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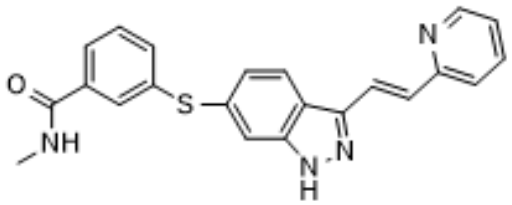
DEVELOPMENT OF A NOVEL HPLC METHOD FOR THE DETERMINATION OF AXITINIB IN HUMAN PLASMA BASED ON FLUORIMETRIC DETECTION AND PRECOLUMN DERIVATIZATION WITH 7-CHLORO-4-NITROBENZOFURAZAN: APPLICATION TO A PROTOTYPE PHARMACOKINETIC STUDY**

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Tyrosine kinase inhibitors (TKIs) are central to modern targeted cancer therapy. Axitinib, a highly selective oral TKI, primarily inhibits angiogenesis. This study aimed to develop a fast, reliable, and simple HPLC method with fluorescence detection to measure axitinib in human plasma. Sample preparation was performed using a simple and efficient liquid-liquid extraction (LLE) procedure. Axitinib was isolated from alkalinized plasma using chloroform, followed by evaporation of the organic phase. Prior to analysis, axitinib was derivatized with 7-chloro-4-nitrobenzofurazan (NBD-Cl). Separation was performed on a reversed-phase C18 column (2.6 µm, 4.6 mm × 150 mm) at 30 °C under isocratic conditions, using a mobile phase of acetonitrile and 0.1% orthophosphoric acid (75:25 v/v) at 0.9 mL min^{-1}. Fluorescence detection was set at 330 nm excitation and 405 nm emission. Axitinib eluted at 3.15 ± 0.02 minutes. The method showed excellent linearity from 0.01 to 30 ng mL^{-1} (r = 0.9998), with LOD and LOQ of 0.003 ng mL^{-1} and 0.01 ng mL^{-1}, respectively. Precision, expressed as RSD, did not exceed 2.65%. Validation confirmed compliance with EMA bioanalytical guidelines. The method was successfully applied in pharmacokinetic studies, enabling determination of AUC_{0-t}, $AUC_{0-\infty}$, Cmax, Tmax, and t½. Thanks to its sensitivity and robustness, this HPLC-fluorescence method is suitable for routine quantification of axitinib in human plasma and can be applied in bioavailability and bioequivalence studies.



INTRODUCTION

Axitinib (Fig. 1) is chemically described as (E)-*N*-methyl-2-((3-(pyridine-2-yl)vinyl)-1H-indazol-6-yl)thio)benzamide. INLYTA (axitinib) got approved in 2012 for the treatment of renal cell carcinoma, is a selective second-generation inhibitor of vascular endothelial growth factor receptors (VEGFR) 1, which is involved in angiogenesis and tumour growth VEGFR 2, which leads to endothelial cell proliferation, migration, and survival as well as angiogenesis and finally

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VEGFR 3, which is involved in lymphangiogenesis by binding to the intracellular ATP site domain of the VEGFR.[1,2] *In vitro*, the compound has been shown to inhibit angiogenesis, vascular permeability and blood flow.[3,4] In phase I and II clinical studies, axitinib showed antitumour activity against different advanced tumours,[5] including renal cell carcinoma (RRC),[6,7] pancreatic cancer,[8] and thyroid cancer.[9] The starting dose of the drug is 5 mg twice daily.

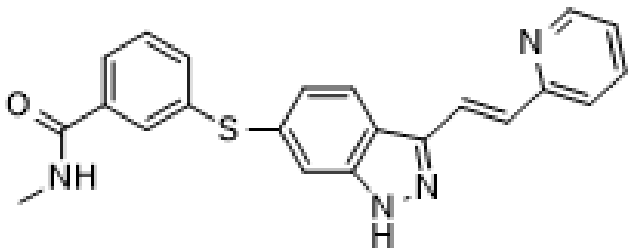


Fig. 1 – Chemical structure of axitinib.

