 hours. Each sample was analyzed six times using UPLC, and the mean accuracy and %CV were calculated. Short-term stability was assessed by keeping the LQC, MQC, and HQC samples at -28 ±5°C for three days. Each day, six replicates of each sample were tested with UPLC, and the accuracy and %CV were recorded. Long-term stability was assessed by storing the samples at -28 ±5°C and -80 ±5°C for a period of 37 days. Six replicates of each sample were analyzed, and the results were used to calculate the mean accuracy and %CV. This testing confirmed the stability of the analyte under different storage conditions.

RESULTS AND DISCUSSION

Optimized method conditions

The CCD study model was considered to optimize the method, with the input independent variables suggested for a quadratic type-III partial model. Optimization of the method was confirmed from the ANOVA test, where the predicted R^2 values of responses were in considerable agreement with the adjusted R^2 values; i.e., the difference is less than 0.2. Adequate precision measures the signal-to-noise ratio. A ratio of more than 4 indicates an adequate signal, which reveals that the model can be desirable to find the way the design space. The results are represented in Table 2. The ramp plots of NTP, TF, resolution, and RT of the recommended method are shown in Figure 2, representing the desirability value and optimized location of methanol, flow rate 0.5ml/min, and temperature (Figure 2).

Table 2: Fit statistic parameters of dependable variables

Parameter	RT of TEP	RT of LIN	Plate count (NTP) of TEP	Tailing Factor (TF)	Resolution (R)
SD	0.0081	0.0051	37.61	0.0094	0.0709
Mean	1.97	1.61	8826.59	1.12	4.23
CV %	0.4118	0.3172	0.4261	0.8382	1.68
R^2	0.9992	0.9993	0.9973	0.9936	0.9977
Adjusted R^2	0.9986	0.9987	0.9948	0.9878	0.9956
Predicted R^2	0.9951	0.9962	0.9856	0.9625	0.9867
Adeq Precision	139.2678	143.2716	63.5403	47.7248	82.7030

