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An LC–MS/MS Analytical Method for Quantifying Tepotinib in Human Liver Microsomes: Application to In Vitro and In Silico Metabolic Stability Estimation

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Abstract: Tepotinib (MSC2156119) is a potent mesenchymal–epithelial transition (MET) factor inhibitor, a receptor tyrosine kinase that plays a crucial role in promoting cancer cell malignant progression. Adverse effects of tepotinib (TEP), such as peripheral edema, interstitial lung disease, nausea and diarrhea, occur due to drug accumulation and lead to termination of therapy. Therefore, the in silico and experimental metabolic susceptibility of TEP was investigated. In the current work, an LC-MS/MS analytical method was developed for TEP estimation with metabolic stability assessment. TEP and lapatinib (LTP) used as internal standards (ISs) were separated on a reversed-phase C18 column using the isocratic mobile phase. Protein precipitation steps were used to extract TEP from the human liver microsome (HLM) matrix. An electrospray ionization multi-reaction monitoring (MRM) acquisition was conducted at m/z 493→112 for TEP, at m/z 581→350, and 581→365 for the IS. Calibration was in the range of 5 to 500 ng/mL (R^2 = 0.999). The limit of detection (LOD) was 0.4759 ng/mL, whereas the limit of quantification (LOQ) was 1.4421 ng/mL. The reproducibility of the developed analytical method (inter- and intra-day precision and accuracy) was within 4.39%. The metabolic stability of TEP in HLM was successfully assessed using the LC-MS/MS method. The metabolic stability assessment of TEP showed intermediate Cl_{int} (35.79 mL/min/kg) and a moderate in vitro $t_{1/2}$ (22.65 min), proposing the good bioavailability and moderate extraction ratio of TEP. The in silico results revealed that the N-methyl piperidine group is the main reason of TEP metabolic lability. The in silico Star Drop software program could be used in an effective protocol to confirm and propose the practical in vitro metabolic experiments to spare resources and time, especially during the first stages for designing new drugs. The established analytical method is considered the first LC-MS/MS method for TEP estimation in the HLM matrix with its application to metabolic stability assessment.

Keywords: tepotinib; human liver microsomes; LC-MS/MS; metabolic stability; P450™ module

1. Introduction

Cancer has a high mortality rate, accounting for approximately one-fourth of deaths internationally [1]. Lung cancer is considered one of the greatest widespread malignancies, as two million patients are diagnosed with it each year globally, causing 20% of all cancer deaths [2]. Among lung cancer cases, 90% are non-small cell lung carcinomas (NSCLC) that have numerous subtypes initiated by a range of activated oncogenes [3–5]. Although progress in developing a new drug series for cancer treatment has been slow, current molecular targeting strategies used in tumor suppressor genes and modulating oncogenes contributed to the improved prognosis of patients [6]. The treatment of NSCLC patients is significantly improved when such personalized targeted therapies are utilized [7,8]. For example, alterations in the mesenchymal–epithelial transition (MET) signaling pathway happen in 3–5% of NSCLC cases and are related to poor clinical prognosis and increased

tumor aggressiveness [9,10]. Tepotinib (MSC2156119; TEP, see Figure 1) is a tyrosine kinase inhibitor that targets such pathways, and the FDA approved a breakthrough therapy designation to this drug for patients with NSCLC [11]. Previously published clinical evidence with TEP showed promising results in metastatic NSCLC [12] as one of two MET inhibitors (capmatinib and TEP) approved by the FDA for the treatment of NSCLC [13].
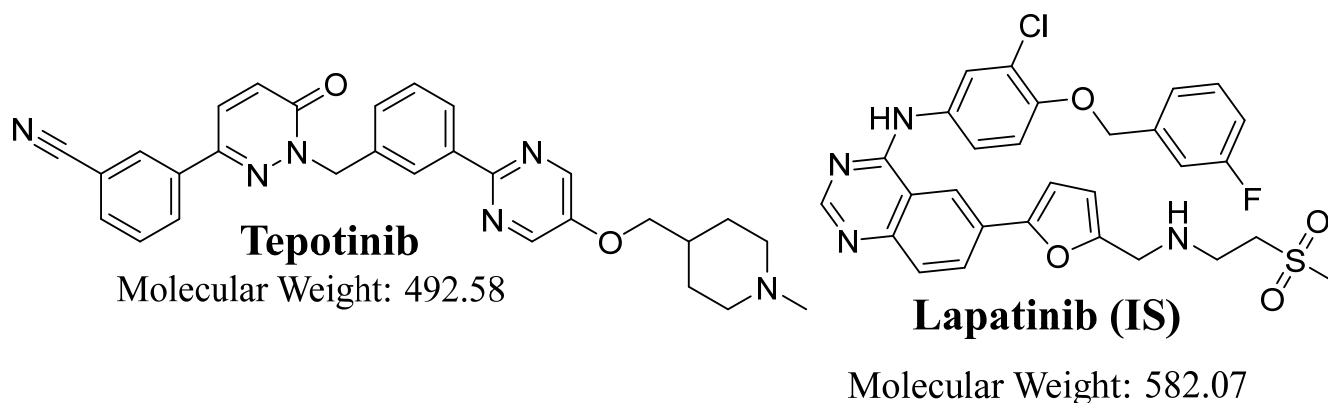
Tepotinib
Molecular Weight: 492.58

Lapatinib (IS)
Molecular Weight: 582.07

Figure 1. Chemical structures of tepotinib and lapatinib (IS).