Several analytical methods have been investigated to determine the concentration of axitinib including spectrophotometric methods,[10–11] thin-layer chromatography,[12] micellar liquid chromatography,[13] nuclear magnetic resonance spectroscopy,[14] high-performance liquid chromatography-UV detector,[15,16] and high-performance liquid chromatography-MS/MS detector.[17–24] No methods have been described yet using the derivatization of axitinib with NBD-Cl compound for biological matrices (human plasma). In the current work, we introduce a novel sensitive and specific high performance liquid chromatographic method combined with fluorescence detection. Axitinib is not a fluorogenic molecule; however, the secondary amine group present in its structure makes it suitable for fluorogenic derivatization reactions. The pre-column derivatization process conducted with a fluorogenic reagent; (NBD-Cl), which is frequently preferred because of its basic reaction procedure, fast reactivity and high sensitivity. It has been used as a fluorogenic agent for the determination of amines, amino acids, thiol and sulfhydryl groups.[25,26] The reagent has also been used for the determination of compounds which contains carbamate, upon hydrolysis in alkali medium.[27] The objective of this work was to quantify axitinib levels in human plasma using a high-performance liquid chromatography (HPLC) technique following derivatization with NBD-Cl. The newly developed method was successfully applied to characterize the pharmacokinetic behavior of axitinib tablets. Pharmacokinetic evaluations were conducted using plasma samples collected from healthy volunteers after axitinib administration, in accordance with approval granted by the relevant ethics committee.

EXPERIMENTAL

Chemicals and Reagents

Axitinib reference material was supplied by Shanghai Yingxuan Pharmaceutical Science & Technology (China), whereas Inlyta® tablets containing 5 mg of axitinib were purchased from a local pharmacy. Acetonitrile and orthophosphoric acid of HPLC grade, along with monobasic dihydrogen phosphate, dibasic monohydrogen phosphate, hydrochloric acid, methanol, and chloroform of analytical grade, were obtained from Merck (Darmstadt, Germany). The derivatizing agent 7-chloro-4-nitrobenzofurazan (NBD-Cl) was sourced from Sigma-Aldrich (St. Louis, USA). Ultrapure water was produced using a Human (Japan) water purification system.

Instrumentation and Chromatographic Conditions

Fluorescence spectral measurements were performed using a Shimadzu RF-1501 fluorescence spectrophotometer. Optimal excitation and emission wavelengths were set at 330 nm and 405 nm, respectively. Solution pH values were monitored with a WTW pH 526 digital pH meter. High-performance liquid chromatographic analyses were performed on a Shimadzu LC-20 system (Japan), comprising an LC-20AT solvent delivery

unit, a SIL-HT autosampler, an SPD-20A UV–Vis detector, and a CTO-10AC column thermostat.

To optimize chromatographic performance, various mobile phase compositions, column chemistries, particle sizes, flow rates, and column temperatures were systematically evaluated. Optimal separation was achieved using an Inertsil® C18 analytical column (150 × 4.6 mm, 2.6 μm particle size) maintained at 30 °C. Isocratic elution was employed with a mobile phase composed of acetonitrile and 0.1% (v/v) orthophosphoric acid in water in a 75:25 ratio, delivered at a flow rate of 0.9 mL min^{-1}.

Solutions

Calibration standards covering a concentration range of 0.01–30 ng mL^{-1} were prepared by appropriate dilution of an axitinib stock solution (0.1 mg mL^{-1}) in methanol. The phosphate buffer was formulated by dissolving 2.0209 g of disodium hydrogen phosphate and 0.3394 g of sodium dihydrogen phosphate in 50 mL of distilled water. The pH was adjusted to 8.5 using 0.1 M hydrochloric acid, after which the solution volume was completed to 100 mL with water. A freshly prepared NBD-Cl solution at a concentration of 5 mg mL^{-1} in methanol was used for derivatization. All remaining solutions were stable for at least two weeks when stored at 4 °C.

