

DEVELOPMENT AND VALIDATION OF A SENSITIVE LC-MS/MS ANALYTICAL METHOD FOR QUANTIFYING AXITINIB WITH AXITINIB D3 AS AN INTERNAL STANDARD

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ABSTRACT

Objectives: An efficient, highly sensitive, and robust Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been optimized, validated for accurately measuring axitinib concentrations in human plasma, utilizing axitinib D3 as an internal standard.

Methods: A liquid-liquid extraction approach was utilized to isolate both the analyte and the internal standard from the plasma matrix. An eclipse phenyl column of 100 × 3.0 mm size with 5 μm particles operated using a mobile phase containing methanol mixed with 0.1% formic acid in 10 mM ammonium formate at a ratio of 60:40 (v/v) following the advanced bioanalytical framework for method establishment and validation.

Results: The complete chromatographic run was accomplished in 4.0 min, with the analyte and internal standard eluting at approximately 1.89 min. The assay demonstrated a validated linear response across the concentration range of ~0.2–125 ng/mL. The developed method was thoroughly validated for key parameters including precision, accuracy, selectivity, matrix effects, and stability. All validation results met the predefined specifications, confirming method's reliability.

Conclusion: The validated method for quantifying axitinib in human plasma complies with regulatory guidelines set by the Food and Drug Administration and European Medicines Agency, making it appropriate for pharmacokinetic studies in human subjects.

Keywords: Axitinib, Axitinib D3, Food and drug administration, European medicines agency, Pharmacokinetics.

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INTRODUCTION

The World Health Organization (2018) identifies cancer as the second foremost cause of mortality globally, contributing to approximately 16% of all deaths [1]. It remains as a leading global health challenge characterized by uncontrolled cell proliferation and resistance to therapy, driving the need for targeted treatment strategies such as tyrosine kinase inhibitors (TKI) and combination regimens to improve outcomes [2-4]. In renal cell carcinoma (RCC), particularly the clear cell subtype, TKIs like Axitinib have therapeutic potential by selectively inhibiting vascular endothelial growth factor receptors (VEGFRs), the key drivers of tumor vascularization and progression [5-7]. Advanced RCC remains challenging due to high relapse rates and metastatic spread, necessitating precision approaches and durable therapeutic combinations [8-10]. Axitinib (marketed as Inlyta®) is a potent oral medication and a selective second-generation inhibitor targeting VEGFRs 1, 2, and 3 [11]. Axitinib possesses the molecular formula $C_{22}H_{18}N_4OS$ and a molecular weight of 386.47 Da, as reflected in its chemical structure (Fig. 1a). This generic drug has been authorized in the US and other nations for the management of advanced kidney cancer. Axitinib exhibits a high degree of plasma protein binding (~99%), with a strong affinity for albumin [12,13].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been extensively developed for the quantification of TKIs, such as asciminib and infigratinib, with proven reliability in pharmacokinetic and clinical applications [14,15]. Among these TKIs, axitinib stands out as a selective second-generation VEGFR inhibitor approved for advanced RCC treatment. This TKI [16-19] has been analyzed using various analytical techniques, including high-performance liquid chromatography (HPLC) [20] and LC-MS/MS methods [21,22].

However, most existing studies have either been limited to animal models or focused on simultaneous quantification methods, often

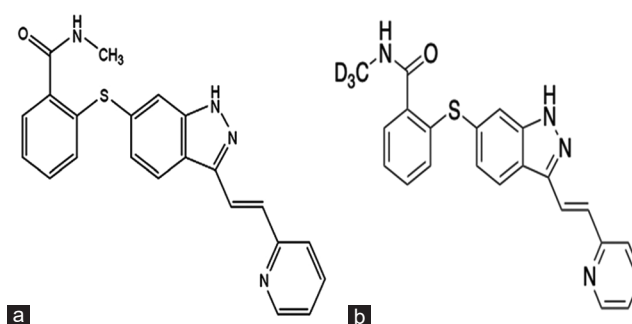


Fig. 1: (a) Structure of axitinib. (b) Structure of axitinib D3

lacking the inclusion of a compound-specific deuterated internal standard [23,24] like axitinib D3 (Fig. 1b). In light of these findings, this study is designed to develop and validate a sensitive, selective, and reliable LC-MS/MS method for measuring axitinib levels in human plasma, using axitinib D3 as a deuterated internal standard. The method is specifically designed to overcome the limitations of previous approaches by ensuring enhanced precision and accuracy. Validation of the method was performed in alignment with the regulatory guidelines set forth by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) [25-27].