The metabolic stability of a drug or a chemical is its vulnerability to metabolism, and it is described as a clearance intrinsic [Cl_{int}] and an in vitro half-life [$t_{1/2}$]. Half-life [$t_{1/2}$] is known as the time needed for eliminating 50% of the parent drug. Intrinsic clearance [Cl_{int}] is the liver's capacity to breakdown the drug in the blood through metabolic reactions. The two parameters were determined using the "in vitro half-life" methodology based on the "well-stirred" model [14,15] as it is the most frequently applied in in vitro drug metabolism expectation, the resulting factors can be utilized for extrapolation to numerous in vivo physiological parameters, involving the potential of accumulation and toxicity [16,17].

TEP treatment terminations were reported due to several adverse events, particularly peripheral edema, nausea, diarrhea, vomiting, fatigue and increased blood creatinine [18]. Consequently, metabolic stability (in silico and practical) experiments are required. Studying the metabolic stability of a TEP is a significant issue in developing drugs with improved metabolic stability profiles in the process of drug discovery [18]. It will be proposed that rapidly metabolized drugs cause a reduction in in vivo bioavailability, resulting in a smaller action duration [19]. TEP is mostly metabolized in the liver (by CYP3A4 and CYP2C8), with common drug–drug interactions with co-administered CYP3A modulators. Consequently, the dose is readjusted when the drug is taken with CYP3A inhibitors [20]. TEP has a rapid clearance rate from the human body if compared to earlier tested TKIs [21,22], with one of the contributing factors being its fast metabolism [23]. So as to determine TEP kinetics in vitro, this study is directed at establishing and validating an LC-MS/MS analytical method. A protein precipitation technique using ACN was used for TEP extraction from the HLM metabolic incubation matrix. All analytical parameters, for example recovery, calibration, precision and accuracy were estimated following the stated FDA guidelines. Finally, the established method was used for determination of TEP in the HLM matrix to calculate the in vitro metabolic stability that was guided by the results in the in silico software program (P450 model of StarDrop software; Cambridge, MA, USA).

2. Experimental

2.1. Materials

Pooled HLM matrix (M0567) from human livers (human male doners of different health states and mixed ages), formic acid (HCOOH), ammonium formate (NH_4COOH) and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HLM matrix was kept at -70 °C; the total protein content of the HLM pool was 20 mg/mL in 250 mM sucrose. Tepotinib (purity: 99.87%) and lapatinib (purity: 99.83%) were procured

from MedChemExpress (Princeton, NJ, USA). Milli-Q plus water filtration system (Millipore company, Burlington, MA, USA) was utilized to produce water for HPLC.

2.2. In Silico TEP Metabolic Vulnerability Prediction

Identification of the TEP metabolism lability was performed using P450 model (StarDrop software) from the Optibrium Ltd. (Cambridge, MA, USA). The composite site lability (CSL) value in the metabolic landscape was utilized as an indication of TEP's metabolic stability [24–26]. To estimate the susceptibility of TEP to metabolism, the site labilities of individual atoms can be collected to determine the CSL generating the overall metabolic lability of TEP. This is computed from the combined determined rates of metabolism for all sites on the molecule:

$$\text{CSL} = \frac{k_{total}}{(k_{total} + k_{water})}$$

where k_{water} is the rate of water formation via the decoupling pathway. The value of k_{water} has been estimated experimentally, utilizing an intrinsic isotope effect method [27]. k_{total} ($k_{total} = \sum$all sites) is the sum of the predicted proportion of metabolism at all active sites.

CSL is estimated as a crucial feature in proposing the rate of metabolism for TEP before performing the in vitro metabolic experiments to verify the value of the study. The TEP (CN1CCC(COC2=CN=C(N=C2)C2=CC(CN3N=C(C=CC3=O)C3=CC(=CC=C3)C#N)=CC=C2)CC1) SMILES format of TEP was uploaded to the StarDrop software program for CSL prediction.