SD: Standard deviation, CV : Co-efficient of Variation

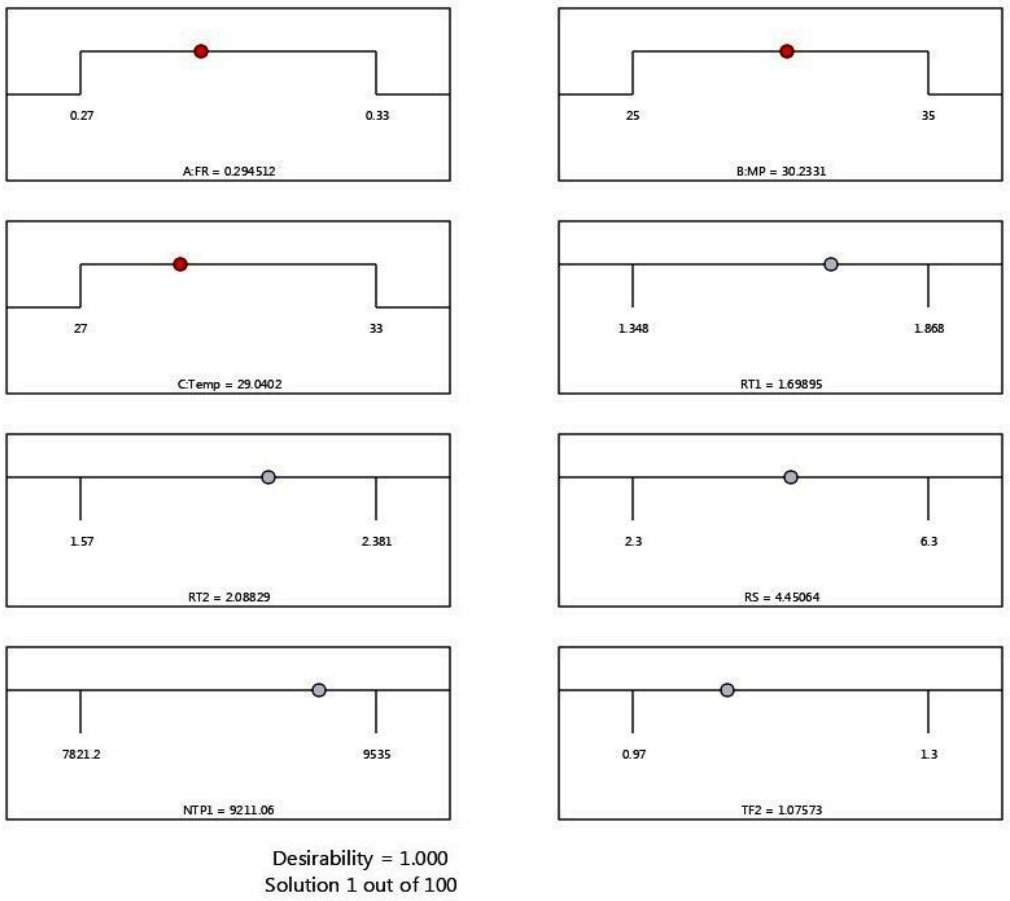


Figure 2: The ramps plots of optimized method conditions with desirability

The successful separation of TEP was achieved by using an ACQUITY UPLC HSS C18 Column (1.8 µm, 2.1 mm×100 mm) with a mobile phase of 0.01N Ammonium formate: Methanol in 70:30 pumped at a flow rate of 0.3ml/min. The separated TEP&its internal standard were detected with a PDA detector at 258nm. The RT of TEP and LIN was observed to be 2.023min and 1.668 min with a resolution of 4.3 and a plate count of 9035 for TEP (Figure-3). The observed RT and plate count, TF, and resolution values were very near to the predicted values. The deviation between predicted & observed values was less than 5%, significantly revealing that the obtained response values obey the design space (Table 3) . The polynomial equations suggested for this model were as follows.

RT of TEP = 2.03 -0.1891 A +0.1396 B -0.0699 C -0.0149 AB +0.0156 AC -0.0084 BC -0.0279 A^2 -0.0200 B^2 -0.0445 C^2

RT of LIN = +1.66 -0.1540 A +0.0227 B -0.0549 C -0.0050 AB +0.0100 AC +0.0015 BC -0.0159 A^2 -0.0187 B^2 -0.0298 C^2

Resolution = 4.35 -0.2253 A +1.23 B -0.0366 C -0.0875 AB -0.0125 AC -0.1125 BC -0.0730 A^2 -0.0199 B^2 -0.0907 C^2

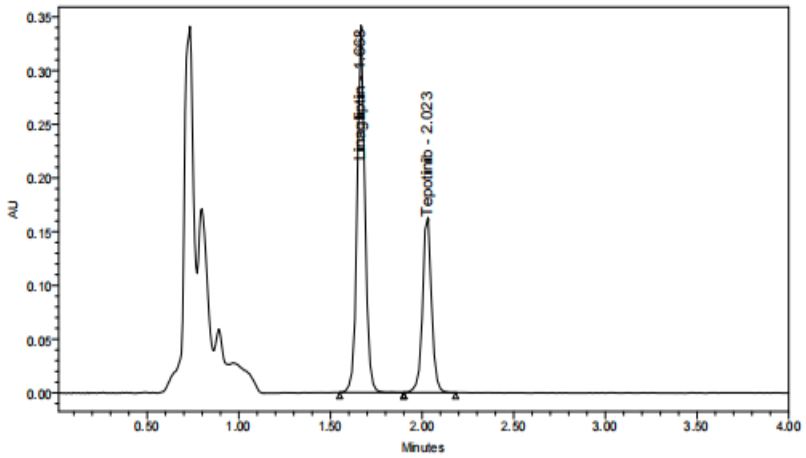
NTP = +9035.24 -451.53 A +42.55 B -343.16 C +97.48 AB -91.72 AC -147.62 BC -148.28 A^2 -64.96 B^2 -92.33 C^2

TF = +1.09 +0.0669 A +0.0044 B +0.0235 C -0.0012 AB +0.0238 AC +0.0312 BC +0.0006 A^2 -0.0117 B^2 +0.0590 C^2 +9035.24 -451.53 A +42.55 B -343.16 C +97.48 AB -91.72 AC -147.62 BC -148.28 A^2 -64.96 B^2 -92.33 C^2