Sample Preparation and General Procedure

Venous blood samples (5 mL) were collected from the peripheral veins of a healthy volunteer following approval by the ethics committee and receipt of written informed consent. The blood was transferred into tubes containing disodium EDTA and centrifuged at 4500 × g for 10 minutes. Separated plasma samples were then stored at −20 °C until analysis. For drug extraction, 1.0 mL of plasma was rendered alkaline by the addition of 500 μL of 0.1 M NaOH, followed by spiking with axitinib working solutions. The mixture was subsequently extracted with 5 mL of chloroform. After vortex mixing at moderate speed for 5 minutes, the samples were centrifuged for an additional 5 minutes at 4500 × g. The aqueous phase was carefully discarded, and the organic layer was evaporated to dryness under a gentle stream of nitrogen at 40 °C. The resulting residue was reconstituted with 500 μL of phosphate buffer (pH 8.5), 500 μL of NBD-Cl solution (5 mg mL^{-1}), and 1 mL of water, then heated at 80 °C for 7 minutes to complete the derivatization reaction. Following cooling in an ice bath, the reaction mixture was acidified by adding 0.2 mL of 1 N hydrochloric acid. After vigorous vortexing for 30 seconds, a 20 μL aliquot of the derivatized sample was injected into the HPLC system for analysis.

RESULTS AND DISCUSSION

Derivatization Process

The derivatization reaction between axitinib and NBD-Cl was systematically investigated and optimized to maximize product formation. Each experimental factor was evaluated individually while all other variables were maintained constant. Optimal conditions were determined for reaction duration, temperature, pH, buffer type, acetonitrile–water ratio, molar proportion of NBD-Cl to axitinib, hydrochloric acid concentration, and the volume of acid required to effectively terminate the derivatization process. The effect of these parameters on derivatization efficiency is depicted in Fig. 2. Although an acetonitrile-water ratio of 1:3 provided optimal results during preliminary experiments, the final procedure employed a buffered aqueous system to maintain the required pH conditions and ensure compatibility with plasma samples.

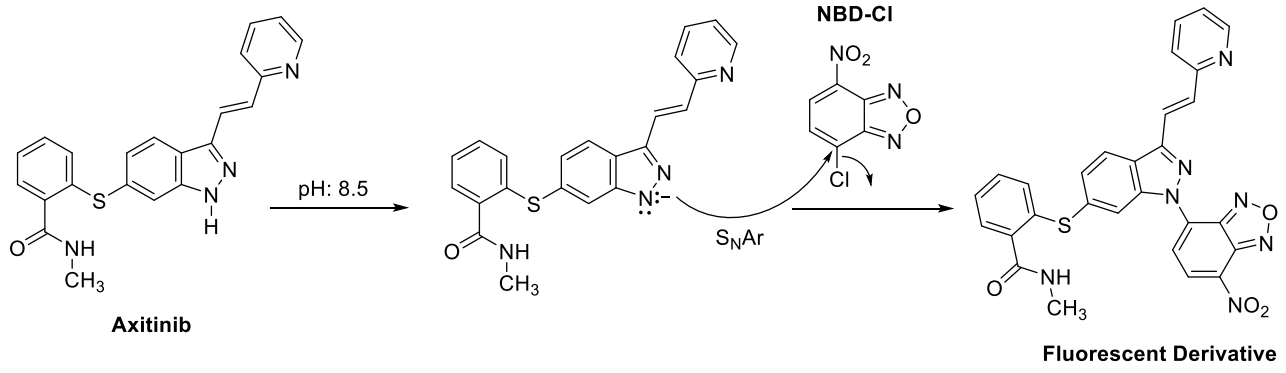


Fig. 2 – Derivatization reaction between axitinib and NBD-Cl.

The derivatization reaction between axitinib and NBD-Cl proceeds via a nucleophilic aromatic substitution mechanism. In the first step, under mildly basic conditions (pH 8.5), the indazole NH group of Axitinib is partially deprotonated, generating a nucleophilic nitrogen-centered anion. In the second step, this nucleophilic indazole nitrogen attacks the electron-deficient aromatic ring of 4-chloro-7-nitrobenzofurazan, which is activated toward nucleophilic substitution by the strongly electron-withdrawing nitro group. This results in a classical S_NAr reaction where the chloride ion is displaced, leading to the formation of the NBD-labeled fluorescent derivative. Importantly, the reaction occurs at the indazole nitrogen rather than the amide nitrogen, as the latter is resonance-stabilized and lacks sufficient nucleophilicity under these conditions.