2.3. LC-MS/MS Method

The analytical parameters of the LC-MS/MS method were attuned to obtain a respectable separation of TEP and LTP (internal standard, IS) (Table 1). Rapid resolution liquid chromatorgraphy (RRLC) with Agilent 1200 instrument was performed using an eclipse plus C_{18} (reversed-phase) column and isocratic mobile phase (70% acetonitrile and 30% 10 mM NH_4COOH in HPLC grade water, pH 3.5) at a flow rate of 0.15 mL/min. A triple quadrupole mass analyzer (Agilent 6410 QqQ) with an electrospray (ESI) ion source was used for mass analysis (ion detection and measurement) in positive ion mode. Nitrogen gas (11 L/min) was used for spray drying in the ESI source and for collision dissociation under pressure of 60 psi. A multiple-injector program was used for optimizing MS features to obtain the highest intensity of ion peaks. Capillary voltage of 4000 V and ESI temperature (T) of 350 °C were utilized. The Mass Hunter software program was used for LC-MS/MS system control, data analysis and data acquisition. Ion peak intensity was measured using MRM mode with mass transitions at m/z 493→112 for TEP (parent to fragment ions) and m/z 581→350 and 581→365 for LTP (SI), as presented in Figure 2. The collision energies and fragmentor voltages were 18 eV and 140 V for TEP and 32 eV and 145 V for LTP, respectively.

2.4. TEP Stock Solutions

TEP and LTP showed a good solubility in the organic solvent (DMSO) at 11.11 mg/mL (22.56 mM; ultrasonic and warming and heat to 60 °C and 125 mg/mL), (215.12 mM; Need ultrasonic), respectively. Stock solutions of TEP and LTP (2 mg/mL) were made in dimethyl sulfoxide (DMSO). Three working solutions of TEP and LTP (WK1, 200 μg/mL, WK2, 20 μg/mL and WK3, 2 μg/mL) were made by sequential dilution in a mixture of DMSO and the mobile phase.

2.5. TEP Calibration Standards

DMSO quenched the in vitro metabolic enzymatic pathways even at a 0.2% concentration [28]. DMSO was utilized, as TEP and LTP are solvable in DMSO and for the deactivation of HLMs purposed at 2% conc with slight heating (at 50 °C for 5 min.) [29,30]. The HLM matrix was made by diluting 1 mg protein/1 mL deactivated HLMs (30 μL) to 1 mL with 0.1 M sodium phosphate buffer (pH 7.4) that contains 1 mM NADPH. Nine cali-

bration levels, 5, 10, 30, 50, 100, 200, 300, 400 and 500 ng/mL, were utilized for generating the calibration line. The solutions were prepared from WK3 of TEP in 30 µL HLM matrix for each point. Four quality control (QCs) levels, lower limit of quantification (LLOQ), low (LQC), medium (MQC) and high (HQC) quality control levels, were chosen at (5, 15, 150, and 400 ng/mL, respectively). Fifty µL of internal standard WK3 (LTP) was then added to each concentration. The protein precipitation using organic solvent (ACN) was used for extraction of TEP and LTP [31–33]. Two ml of ACN was added to 1 mL of each calibration level and QC; they were vortex-mixed for 1 min and centrifuged at 14,000 rpm (at 4 °C for 12 min); then, the supernatant was filtered (0.22 µm syringe filter) into 1.5 mL HPLC vials, and 1 µL was injected into the LC-MS/MS instrument. A calibration plot was established by plotting the concentration of TEP (*x*-axis) against the peak area ratio of TEP to LTP (*y*-axis). The analytical method linearity was proven from the estimated linear regression parameters.

Table 1. Analytical features of liquid chromatography and mass detection.

	LC (Agilent 1200)			MS/MS (Agilent 6410 QqQ)
Mobile phase (isocratic)	70% acetonitrile			ESI (positive mode)
	10 mM NH$_4$COOH	30%	ESI	Drying gas: nitrogen of high purity at 11 L/min flow rate and pressure of 60 psi
		pH 3.5		
	0.15 mL/min.			
	5 µL			
Agilent eclipse plus C$_{18}$ Column	T: 20 ± 2 °C.			Capillary voltage: 4000 V
	1.8 µm particle size			Source T: 350 °C
	2.1 mm i.d.		Collision cell	Nitrogen gas (high purity)
	50 mm long		Mode	MRM
Mass spectra segment	0.0 to 1.5 min	TEP (0.86 min)	Analyte: Tepotinib (TEP)	*m/z* 493→ *m/z* 112, FV [a]: 140 V CE [b]: of 18 eV
	1.5 to 3.0 min	LTP (2.0 min)	Lapatinib (LTP, IS)	*m/z* 581.1→ *m/z* 350, FV [a]: 145 V, CE [b]: 32 eV
				m/z 581.1→ *m/z* 365, FV [a]: 140 V, CE [b]: 30 eV

[a] Fragmentor voltage. [b] Collision energy.

2.6. Method Validation

The validation of the established methodology was verified by calculating various analytical parameters. The details of the parameters were the same as those published in our previous work [34,35].