The provided second-order polynomial equations, derived from response surface methodology (RSM), describe the influence of independent variables (A, B, and C) on key chromatographic responses, including the retention time (RT) of Tepotinib and Linagliptin, resolution (Rs), number of theoretical plates (NTP), and tailing factor (TF). These equations incorporate linear, interaction, and quadratic terms to model the complex relationships affecting drug separation. The root method, likely involving optimization techniques such as partial derivative calculations, Newton-Raphson, or response surface analysis, helps determine the ideal chromatographic conditions. The equations show that RT decreases with increasing A (organic phase) and C (temperature**)** but slightly increases with B (flow rate)**.** Rs (resolution) improve with B but decreases with A and C, indicating higher flow enhances separation while excess organic content and temperature reduce it. NTP (column efficiency) drops with increasing A and C**,** while B has a minor positive effect. TF (tailing factor) increases slightly with all three, especially A and C, affecting peak symmetry. Interaction and quadratic terms suggest extreme changes worsen performance, emphasizing the need for balanced parameter optimization.

Table 3: Correlation between predicted and observed responses by optimized conditions

Solution 1 of 100 Response	Predicted Mean	Predicted Median	Observed Mean	SD	n	SE Predicted	95% PI low	95% PI high
RT of LIN	1.699	1.699	1.668	0.005	1	0.006	1.68	1.71
RT of TEP	2.088	2.088	2.023	0.008	1	0.009	2.069	2.10
R	4.451	4.451	4.32	0.071	1	0.076	4.280	4.62
NTP	9211.06	9211.06	9035	37.607	1	40.541	9120.73	9301.39
TF	1.076	1.076	1.0	0.009	1	0.010	1.05	1.09



S.No	Peak Name	RT	Area	USP Plate Count	USP Tailing
1	LIN	1.668	450641	7276.4	1.1
2	TEP	2.023	139760	9035.0	1.0

Figure 3: Optimized chromatogram of TEP with internal standard LIN

METHOD VALIDATION

System suitability

The % CV of the peak areas of the six injections of MQC solution of TEP was assessed to be 0.50, while the % CV of the peak area ratio of TEP and its internal standard was found to be 0.72 (Table 4). Those results, within the acceptable limits, ensure the system suitability of the method.

Linearity

The R^2 value addressed for the stated series of concentrations was 0.999, which depicts the linear response of the proposed method for the stated range of concentrations (Figure 3).

Recovery

The %CV of TEP recovery at each QC level was ≤ 15.00%, with an overall mean %CV across all QC levels remaining ≤ 20.00% (Table 5). These findings confirm the accuracy of the current method, as evidenced by the consistently low %CV values in the recovery of all QC samples.

Precision and accuracy

The %CV for TEP at HQC, MQC, LQC & LLQC levels were found to be 0.39%, 0.56%, 3.14%, and 4.41%, respectively. These values fall within the acceptable range, as indicated in Table 6, confirming the precision of the UPLC method for both intra-day and reproducibility precision, aligning with USFDA guidelines. Furthermore, the mean accuracy across all QC levels was determined to be within 100±5%.

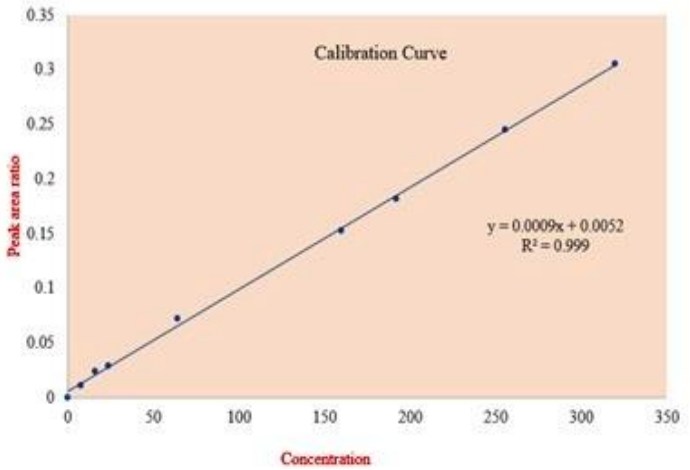
Specificity

No other interfering peaks were detected in six different randomly selected blank plasma samples at the retention times of either TEP or the internal standard (ISD), as shown in Figure 5. This demonstrates the method's specificity for analyzing TEP in biological samples.