METHODS

Chemicals and reagents

Working standards for axitinib (analyte) and axitinib D3 (internal standard) were procured from Daicel Chiral Technologies, Hyderabad, India. LC-MS grade methanol, acetonitrile, and other reagents were

obtained from Merck (Mumbai, India) and high-purity water was obtained from the Milli-Q system.

Chromatographic conditions

An HPLC system (Exion LC) consisting of a solvent degasser, an autosampler manager for sample injection, and a column oven maintained at 40°C with an LC controller. The Eclipse Phenyl column (100×3.0 mm, 5 µm) serves as the basis for the chromatographic method employing isocratic elution mode. A solution containing 60% methanol and 40% 0.1% formic acid in 10 mM ammonium formate served as the mobile phase. Samples were analyzed at a flow rate of 0.9 mL/min which required 4.0 min for each analysis.

LC-MS/MS instrumentation

The AB Sciex triple quad MS instrument (California, USA) was used to perform mass spectrometric detection through a turbo ionspray. Data processing was carried out using Analyst software version 1.7.2. The multiple reaction monitoring conditions were summarized in Table 1.

Preparation of analyte and internal stock solutions

Master stocks of both the analyte and internal standard were prepared at 1 mg/mL concentration using a solvent mixture of 0.01% dimethyl sulfoxide (DMSO) in methanol.

Preparation of internal standard dilutions

From the stock solution, pipette 50.0 µL and make up the volume to 1 mL using appropriate dilution solution (CH₃OH: H₂O: 50:50, v/v). From this, pipette out 20.0 µL and make up the volume to 100 mL to prepare a 10 ng/mL concentration of working dilution for further analysis.

Preparation of calibration curve (CC) samples

Prepare the calibration curve and quality control samples (QC's) from analyte stock solutions using the diluent solution of methanol: water: 50:50 (v/v) within the linearity range of 0.20–125.30 ng/mL.

Extraction protocol

Blank plasma, CC, and quality controls samples were retrieved from appropriate storage conditions, thawed at room temperature, and vortexed. Aliquot 100.0 µL of plasma into a pre-labeled tube and add 25.0 µL of internal standard dilution (10 ng/mL) to all the samples except the blank and vortex. Add 100.0 µL of extraction buffer (5 mM ammonium bicarbonate) and then add 2.0 mL of tert-butyl methyl ether to all the samples, vortex them for 10 min at 2000 revolutions/min. Centrifuge the samples for 5 min at 4000 rpm at 5°C. Transfer the supernatant and evaporate to dryness using nitrogen gas at 50°C. The dry extracted samples were reconstituted with 300.0 µL of mobile phase, vortexed thoroughly, and then injected.

Statistical analysis

The mean, standard deviation, and coefficient of variation (% CV) were calculated to assess data variability and consistency. A comprehensive statistical evaluation using Analyst software version 1.7.2 was performed to compare three regression weighting models: None, 1/X, and 1/X². This analysis was conducted across three independent precision and accuracy runs to determine the optimal model for data fitting. The results consistently demonstrated that the 1/X² weighting model produced the lowest cumulative relative error (% RE), indicating it as the most accurate and reliable regression statistical approach during the method validation (Fig. 2).