2.7. TEP Metabolic Stability

TEP metabolic stability was calculated by estimating the TEP remaining concentration after HLM pool incubation. One µM TEP was incubated with 30 µL HLM pool (containing 1 mg protein of microsomes) in phosphate buffer (100 mM) pH 7.4 (1 mL) and 3.3 mM magnesium chloride (MgCl$_2$). Negative controls (absence of TEP or NADPH) were used. The incubation was performed at 37 °C for 10 min; then, the metabolic pathways were started by adding NADPH (1 mM). Fifty µL of internal standard WK3 (LTP) was added to the incubation mixture just before the metabolic pathway termination so as to avoid the metabolism effect on the IS concentration. The reaction was stopped at specific time points, 0, 0.5, 2.5, 7.5, 15, 30 and 50 min, by adding 2 mL acetonitrile. The TEP metabolic stability curve was developed from the experimental measurements using the established LC-MS/MS method. Negative controls were repeated (absence of NADPH or HLM) to confirm the data of the metabolic study. The % of the remaining TEP was plotted against

incubation time. The linear portion of the plot was utilized to calculate the natural logarithm (ln) of % the remaining TEP against time (slope), which translates to the rate constant of TEP disappearance. This was used to determine in vitro $t_{1/2}$, following the equation

$$\text{In vitro } t_{1/2} = \frac{ln2}{Slope}$$

Then, the Cl_{int} (mL/min/kg) of TEP was estimated [36] using the following equation

$$Cl_{int} = \frac{0.693}{\text{in vitro } t_{\frac{1}{2}}} \times \frac{\text{mg HLMs}}{\text{g liver}} \times \frac{\text{mL incubation}}{\text{mg microsomes}} \times \frac{\text{g liver}}{\text{Kg b.w.}}$$

where a value of 45 mg of microsomal protein (mg HLMs) was considered in gram of liver tissue (g liver), and 26 g for liver tissue was considered in Kg of the body weight (Kg b.w.) [37,38].
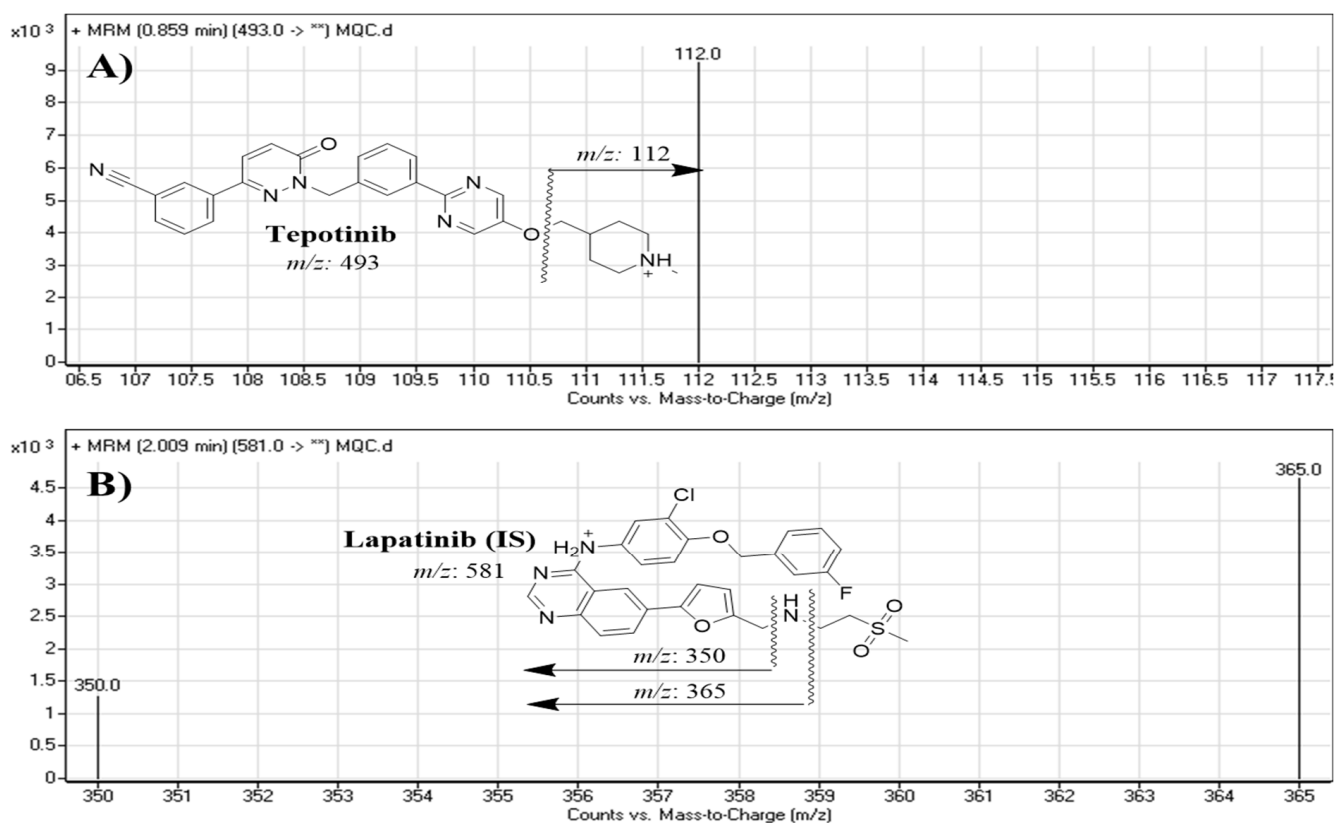

Figure 2. MRM mass transitions of (**A**) tepotinib (TEP) and (**B**) lapatinib (IS).