Effect of pH

NBD-Cl itself does not exhibit fluorescence; however, upon reaction with amino or thiol functional groups, it forms strongly fluorescent derivatives. In addition, NBD-Cl provides a simple and reliable approach for the detection of N-terminal amino acids, and differences in fluorescence intensity and color can be exploited for the identification of prolyl-containing peptides.[28] Because fluorescence emission was observed exclusively under alkaline conditions, the influence of pH on the derivatization reaction was evaluated within the range of 7 to 11 using phosphate buffer.[29] The highest fluorescence response for the proposed method was obtained at pH 8.5. The effect of pH on the derivatization efficiency is illustrated in Fig. S2, which is provided in the Supplementary Material.

Effect of time and temperature

To determine the optimal conditions for fluorophore formation, the derivatization process was examined over a range of temperatures and reaction times. The reaction mixture was heated in a thermostatically regulated water bath, and maximum fluorescence response was achieved by maintaining the temperature at 80 °C for 7 minutes. Temperature effects were evaluated across a range of 30–100 °C, while reaction times from 0 to 10 minutes were investigated. The influence of temperature on peak area and the selection of the optimal reaction temperature is illustrated in Figure S3, which is included in the Supplementary Material.

Effect of NBD-Cl concentration

The effect of NBD-Cl concentration on the derivatization efficiency was systematically investigated. A total of 0.025 mmol (corresponding to 500 µL of a 0.5% w/v solution) was found to provide the maximum fluorescence intensity. During optimization, NBD-Cl amounts of 0.005, 0.010, 0.015, 0.020, 0.025, and 0.030 mmol were tested. No derivative formation was observed at concentrations below 0.025 mmol, resulting in the absence of a detectable peak in the chromatogram. Increasing the concentration above 0.025 mmol did not further enhance the peak area. The relationship between NBD-Cl concentration and peak area, as well as the determination of the optimal concentration, is illustrated in Figure S1 in the Supplementary Material.

Effect of acetonitrile to water ratio in derivatization medium

Different proportions of acetonitrile and water were evaluated while keeping the concentrations of the drug, buffer, and NBD-Cl solution constant. The highest peak area was achieved using an acetonitrile-to-water ratio of 1:3.

Stoichiometry of the reaction

The molar ratio of NBD-Cl to axitinib in the derivatization reaction was evaluated using Job's method of continuous variation.[30] By employing equimolar solutions of axitinib and NBD-Cl, the reaction stoichiometry was determined to be approximately 1:1. Chromatographic peak analysis confirmed that this ratio neither leaves the reagent in excess nor limits its availability, indicating complete consumption of NBD-Cl. To identify the optimal conditions for the derivatization, all prepared solutions were injected into the HPLC system and the corresponding peak areas were recorded. Under these optimized conditions, the resulting derivatives remained stable for at least 24 hours.

Effect of HCl concentration for acidification

Optimal results were achieved by adding 0.2 mL of 1.0 N HCl, which facilitated the formation of NBD-OH and effectively quenched any excess NBD-Cl. Acidification also minimizes potential secondary reactions with endogenous plasma components.

Chromatographic Process

Using the previously described HPLC–fluorescence method with isocratic elution, clear separation of the derivatized axitinib and endogenous plasma components was achieved.

Representative chromatograms of blank plasma, plasma spiked with 10 ng mL^{-1} axitinib, and plasma collected at tmax from a volunteer administered 5 mg Inlyta® tablets are presented in Figs. 3a–d. No interference from endogenous plasma constituents was observed. Axitinib was eluted with a retention time of 3.15 ± 0.02 minutes. Although axitinib metabolites such as glucuronide (M7) and sulfoxide (M12) have been reported in plasma, no additional peaks corresponding to these compounds were observed under the applied chromatographic conditions. The glucuronide metabolite is expected to elute near the void volume due to its high polarity, while the sulfoxide metabolite may be present at low concentrations and/or exhibit different derivatization behaviour. Key chromatographic system suitability parameters are summarized in Table 1, which were assessed between the axitinib peak and a subsequent peak at 5.5 minutes.