RESULTS AND DISCUSSION

Bioanalytical method development

The MRM conditions were optimized using a tuning solution containing 200.0 ng/mL of axitinib and axitinib D3. The mass fragmentation spectra of the tune files were represented in Fig. 3a and b, and MRM transitions were presented in Table 2. The chromatographic

Table 1: Mass spectrometric conditions

Parameters	Value (s)
Declustering potential	40 V
Entrance potential	10 V
Collision energy	30 V
Collision cell exit potential	10 V
Curtain gas	30 psi
Collision gas	Nitrogen
Source temperature	500°C
Ion source voltage	5500 V
Nebulizer gas	45 psi
Turbo gas	50 psi
Dwell time	200 msec
Resolution	Unit

Table 2: MRM transitions

Parameters	Axitinib (Analyte)	Axitinib D3 (Internal standard)
Q1 mass (m/z)	387.2	390.2
Q3 mass (m/z)	356.0	356.2
Polarity	Positive	Positive

MRM: Mass spectrometric conditions

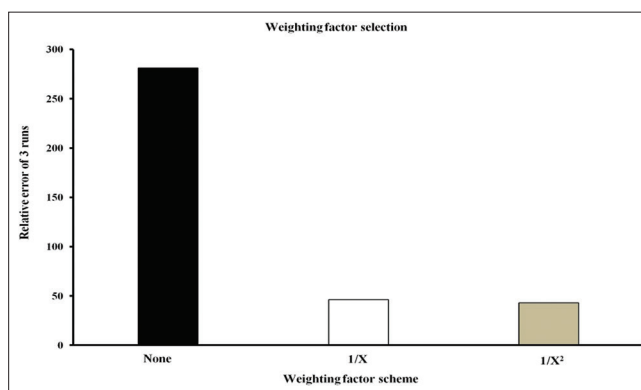


Fig. 2: Selection of weighting factor based on cumulative relative error

optimization was performed using different ratios of organic solvents and buffer solutions using acetonitrile, methanol, formic acid, and ammonium formate in order to optimize the mobile phase pH.

A post-column infusion test was performed to evaluate the matrix effect by infusing the neat upper limit of quantification (ULOQ) sample sample post-column into the MS source while acquiring an extracted blank sample. The sample preparation method was developed using an appropriate extraction buffer, like ammonium bicarbonate solution. Carryover is the appearance of an analyte of interest in an injected sample from a preceding injected sample and was successfully evaluated without any significant interference at the retention time (RT) of the analyte.

Method validation-bioanalysis

The method validation was conducted to assess specificity, precision, accuracy, sensitivity, matrix factor (MF), selectivity, linearity of dilution, recovery, and stability. The inclusion and exclusion criteria for each analytical parameter are summarized in Table 3.

Selectivity

The method's selectivity was assessed, and it was found that none of the plasma lots showed interference at the RTs of the analyzed chemical species. Chromatograms of the control sample (blank), control sample

Table 3: Inclusion and exclusion criteria for analytical parameters

Parameter	Inclusion criteria	Exclusion criteria
Selectivity	Interference at analyte RT within $\pm 20\%$ of LLOQ	$>20\%$ interference at analyte RT $>5\%$ response in blank at deuterated ISTD RT with that of mean ISTD response of calibrators and QC's
Specificity	Area response $<20\%$ of LLOQ at analyte RT	Follows selectivity criteria
Precision and accuracy	Accuracy: $\pm 15\%$ of nominal value ($\pm 20\%$ at LLOQ) Precision: $\leq 15\%$, except $\leq 20\%$ at LLOQ	Overall 67% of QC's should meet the inclusion criteria
Sensitivity and linearity of dilution	Linearity with $r^2 \geq 0.98$ Back-calculated concentrations within $\pm 15\%$ ($\pm 20\%$ at LLOQ)	Calibration curve standards exceeding specification limits or $r^2 < 0.98$
Recovery	% CV across QC levels $\leq 15\%$	Recovery difference between analyte and ISTD $> 15\%$
MF	Normalized MF between 0.85 and 1.15% CV $\leq 15\%$ across different matrix lots	MF outside 0.85–1.15 range and % CV $> 15\%$
Stabilities	Accuracy: $\pm 15\%$ of nominal concentration Precision: $\leq 15\%$	Fails to meet accuracy or precision thresholds

RT: Retention time, CV: Coefficient of variation, LLOQ: Lower limit of quantification, ISTD: Internal standard, QCs: Quality controls, MF: Matrix factor

