

Development and Validation of LC-MS/MS Method for the Determination of Sorafenib in Plasma

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ABSTRACT

A simple, quick, accurate, and highly specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was established and validated for measuring Sorafenib levels in human plasma. Erlotinib, serving as the internal standard, was added to the plasma sample before performing extraction with acetonitrile as the precipitating agent. The chromatographic separation was performed using a Luna C18 column (50 mm x 1 mm, 5 µm) and an isocratic mobile phase consisting of acetonitrile and 10 mM ammonium formate buffer in an 80:20 (v/v) ratio, with a flow rate of 0.5 mL/min. Quantification was carried out using the transitions 465.2 → 252.2 (m/z) for Sorafenib and 394.0 → 278.0 (m/z) for Erlotinib. The minimum quantitation level is determined to be 20 ng/mL with a 100 µL plasma sample. The nine working standards demonstrated linearity within the range of 20 to 8000 ng/mL, with an r^2 value of at least 0.9998. The chromatographic column achieved separation in just 2.0 minutes. The recovery rates for three quality control concentration trials averaged 94.25% for Sorafenib and 93.55% for Erlotinib. The intra- and inter-batch assays exhibited a coefficient of variation of 15% or less. The method developed was effectively utilized to analyze Sorafenib pharmacokinetics following oral administration.

Keywords: LC-MS/MS; Sorafenib; Erlotinib; Validation; Human Plasma.

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Introduction

Sorafenib is an oral multikinase inhibitor utilized as a targeted treatment for specific forms of advanced liver, kidney, and thyroid cancers. It functions by inhibiting proteins that instruct cancer cells to proliferate and divide, in addition to those that facilitate the development of new blood vessels essential for tumor growth. Sorafenib 4-[4-({[4-Chloro-3-(trifluoromethyl) phenyl] carbamoyl} amino) phenoxy]-N-methyl-2-pyridinecarboxamide is a multi-kinase inhibitor initially developed to target the Raf1 kinase pathway. However, in addition to the RAF/ MEK/ERK pathway, this drug also targets receptor tyrosine kinases such as VEGFR (Vascular endothelial growth factor receptor)-2 and -3, PDGFR (Platelet-derived growth factor receptor)-β, FLT (Fms-like tyrosine kinase)-3, and c-KIT.¹⁻³

Several methods have been reported for the determination of Sorafenib in plasma, such as high-performance liquid chromatography with ultraviolet detection (HPLC-UV)⁴⁻⁶ and gas chromatography-mass spectrometry (GC-MS). From the literature survey, it was concluded that the developed methods showed a lack of sensitivity and internal standard usage. There are very limited methods that have been reported for the estimation of Sorafenib in biological samples. The HPLC-MS/MS approach for Sorafenib analysis in plasma has been described in recent publications as being quicker and easier. A novel HPLC-tandem mass spectrometry technique was created and verified in the current study to analyse the amounts of sorafenib in plasma. The use of liquid chromatography with mass spectrometry (LC-MS/MS) is now generally accepted as the preferred technique for the determination of small

molecules, metabolites, and enantiomers in biological samples, since this technique is highly selective and sensitive. In the present work, a new LC-MS/MS method for the determination of sorafenib in plasma is presented utilizing a deuterated internal standard (Erlotinib) in positive ionization mode. The LC-ESI MS/MS (liquid chromatography and tandem mass spectrometry with electron spray ionization approach) was measured using the multiple reaction monitoring (MRM) method.⁷⁻⁹

Erlotinib is used as an internal standard for the estimation of sorafenib as it exhibits similar physicochemical properties and chromatographic behavior to sorafenib, but can be detected separately, allowing it to accurately account for potential variations during sample preparation and analysis.¹⁰

Materials and Methods

Chemicals and reagents

Sorafenib and Erlotinib were procured from Samed Labs Ltd. Acetonitrile (HPLC grade) was purchased from Merck Millipore India Ltd. Milli-Q water from Millipore water system (Billerica, USA) was used throughout the experiments. Analytical grade Ammonium Formate was obtained from Sigma Aldrich (Bangalore, India). Blank plasma was purchased from Kaveri blood bank (Hyderabad, India).

Equipment

The equipment's used in the present study are vortex mixer CM-101 plus (Remi Laboratory Instruments, Mumbai); analytical balance Mettler Toledo® model XS 205 (Greifensee, Switzerland); refrigerated centrifuge 5702 R (Eppendorf India Limited, Chennai); Analysis was carried on Agilent (Santa Clara, CA, USA) 1200 series HPLC coupled with tandem quadrupole mass spectrometer (MS/MS) API 4000 (Applied Biosystems/AB Sciex).

Chromatographic and mass spectrometry conditions

Samples were separated on a Luna C18 column (50 mm x 1 mm, 5 µm). The column temperature was set at 40°C. The mobile phase was acetonitrile: 10mM ammonium formate buffer (80:20 v/v) as an isocratic mobile phase with a flow rate of 0.5 mL min⁻¹, and the total run time was 2.0 minutes. Sample analyses were performed in positive electrospray ionization mode. The MS/MS detection was conducted by multiple reaction monitoring at a transition of 465.2 → 252.2 (m/z) for Sorafenib and 394.0 → 278.0 (m/z) for Erlotinib. For both Sorafenib and Erlotinib, the source-dependent parameters were as follows: Gas 1 (nebulizer gas):

50psig; Gas 2 (heater gas flow): 55 psig; ion spray voltage: 4500 V; turbo heater temperature: 450°C; interface heater: On; entrance potential: 10V; collision activation dissociation gas: 6 psig. The optimized values for analyte-dependent parameters were set as follows: declustering potential, collision energy, and cell exit potential were set to 80, 20, and 10 V for Sorafenib and 80, 20, and 10 V for Erlotinib. Quadrupoles 1 and 3 were maintained at unit mass resolution, and the dwell time was set at 200ms.