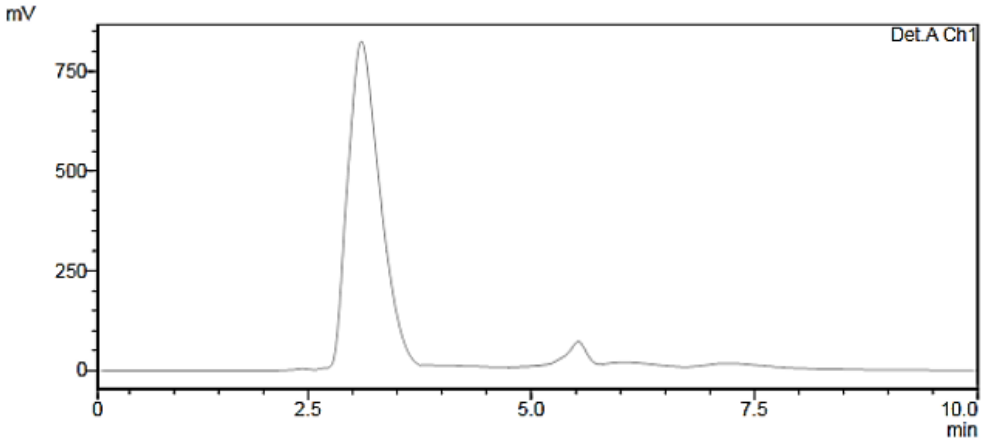
Table 1

Chromatographic system suitability parameters

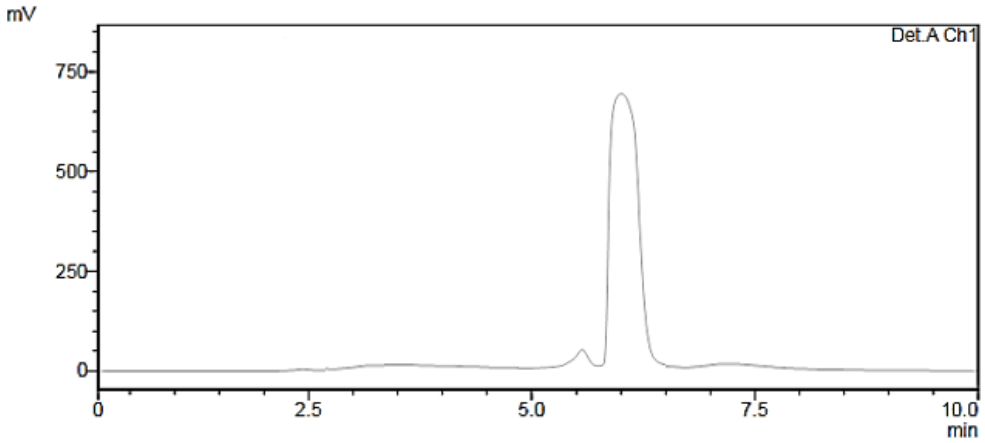
Retention time (tR, min)	Resolution (Rs)[a]	Retention factor (k)[b]	Theoretical plate Number (N)	Tailing factor	Asymmetry factor
3.15	2.44	2.15	1850	1.2	1.4

*Resolution calculated between the axitinib peak and the adjacent peak eluting at approximately 5.5 min.
[b]The void time (t_0) was estimated based on column dimensions and flow rate.