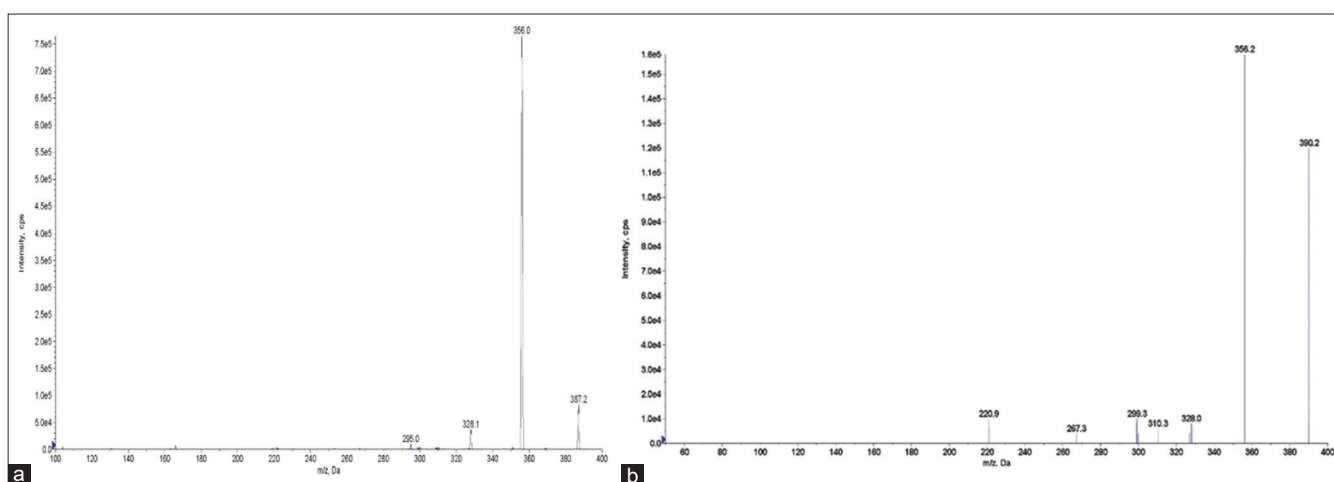


Fig. 3: (a) Mass fragmentation of axitinib. (b) Mass fragmentation of axitinib D3

added with internal standard, and lowest calibrator are shown in (Fig. 4a-f).

Specificity

No notable interference was detected at the RT of the molecule(s) of interest.

Precision and accuracy

The assay showed linear response over the range 0.20 ng/mL to 125.30 ng/mL during validation, and all " r^2 " values obtained for the three CCs were ≥ 0.98 . The method's linearity evaluation involved creating a ten-point standard curve (Fig. 5), which underwent regression analysis using $1/x^2$ weighting.

The results for intra and inter-assay with respect to precision and accuracy met the acceptance criteria specified in FDA and EMA guidelines, with the data briefed in Tables 4 and 5.

Sensitivity

Lower limit of quantification (LLOQ) defines the method sensitivity. The method was found to be sensitive (0.20 ng/mL) based upon the evaluation of the LLOQ quality controls (QC) samples of three precision and accuracy batches.

Linearity of dilution

The precision and accuracy of the dilution quality control (DQC) were checked as an indicator of dilution integrity, which was prepared by

spiking 1.6 times the concentration of the ULOQ. 6 samples each of these were prepared by diluting them 4 times before extraction using screened blank matrix along with CC and QC. The precision (% CV) for the DQC sample was 1.56%, and the % mean accuracy was 101.59%.

Recovery

The method's recovery was assessed by testing six sets of extracted and post-extracted quality control samples representing low, medium, and high concentration levels (lower quality control [LQC], middle quality control, higher quality control [HQC]). Recovery was calculated based upon the peak areas of these samples, and the % recovery was calculated. Extraction efficiency at low, middle, and high QC levels was found to be 65.1%, 69.8%, and 65.3% for analyte and 67.7%, 63.5%, and 66.2% for internal standard, respectively. The overall mean recovery of axitinib and axitinib D3 was found to be 63.41% and 67.00%, respectively.

MF

MF was assessed from a total of 8 different lots from previously screened plasma lots. To assess the MF, the peak area signals/responses of fortified (post-spiked) and neat samples were compared. The %CV of the internal standard (IS)-normalized MF calculated at LQC and HQC levels was reported to be $<15.0\%$, which meets the acceptance limits.