3. Results and Discussions

3.1. In Silico TEP Metabolic Lability

The lability of each site on the TEP structure with respect to the activity of CYP3A4, which plays an important role in the TEP metabolism [20], is indicated by the metabolic landscape, which also allows for the prediction of the TEP metabolites to be confirmed experimentally. The obtained results indicated that the N-methyl piperidine (C1, C3, and C7) is the main reason of TEP metabolic instability, as shown by the CSL presented in Figure 3 (the value of 0.9983, revealing high lability to metabolism), which is in line with the experimental work (see below).
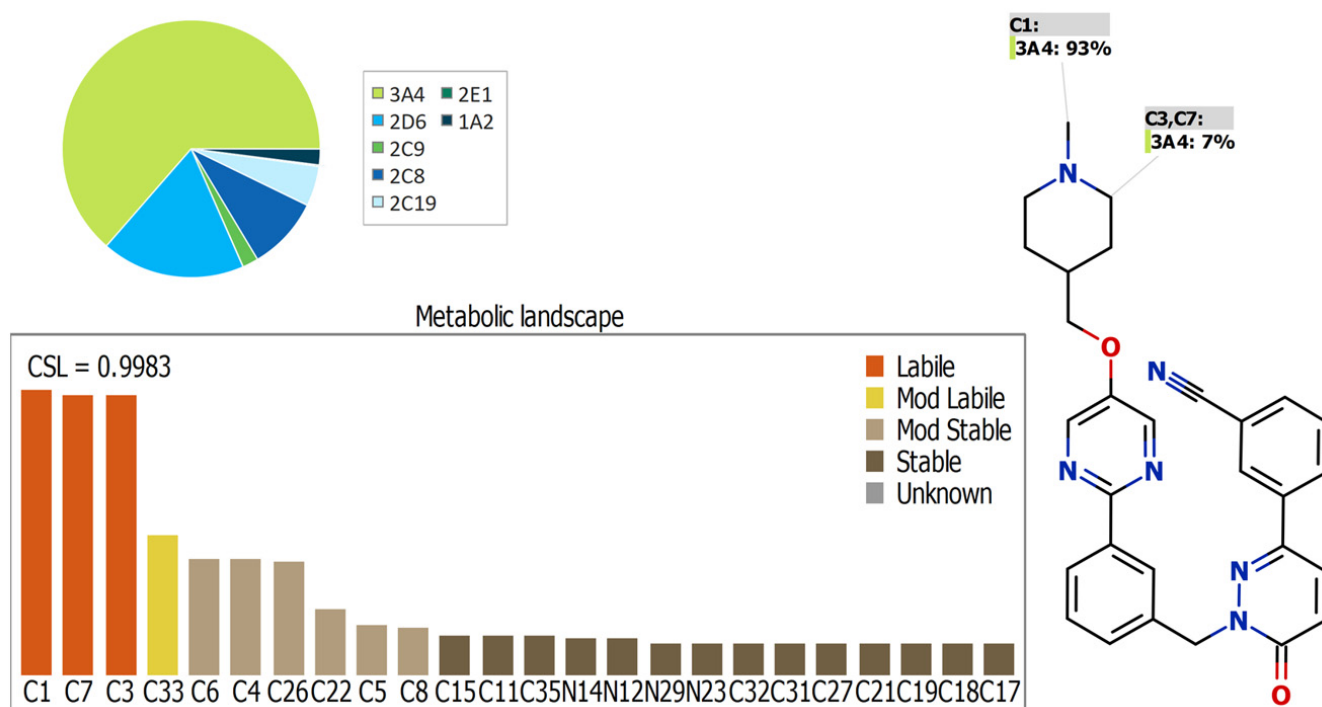
Figure 3. Predicted metabolic lability of TEP predicted by StarDrop software package (P450 model).

3.2. Quantification of TEP with the Developed LC-MS/MS Method

LTP was selected as the IS for quantifying TEP in the HLM matrix in the in vitro metabolic incubation experiments due to the following reasons. First, TEP and LTP were extracted from the HLM matrix using the same method of extraction (protein precipitation), with a high yield for TEP (99.43 ± 2.21%) and LTP (96.9 ± 1.27%). Second, the chromatographic peaks of TEP (0.86 min) and LTP (2.0 min) were well-separated in 3 min., which resulted in a quickly established LC-MS/MS method, saving time and using less can, which resulted in a green chemistry method. Third, TEP and LTP are not administered together to the same patient. Therefore, the current LC-MS/MS could be used for the pharmacokinetic studies and therapeutic drug monitoring of TEP.