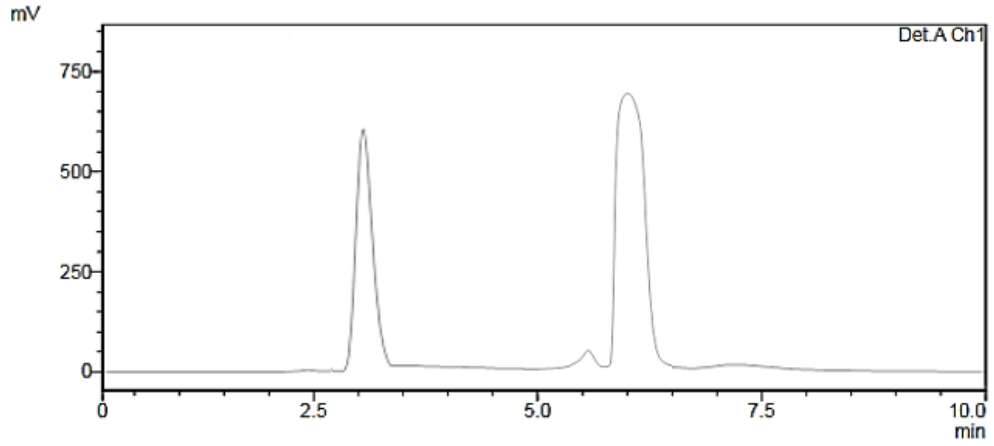
Chromatographic system suitability parameters were recalculated using standard equations based on experimentally observed peak characteristics. The void time (t_0) was estimated to be approximately 1.0 min under the applied chromatographic conditions. Theoretical plate number (N) was calculated from peak width, and resolution was determined between the axitinib peak and the nearest eluting peak.



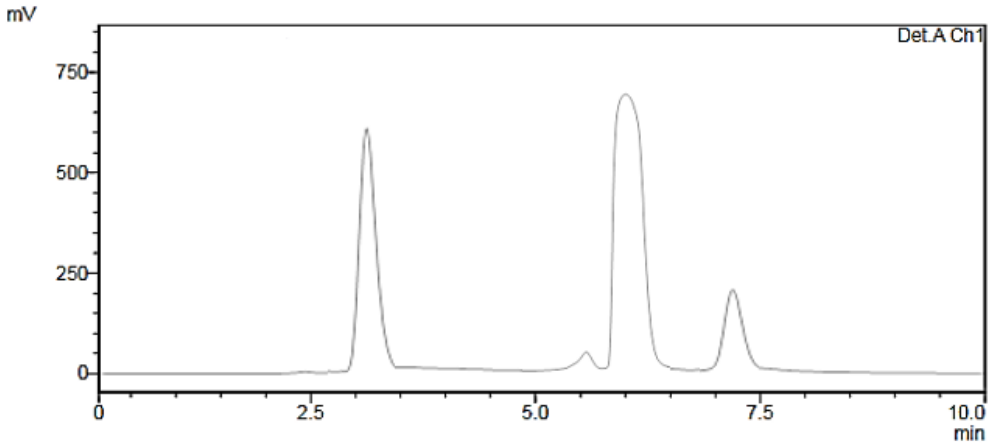
(a)



(b)



(c)



(d)

Fig. 3 – a) standard solution (10 ng/mL standard axitinib solution); b) blank plasma sample; c) 10 ng/mL axitinib spiked to plasma; d) plasma sample of volunteer after t_{max}.

Validation of the Method

The method was validated in accordance with European Medicines Agency (EMA) guidelines.[31] Based on the validation results, the method demonstrated sufficient reproducibility, and the inclusion of an internal standard was considered unnecessary.

Linearity and sensitivity

The linearity of the method was evaluated using a calibration range of 0.01–30 ng mL^{-1} (n = 5). Plasma samples (5 mL) were spiked with varying amounts of axitinib working solutions to generate the calibration standards. These samples were then processed following the described extraction, derivatization, chromatographic separation, and fluorescence detection procedures. Calibration curves were constructed by plotting the peak areas of the axitinib–NBD-Cl derivatives against the corresponding axitinib concentrations and analyzed using linear least-squares regression. The resulting five-point calibration curve (n = 5) was described by the equation: y = 233165 x + 16667, with a correlation coefficient (r^2) of 0.9998, where x represents the axitinib concentration and y corresponds to the peak area.