Table 4: System suitability results of TEP

Parameter	TEP MQC (160ng/mL)) area	TEP RT (min)	LIN Area (50ng/mL)	LIN RT (min)	Area Ratio
Mean (N=6)	**69523.83**	**2.04**	**454669.67**	**1.68**	**0.15**
SD	**349.37**	**0.01**	**4581.24**	**0.01**	**0.00**
%CV	**0.50**	**0.41**	**1.01**	**0.31**	**0.72**
Acceptance limit (%CV)	**≤ 15**	**≤ 2**	**≤ 15**	**≤ 2**	**≤ 5**

SD: Standard deviation, CV: Co-efficient of Variation



Concentration (ng/mL)	Peak area ratio (TEP/LIN
8	0.011
16	0.023
24	0.028
64	0.071
160	0.153
192	0.180
256	0.247
320	0.303

Figure 4: Linear graph of TEP

Table 5 : Recovery results of TEP at various levels

QC level	Parameter	Extracted Response	Un Extracted Response
HQC (256 ng/mL)	Mean (n=6)	113273	102897
	SD	1122.99	1431.79
	%CV	**0.99**	**1.39**
	%Mean Recovery	90.84	
MQC (160 ng/mL)	Mean (n=6)	69237	64032
	SD	668.07	1558.67
	%CV	**0.96**	**2.43**
	%Mean Recovery	92.48	
LQC (24 ng/mL)	Mean	12676	12078
	SD	196.09	179.34
	%CV	**1.55**	**1.48**
	%Mean Recovery	95.28	
	Overall % mean recovery ± SD	92.86 ±2.245	
	%CV	2.42	

Table 6: Precision and accuracy results of TEP

Precision		HQC	MQC	LQC	LLQC
		Nominal Concentration (ng/ml)			
		256	160	24	8
Intra day Precision and Accuracy	Calculated Concentration Mean (n=6)	255.4602	158.7618	23.7816	8.0589
	SD	0.99001	0.88581	0.74682	0.43634
	% CV	0.39	0.56	3.14	4.41
	% Mean Accuracy	99.79	99.23	99.09	100.74
Reproducibility and Accuracy	Calculated Concentration Mean (n=18)	255.5219	158.9111	23.8238	8.0303
	SD	0.82300	1.07008	0.66589	0.46107
	% CV	0.32	0.67	2.80	5.74
	% Mean Accuracy	99.81	99.32	99.27	100.38

Sensitivity

The %CV & % mean recovery of analyte from LLQC samples were 3.54% % 99.80%, respectively, indicating the sensitivity of the method with good precision and accuracy

Ruggedness

The percentage mean accuracy for LQC, MQC, and HQC samples should be within 100±15%. The observed results of 96.24%,101.30%, and 100.13% for HQC, MQC, and LQC correspondingly confirm the ruggedness of the procedure (Table 7).

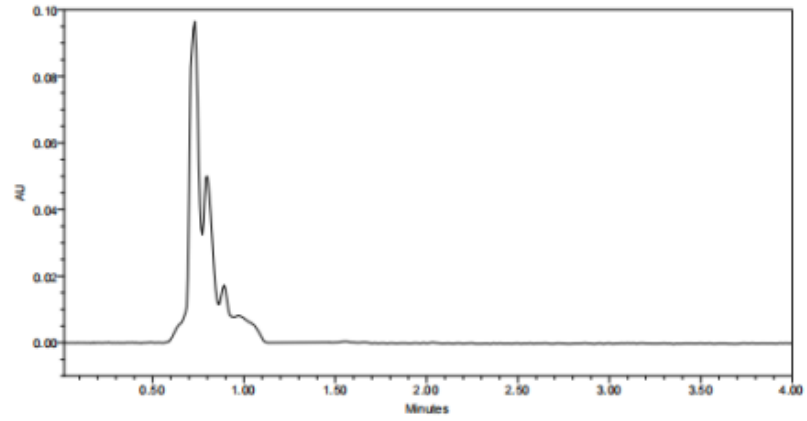
Matrix effect

A batch lot is to be acceptable when at least two out of three samples must fall within the range of 85% to 115%. Additionally, a minimum of 80% (5 out of 6) of the matrix lots must meet