The chromatographic features (mobile phase, pH and nature of the stationary phase) that can affect the separation process were adjusted. The mobile phase was optimized to pH 3.5; increasing the pH of the mobile phase led to increased elution time and chromatographic peak dissymmetry (tailing). The organic/aqueous solvent ratio was fixed at 70: 30%; increasing the organic solvent led to overlapped peaks with low resolution, while a reduction in acetonitrile resulted in increased elution time with good resolution. Hydrophilic interaction liquid chromatography (HILIC) columns were tested as a stationary phase, which resulted in poor retention and low-quality separation (data not shown). Good separation was achieved using a C18 column. Under the final conditions, the elution times for TEP and LTP were 0.86 min and 2.0 min, respectively, with respectable chromatographic peak separation (Figure 4). The LC-MS/MS method run time was 3 min. No carryover of blank HLM were observed.

3.3. Validation Parameters

3.3.1. Specificity

The chromatograms in Figure 4 show respectable separation of TEP and LTP without interference from the blank HLM matrix constituents at the specific elution times of TEP and LTP. No carryover influence appeared in the resulting MRM chromatograms. The linearity range of the proposed method was 5–500 ng/mL with a correlation coefficient of R^2 = 0.9999. The linear calibration equation was y = 0.5635714x + 0.4066 (Table 2). The LLQ and LOD peaks exhibited favorable signal-to-noise ratios (S/N) and respectable peak

symmetries. The slope of the calibration graph was 0.5635714, whereas its relative error was 0.002105. The intercept of the line was 0.4066 and its relative error was 0.1123472. The LOD was 0.4759 ng/mL and the LOQ was 1.4421 ng/mL (Table 2). The recovery of TEP based on the calibration and QC concentrations in the HLM matrix confirmed the success of the proposed LC-MS/MS analytical method.
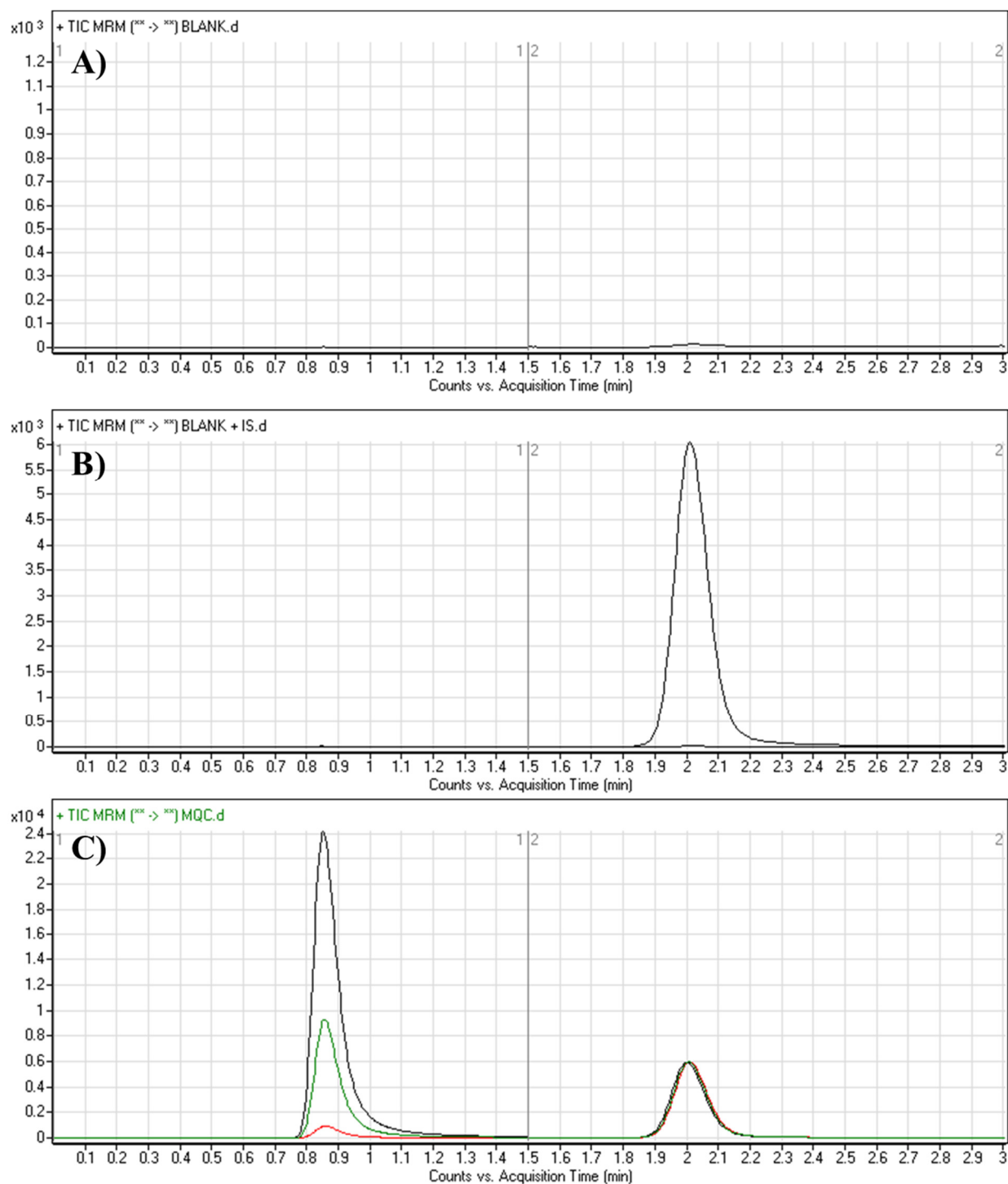