Stability experiments

All the matrix stability experiments (freeze-thaw, benchtop, autosampler, and wet extract at refrigerator temperature) were performed at low and high QC levels with $n=6$. The benchtop stability

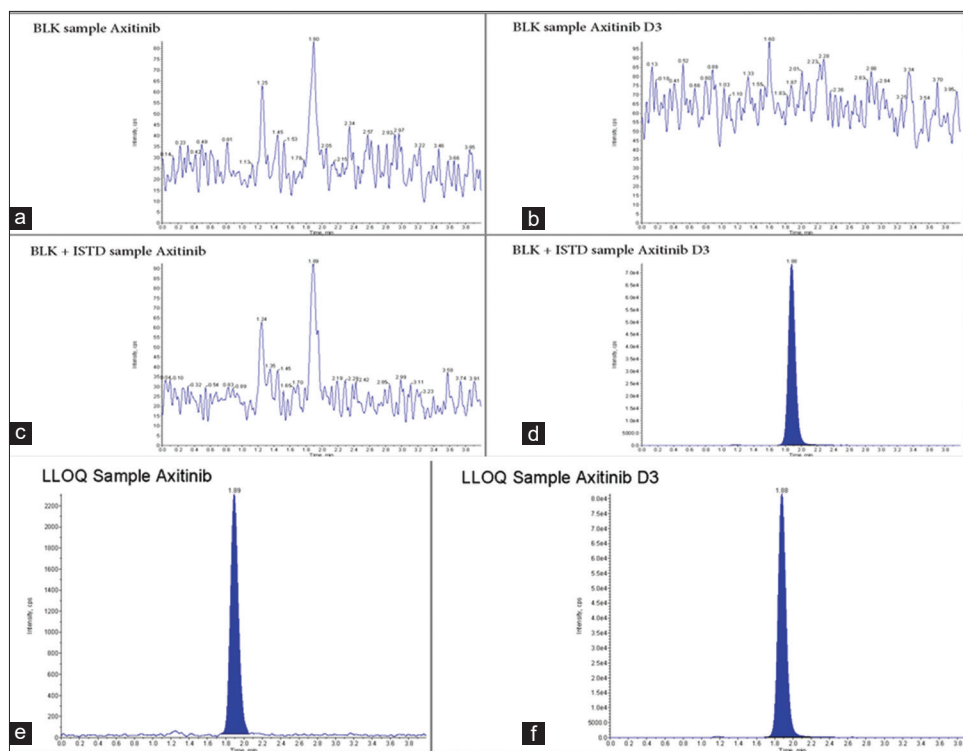


Fig. 4: Representative chromatograms of BLK sample (a and b), BLK+ISTD Sample (c and d), LLOQ sample (e and f). BLK: Blank, ISTD: Internal standard, LLOQ: Lower limit of quantification