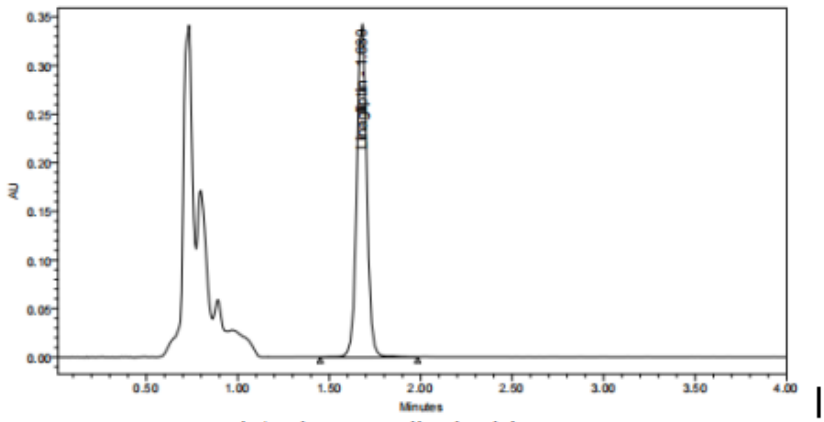
these criteria for approval. The mean accuracy for back-calculated concentrations of LQC and HQC samples prepared from different biological matrix batches should also fall within the 85% to 115% range. The mean accuracy for HQC & LQC was determined to be 99.64% &99.05%, respectively. (Table 8)

Stability studies

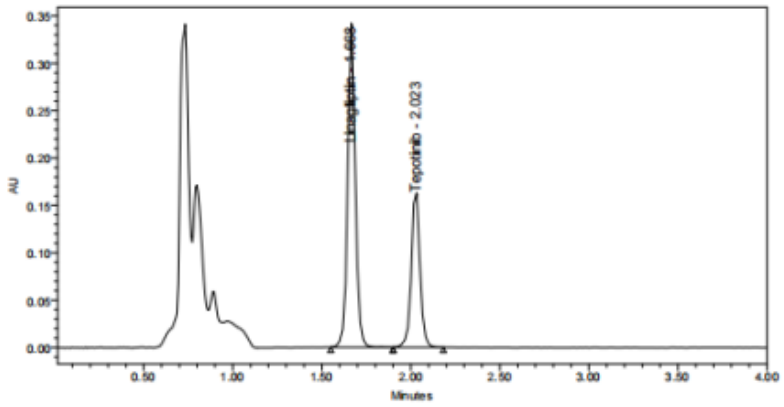
The (%) mean accuracy and (%) CV of repeated injections should not deviate from the acceptance criteria of 100±15% and ≤15%, respectively (Table 9). The attained results indicate that the concentration of the TEP is not changed by any of the processes of analysis and storage.



a) Blank Plasma



b) Plasma spiked with ISD



c) Plasma with analyte and ISD

Fig: 5 Chromatograms representing the specificity of the method

Table 7: Ruggedness on reinjection results of TEP

Injection number	HQC (256 ng/ml)	MQC (160 ng/ml)	LQC (24 ng/ml)
	Nominal Concentration (ng/ml)		
	15.528	10.416	5.357
	Calculated amount in concentration		
Mean (n=6)	255.4190	158.5231	23.7782
SD	0.76773	0.82915	0.46934
% CV	0.30	0.52	1.97
% Mean Accuracy	99.77	99.08	99.08
Acceptance criteria :	±15%	±15%	±15%