Figure 4. MRM chromatograms of (**A**) blank HLM matrix, (**B**) blank HLM matrix with LTP and (**C**) QCs of TEP: LQC (15; red color), MQC (150; green color) and HQC (400; black color) revealing TEP peak (0.86 min) and LTP peak (2.0 min).

Table 2. Analytical characteristics of the proposed LC-MS/MS analytical method (average data for six replicates).

Parameter	Value
Slope	0.5635714
Intercept	0.4066
Standard deviation of the intercept	0.1123472
Standard deviation of the slope	0.002105
Correlation coefficient (R^2)	0.999
Standard deviation of the residuals (Sy.x)	0.0812733
Limit of detection (LOD), ng/mL	0.4759
Retention time of TEP, min	0.86
Retention time of IS, min	2.0
Lower limit of quantification (LOQ), ng/mL	1.4421
Linearity range, ng/ml	5–500

3.3.2. Precision and Accuracy

The intra- and inter-day precisions of the adopted LC-MS/MS were 4.19% to 1.05% and 4.39% to 1.24%, respectively (Table 3). Intra- and inter-day accuracy and precision were in the range recommended by the FDA guidelines [32]. The relative error for spiked HLM (1.84% to −0.28) with a relative standard deviation of range 4.19% to 0.72% is presented in Table 4. LTP quality control samples show relative error range from 1.84% to −0.55%, whereas the RSD % was in the range from 4.19% to 1.05% (Table 4).

Table 3. Intra-day (data for twelve replicates on one day) and inter-day (data for six replicates on three days) precision and accuracy of the proposed LC-MS/MS analytical method.

HLM Matrix	Intra-Day Assay *			
	5 ng/mL (LLQC)	15 ng/mL (LQC)	150 ng/mL (MQC)	400 ng/mL (HQC)
Mean	5.09	14.92	151.04	401.36
SD	0.21	0.19	2.12	4.22
Relative error	1.84	−0.55	0.69	0.34
Precision (RSD), %	4.19	1.24	1.40	1.05
	Inter-day assay **			
Mean	5.17	15.02	146.73	397.46
SD	0.23	0.31	2.67	4.94
Relative error	3.48	0.11	−2.18	−0.63
Precision (RSD), %	4.39	2.05	1.82	1.24

* Average of 12 repeats in one day. ** Average of 6 repeats in 3 days.

Table 4. Back-calculated calibration levels of TEP (data for six replicates).

TEP Nominal Concentrations (ng/mL)	Mean *	SD	RSD %	Relative ERROR %	Recovery %
5	5.09	0.21	4.19	1.84	100.8
10	10.03	0.19	1.92	0.28	100.3
30	29.92	0.72	2.41	−0.28	99.73

Table 4. *Cont.*

TEP Nominal Concentrations (ng/mL)	Mean *	SD	RSD %	Relative ERROR %	Recovery %
50	50.38	2.23	4.43	0.75	100.76
100	98.90	3.18	3.21	−1.10	98.9
200	201.31	4.26	2.12	0.66	100.65
300	297.44	7.07	2.38	−0.85	99.15
500	500.81	3.61	0.72	0.16	100.16

* Mean of six replicates.

3.3.3. Extraction Recovery and Matrix Effects

The extraction recovery of TEP in spiked HLM matrix was 100.58 ± 0.97%, and the relative standard deviation was (RSD) < 4.18%. The results are displayed in Table 5. The matrix effect (ME) of the developed method for TEP or LTP was estimated by preparing two sets of HLM batches that were analyzed and injected into the LC-MS/MS system, Set 1 and 2, which were spiked with the LOQ concentration of TEP in addition to 100 ng/mL of IS (LTP). The matrix effects for TEP and LTP were calculated according to the following equations:

$$Matrix\ effect\ (\text{TEP}) = Mean\ peak\ area\ ratio\ {}^{Set\ 1}\!/_{Set\ 2} \times 100$$

$$Matrix\ effect\ (\text{LTP}) = Mean\ peak\ area\ ratio\ {}^{Set\ 1}\!/_{Set\ 2} \times 100$$

The HLM matrix containing TEP and LTP exhibited matrix effects of 101.46 ± 1.2% and 98.05 ± 2.1%, respectively. The IS normalized matrix effect was computed using the next equation:

$$IS\ normalized\ ME = {}^{Matrix\ effect\ (\text{TEP})}\!/_{Matrix\ effect\ (\text{LTP})}$$

The IS normalized ME was 1.04, well within the satisfactory range [39]. The normalized factor indicates that HLM has no clear influence on the ionization of either TEP or LTP.