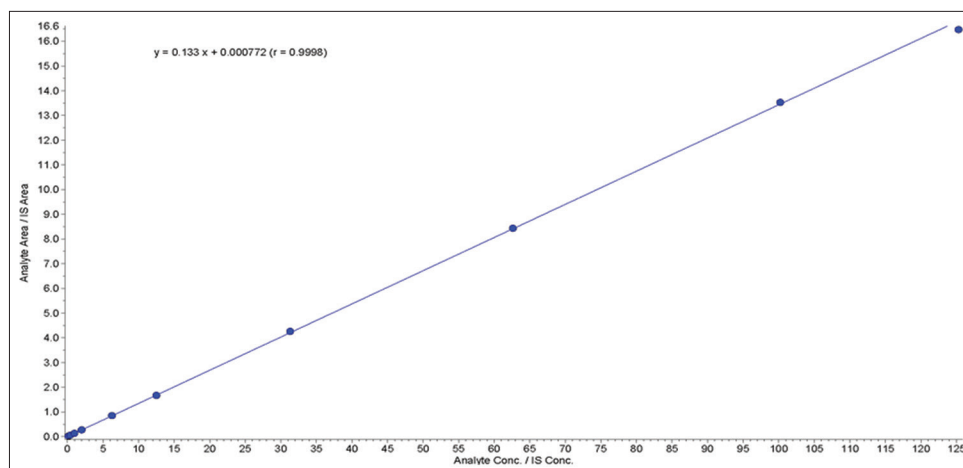


Fig. 5: Linearity curve of axitinib

Table 4: Intraday assay precision and accuracy

QC name	Nominal concentration (ng/mL)	Intraday assay		
		Mean±SD	% Nominal	% CV
Sensitivity QC	0.20	0.21±0.01	104.98	3.82
Lower QC	0.50	0.54±0.02	104.67	2.86
Middle QC	57.57	59.23±1.20	102.89	2.02
Higher QC	98.20	97.21±1.50	99.00	1.54

SD: Standard deviation, CV: Coefficient of variation, QC: Quality control

Table 5: Interday assay precision and accuracy

QC name	Nominal concentration (ng/mL)	Interday assay		
		Mean±SD	% Nominal	% CV
Sensitivity QC	0.20	0.21±0.01	105.92	4.71
Lower QC	0.50	0.55±0.02	105.73	4.29
Middle QC	57.57	59.75±2.46	103.79	4.12
Higher QC	98.20	98.47±3.12	100.27	3.17

SD: Standard deviation, CV: Coefficient of variation, QC: Quality control

was evaluated for 18 h by calculating the stability samples retrieval time and extraction start time. Autosampler stability of the analyte was carried out at 10°C and found to be stable for 1 day 16 h. Three freeze-thaw cycles were performed to validate a series of repeated

freeze-thawing. The wet extract stability at refrigerator temperature (2°C–8°C) was proved for 1 day and 17 h based on the time of storage and retrieval of stability samples from the refrigerator. Stability results and assessment (Fig. 6) are presented in Table 6.

Table 6: Matrix stabilities

Stability parameter	Stability sample at low QC		Fresh sample at low QC		Stability sample at high QC		Fresh sample at high QC	
	% Nominal	% CV	%Nominal	% CV	% Nominal	% CV	% Nominal	%CV
Benchtop	104.21	5.00	101.13	4.77	106.58	1.42	106.56	1.53
Freeze-thaw at -70°C	96.17	4.37			108.04	1.12		
Autosampler	109.26	1.54			106.62	1.97		
Wet extract at RF	107.26	1.15			105.96	0.83		

QC: Quality controls CV: Coefficient of variation, RF: Refrigerator

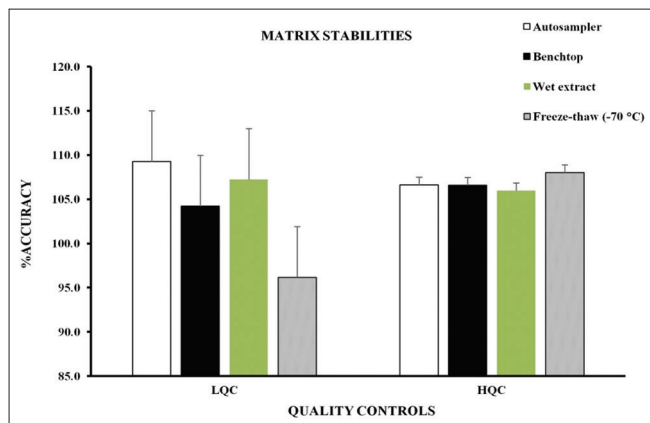


Fig. 6: Stability assessment of axitinib quality control samples in human plasma

CONCLUSION

This research work successfully achieved its key objective of developing and validating an accurate, precise, and efficient LC-MS/MS technique for measuring axitinib levels in human plasma, facilitating its application in pharmacokinetic studies. The utilization of axitinib D3 as an internal standard is a novel aspect of this method, effectively minimizing matrix effects and enhancing analytical precision. Notably, the method exhibited no significant matrix effects or carryover, highlighting its accurate and precise quantification of Axitinib in human plasma with a shorter run time. This validated method aligns with FDA and EMA guidelines and is well-suited for pharmacokinetic profiling and bioequivalence studies involving Axitinib.

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AUTHOR'S CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTEREST STATEMENT

The author declares that there are no conflicts of interest related to this work.

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