The limits of detection (LOD) and quantification (LOQ) were calculated using the formula LOD or LOQ = k·SDa/b, where k = 3 for LOD and k = 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope of the calibration curve. Analytical performance parameters are summarized in Table 2. The method demonstrated a lower limit of quantitation (LLOQ) of 0.02 ng mL^{-1} and an upper limit of quantitation (ULOQ) of 30.0 ng mL^{-1}. A weighting factor of $1/x^2$ was applied during regression analysis to improve accuracy at lower concentrations.

The limits of detection (LOD) and quantification (LOQ) were calculated using the expressions LOD = (3 × SDa) / b and LOQ = (10 × SDa)/b. Theoretical LOQ values calculated from calibration data were lower; however, the reported LOQ (0.01 ng mL^{-1})

corresponds to the experimentally validated lower limit of quantification (LLOQ), determined based on accuracy, precision, and signal-to-noise criteria in accordance with EMA guidelines. A representative chromatogram at the LOQ level (0.01 ng mL^{-1}) is provided (Fig. S4), demonstrating that the signal-to-noise ratio is sufficient for reliable quantification.

Table 2

Analytical parameters of the method

Parameters	Method
Concentration range [a] (ng mL^{-1})	0.01–30
Regression equation[b]	y=233165×+16667
Slope± SD	233165± 381
Intercept± SD	16667± 28
Correlation coefficient (r^2)	0.9998
LOD (ng mL^{-1})	0.003
LOQ (ng mL^{-1})	0.01

[a] Average of six determinatons
[b] $y=xC$ +b where C is the concentration in ng mL^{-1} and y is the peak area

Accuracy, precision and recovery

Accuracy and precision of the method were assessed using quality control (QC) samples at three concentration levels. QC samples were prepared in both plasma and aqueous matrices at low, medium, and high concentrations (0.01, 1.0, and 30 ng mL^{-1}; n = 3). Accuracy was expressed in terms of recovery and relative mean error (RME), while precision was reported as relative standard deviation (RSD).

The absolute recovery of axitinib from plasma was evaluated by comparing peak areas of extracted and derivatized plasma samples to those of equivalent aqueous solutions subjected only to derivatization. The mean absolute recovery was 99.74%, and the mean relative recovery, calculated by comparing measured versus spiked concentrations using the calibration curve, was 98.08%. Intraday precision and accuracy were determined by analyzing three replicates of each concentration within a single day, while interday assessments were performed over three separate days. All RSD values for intraday and interday measurements were below 2.65%. These results, summarized in Table 3, demonstrate that the method exhibits excellent accuracy and precision.

Table 3

Accuracy and precision of the method

Existing concentration (ng mL^{-1})	Added concentration (ng mL^{-1})	Found concentration (ng mL^{-1}) (Mean±SD[1])	Recovery (%)	RSD of recovery	RSD of intraday variation	RSD of interday variation
1	0.01	1.00 ± 0.03	99.00	1.03	1.73	2.53
	1	1.97 ± 0.02	98.50	1.21	1.94	2.65
	30	30.92 ± 0.07	99.74	0.97	1.46	2.32

Mean relative recovery = 99.08
For each concentration n=3

Robustness

As described in the validation section, method robustness was assessed using QC samples at three concentration levels (n = 3). The effect of deliberate variations in chromatographic conditions – including flow rate, column oven temperature, and the acetonitrile-to-water ratio in the mobile phase—was evaluated. Specifically, the column temperature was adjusted from 30 °C to 25 °C and 35 °C, the mobile phase composition was varied from 75:25 (v/v) to 80:20 and 70:30, and the flow rate was changed from 0.9 mL min^{-1} to 0.8 and 1.0 mL min^{-1}. These modifications had negligible impact on peak area and resolution. Low RSD values, summarized in Table 4, confirm the method's robustness.