Table 5. TEP extraction recovery (data for six replicates).

TEP (ng/mL)	Mean ± SD	RSD, %	Recovery %
5 (LLOQ)	5.09 ± 0.21	4.19	101.80
15 (LQC)	14.92 ± 0.19	1.24	99.47
150 (MQC)	151.04 ± 2.12	1.40	100.69
400 (HQC)	401.36 ± 4.22	1.05	100.34
TEP extraction recovery± SD		100.58 ± 0.97	

3.4. Metabolic Stability

One μM TEP was metabolically incubated with HLM (1 mg/mL); the concentration was less than the Michaelis–Menten constant to achieve the linearity between the time of the metabolic incubation and the metabolic rate. The microsomal protein concentration (1 mg/mL) was used to ensure minimal protein binding. The concentration of TEP in the incubation samples was recorded using the peak area ratio based on the pre-constructed calibration curve against incubation time. The TEP metabolic stability curve (Figure 5) was established by recording the remaining concentration of TEP (percentage compared to zero time) against the time of metabolic incubation. From this curve, time points in the linear range of 0–15 min were selected to plot the natural logarithm (ln) of the percentage of the

remaining TEP against time. The results (Table 6) exhibit that the linear regression equation of the straight line portion of the curve was y = −0.0306x + 4.5813, with R^2 = 0.9914, which can be utilized to estimate the in vitro $t_{1/2}$, utilizing the equation:

$$\text{In vitro } t_{1/2} = \frac{ln2}{Slope}$$

The slope was 0.0306.

$$\text{In vitro } t_{1/2} = \frac{ln2}{0.0306}$$

$$\text{In vitro } t_{1/2} = 22.65 \text{ min.}$$

The clearance intrinsic to TEP was computed using the in vitro $t_{1/2}$ method [19], so the Cl_{int} of TEP was 35.79 mL/min/kg [38]:

$$Cl_{int} = \frac{0.693}{22.65} \times \frac{1}{1} \times \frac{45}{1} \times \frac{26}{1}$$

In vitro $t_{1/2}$ and Cl_{int} were 22.65 min and 35.79 mL/min/kg, respectively. Using the scoring published by McNaney et al. [36], it is proposed that TEP has an intermediate clearance ratio character. By using other in silico software (the simulation and Cloe PK software programs; Framingham, MA, USA), these results could also be used to predict the in vivo TEP pharmacokinetics [40,41].

Figure 5. (**A**) The curve of TEP metabolic stability and (**B**) the linear portion of TEP metabolic stability curve.

Table 6. Parameters of TEP metabolic stability (data for three replicates).

Time (min)	Mean (ng/mL) [a]	X [b]	ln (X)	Analytical Features
0.00	467.74	100.00	4.61	
0.50	455.73	95.96	4.56	Regression equation:
2.5	450.58	88.12	4.48	y = −0.0306x + 4.5813
7.5	439.12	77.65	4.35	R^2 = 0.9914
15.00	375.32	62.00	4.13	Slope: −0.0306
30.00	347.06	54.30	3.99	$t_{1/2}$: 22.65 min
50.00	336.11	47.97	3.87	Cl_{int}: 35.79 mL/min/kg

[a] Mean of three injections. [b] X: mean of % TEP remaining.

4. Conclusions

The in silico metabolic experiment was performed using the P450 metabolism model, which was expressed in the form of CSL (09983), which revealed the high lability of TEP to metabolism. A reliable LC-MS/MS method was established for the TEP estimation. The method was based on liquid chromatography separation with an isocratic mobile phase and a reversed C18 stationary phase in conjunction with mass detection using an MRM with an ESI source. Protein precipitation was applied for the extraction of TEP from the HLM matrix. The established LC-MS/MS method was adjusted, validated using the FDA guidelines and applied in a practical metabolic stability evaluation of TEP in an HLM matrix. The reproducibility of the LC-MS/MS method (inter- and intra-day accuracies and precisions) was within 4.39%, and linearity was established over a wide range (5–500 ng/mL). The metabolic stability assessment of TEP showed intermediate Cl_{int} (35.79 mL/min/kg) and in vitro $t_{1/2}$ (22.65 min), indicating the moderate clearance character of TEP and the predicted optimum in vivo bioavailability. The metabolic experimental data of TEP agreed with the outcomes of the in silico software that revealed the value of in silico metabolic experiments before performing the practical work so as to save effort and time. The established LC-MS/MS method could be used for therapeutic drug monitoring (TDM) or pharmacokinetic studies for TEP after reoptimizing the extraction procedure, utilizing the same optimized chromatographic features.

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