.Table 8: Results representing the matrix effect on TEP

S.No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration(ng/ml)	
		256.000	24.000
		Back calculated amount	
Mean (n=18)		255.0729	23.7711
SD		1.38975	0.62466
%CV		0.54	2.63
% Mean Accuracy		99.64	99.05
No. of QC Failed		0	0

DISCUSSION

Bioanalytical methods play a crucial role in preclinical and clinical studies by evaluating the pharmacokinetic properties of drug substances and products in biological fluids [22,23]. A well-optimized UPLC method ensures effective separation, identification, and quantification of pharmaceutical compounds. Previously, only one single LC-MS/MS method with a longer retention time was reported [16]. The existing liquid chromatographic (LC) methods cannot be applicable to analyze TEP in human plasma samples [11-15]. Moreover, the existing LC has a few drawbacks, like longer RT and expensive solvent systems leading to more expensive and more time-consuming [11-13]. To overcome the drawbacks of the reported method, a new UPLC method was created with a shorter RT of 2.02 min and a mobile system of Ammonium formate: Methanol in 70:30 v/v. The developed method was validated in terms of USFDA guidelines. The obtained results of validation parameters were within the acceptance limit. Based on the results, it was confirmed that the matrix effect was very minimal within allowable limits. Implementation of the analytical QbD approach confirms the robustness of the method as per ICH specifications. The developed method determines the TEP with short retention time, simple mobile phase, sensitivity, and specificity. Hence, the proposed method is adoptability in the analysis of biological samples of TEP in the quality control department of the pharmaceutical production sector.

CONCLUSION

A specific and sensitive RP-UPLC method was developed for the quantification of TEP in human plasma samples. The method has features like a shorter retention time for both TEP and its internal standard LIN. It confirmed accuracy, precision, and sensitivity in compliance with ICH guidelines. The stability study results deep-rooted that the analyte concentration remained stable throughout the preparation, processing, analysis, and storage

stages. The robustness and fitness of the established method were further authenticated through QbD studies, making it compatible with therapeutic drug monitoring and determining the pharmacokinetic profile of TEP in *In-Vivo* studies.

Table 9: Stability testing of TEP in different conditions

Stability type	Parameter	HQC	LQC
		Nominal Concentration(ng/mL)	
		256 ng/mL	24 ng/mL
		Calculated amount	
Bench Top	Mean (n=6)	255.51	158.9239
	SD	0.94439	0.70850
	%CV	0.37	0.49
	% Mean Accuracy	99.81	99.38
Auto Sampler	Mean (n=6	255.2778	158.9239
	SD	0.70133	0.70850
	%CV	0.27	0.45
	% Mean Accuracy	99.72	99.33
Freeze-Thaw	Mean (n=6)	253.2381	157.8317
	SD	0.02696	0.01968
	%CV	0.80	1.70
	% Mean Accuracy	96.67%	99.87%
Short term	Mean	254.8063	23.9736
	SD	0.96467	0.67428
	%CV	0.38	2.81
	% Mean Accuracy	99.53	99.54
Long term (Day-37,-28 ±5°C**)**	Mean	254.9805	24.0057
	SD	1.01643	0.95366
	%CV	0.40	3.97
	% Mean Accuracy	99.60	100.02
Long term (Day-37,-80 ±5°C**)**	Mean	255.1403	24.0531
	SD	0.93321	0.45303
	%CV	0.37	1.88
	% Mean Accuracy	99.66	100.22

ABBREVIATIONS

RP-UPLC: Reverse Phase Ultra Performance Liquid Chromatography; **TEP:** Tepotinib; **LIN:** Linagliptin; **QbD:** Quality by Design; **MET:** Mesenchymal-Epithelial Transition Factor; **NSCLC:**Non-Small Cell Lung Cancer; **CCD:** Central Composite Design; **CQA:** Critical Quality Attributes; **QTPP:** Quality Target Product Profile; RT: Retention Time; **HQC:** High quality control; **MQC:** Medium quality control; **LQC:** Low quality control; **ULOQ:** Upper limit of quantification; **LLOQ:** Lower limit of Quantification;

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Conflict of Interest

The authors affirm that there are no conflicts of interest associated with the publication of this paper.

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