Table 4

Robustness of the method

Condition	Value	Recovery %	RSD %
Flow rate mL min^{-1}	0.8	97.8	1.62
	1.0	98.7	1.44
Mobile phase composition (acetonitrile:aqueous phase)	70:30	98.0	2.11
	80:20	99.1	1.96
Column temperature	25	99.7	1.06
	35	99.8	1.03

n=3 for all QC sample levels

Stability

The stability of axitinib working standard solutions at QC levels was evaluated under different storage conditions using three replicates. The tested conditions included 24 hours at room temperature in the dark, 24 hours under autosampler conditions, and refrigeration at 4 °C for 1 month. Recovery rates under these conditions were 99.2%, 98.7%, and 99.6%, respectively, exceeding those reported in previous studies. The highest observed RSD across all conditions was 2.83%. These results demonstrate that axitinib remains stable under all evaluated storage conditions.

Application of the Method to Pharmacokinetic Analysis

The developed method was applied to determine axitinib levels in plasma for a pharmacokinetic study. A single oral dose of 5 mg axitinib was administered to a healthy 28-year-old male volunteer. On the first day, approximately 5 mL of venous blood was collected prior to dosing and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, and 8 hours post-dose. Additional blood samples were taken once daily for the following five days. Plasma was separated from the collected blood as described previously. A representative chromatogram of a plasma sample collected 2 hours after administration is shown in Figure 3d. All samples were stored at −20 °C until analysis. Pharmacokinetic parameters were calculated based on the measured plasma concentrations using the proposed method. The TOPFIT 2.0 pharmacokinetic and pharmacodynamic data analysis software was employed to determine the areas under the plasma concentration-time curves ($AUC_{0–12}$ and $AUC_{0–\infty}$).[32] The plasma concentration-time profile of axitinib following a single 5 mg oral dose is presented in Fig. 4. The pharmacokinetic parameters, summarized in Table 5, were consistent with previously reported values.[33]

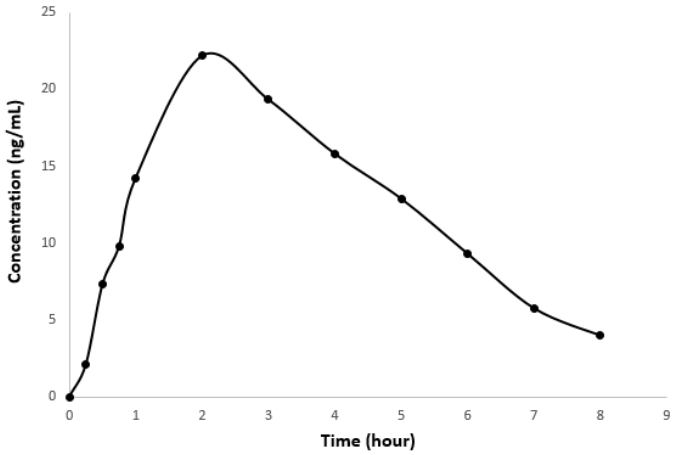


Fig. 4 – Pharmacokinetic curve of axitinib after administration of 5 mg dose oral.

Table 5

Pharmacokinetic parameters of axitinib after administration of single oral dose of 5 mg

Parameter	Found value
T_{max} [a](h)	2.0
C_{max} [b](ng mL^{-1})	22.18
$t_{1/2}$[c](h)	5.1
$AUC^{d}_{0\text{-}12}$ (ng h mL^{-1})	55.8
$AUC^{d}_{0\text{-}\infty}$ (ng h mL^{-1})	59.3

[a]Time to maximum concentration
[b]Maximum concentration,
[c]Elimination half life,
[d]Area under the concentration-time curve

CONCLUSION

As axitinib is a relatively new therapeutic agent, further studies on its potential drug–drug and drug–food interactions, as well as safety profile, are required. The HPLC–fluorescence (HPLC–FL) method developed in this study is sensitive, cost-effective, and highly reproducible. The drug

exhibits a short retention time of approximately 3.15 minutes, enabling rapid analysis. The formation of highly fluorescent axitinib–NBD-Cl derivatives allows straightforward fluorometric detection using a simple HPLC–FL system, making this approach accessible in most analytical laboratories. To the best of our knowledge, this represents the first fluorimetric detection method for axitinib reported in the literature. The method is well suited for routine plasma analysis, as well as for bioequivalence and bioavailability studies.

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