Standard and quality control sample preparation

Sorafenib and Erlotinib stock solutions were prepared in DMSO (Dimethyl Sulfoxide) to a final concentration of 1 mg mL⁻¹. The appropriate dilutions with acetonitrile: Water (50:50 v/v) were prepared to produce 9 working standards of 20 to 8000 ng/mL of Sorafenib and 100 µg mL⁻¹ sample of Erlotinib. The standard solutions for calibrations in the range of 20 to 8000 ng mL⁻¹ were prepared by spiking 50 µL of the working standards into 1 mL of blank plasma. The QC samples were prepared with a different Sorafenib working solution and diluted in blank plasma. The QC Sorafenib concentrations included 20ng mL⁻¹ for the lower limit of quantity (LLOQ QC), 60 ng mL⁻¹ for a low-quality control (LQC), 1500 ng mL⁻¹ for a middle quality control (MQC), 7000 ng mL⁻¹ for a high-quality control (HQC), and 14400 ng mL⁻¹ for a Dilution quality control (DIQC). All spiked plasma samples were stored at -70°C.

Sample preparation

Before extraction, the aliquots of frozen plasma samples (100 µL) were thawed at room temperature and thoroughly vortexed. The calibration curve consists of a blank sample (matrix sample processed without IS), a zero sample (matrix sample processed with IS), and nine non-zero samples covering the expected range, including LLOQ. To each calibration sample and quality control sample, 25µL of IS was added, followed by 0.6 mL of acetonitrile as a precipitating agent in an Eppendorf tube. The samples were vortexed thoroughly and centrifuged at 4000 rpm for 5 min at 5°C. The supernatant layer was transferred to autosampler vials and 10 µL was injected into LC-MS/MS.

Data Collection and Integration

The data were gathered and processed with Sciex Analyst version 1.6 data collection and integration software on a Microsoft Windows-compatible computer.

Bioanalytical Method Validation

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ICH guidelines and USFDA guidelines were followed for method validation.¹¹⁻¹² Assay performance was evaluated through the determination of specificity, linearity, precision, accuracy, recovery, and stability. Specificity of the method was carried out by analyzing six drug-free plasma and volunteer samples for interference of endogenous compounds. The standard curve was calculated by plotting a calibration plot with peak area versus the corresponding drug concentrations. Linearity of the standard curve was evaluated using least-squares linear regression analysis with weighting 1/x². Intra and inter-day precision were determined by analyzing quality control plasma samples on the same day and on different days. For determining the method precision, six replicates of samples from three quality control standards and the lowest calibrator were analyzed in a batch, and the % relative standard deviation was determined. Recovery studies were carried out by comparing the observed concentration with the actual values of spiked controls. The recoveries of Sorafenib and Erlotinib were determined by comparing the response of plasma samples with the response of identical standards prepared in the mobile phase, which did not undergo sample pretreatment.

Stability

The sample stability of Sorafenib on refrigerated stock solution, stock solution stability at room temperature, Short-Term room temperature stock dilution stability, Auto sampler stability, Freeze-Thaw stability, Long-Term stability of plasma sample for below -70°C, and bench top stability were investigated. For evaluating the stability of spiked plasma samples, higher and lower quality control standards were used in the study.¹³⁻¹⁴

Clinical Study

Eighteen volunteers were enrolled in the study. The study protocol was approved by the Ethics committee and was carried out in accordance with the principles of the Declaration of Helsinki and ICH guidelines. The blood samples were collected at designated time points and analyzed.

Results and Discussion

The main aim of this work was to develop a rapid, precise, selective, and sensitive LC-MS/MS method for the quantification of Sorafenib in human plasma. Erlotinib was used as an internal standard. Chromatographic separation was achieved on a Luna C18 column (50 mm x 1 mm, 5 µm) with Acetonitrile: 10mM ammonium formate buffer (80:20 v/v) as an isocratic mobile phase with a flow rate of 0.5 mL min⁻¹.

Quantitation was performed by transition of 465.2 → 252.2 (m/z) for Sorafenib and 394.0 → 278.0 (m/z) for Erlotinib.

Chromatography

Representative chromatograms of Drug-free blank plasma, LLOQ QC sample, and HQC sample are given in Figure 3 (A), (B), and (C), respectively.

Selectivity and sensitivity

Six distinct lots of plasma were collected under controlled conditions, extracted, and analyzed using the established method. No interferences were detected at the retention times for Sorafenib and Erlotinib. The samples were compared with those derived from an extract of a previously spiked plasma sample at LLOQ that contained Sorafenib and Erlotinib. The impact of potentially interfering drugs (PID) such as caffeine, nimesulide, paracetamol, ibuprofen, and aspirin on the analysis of Sorafenib was assessed by spiking PIDs at their approximate C_{max} concentration in the MQC sample in triplicate. Samples were prepared by adding 25µL of each interfering drug to the MQC containing Sorafenib. Likewise, blank samples were prepared by taking 100 µL of blank plasma and 25 µL of the potentially interfering drug. The samples were quantified using a calibration curve. The back-calculated concentrations of the MQC sample spiked with PID were found to be within +15% of the actual concentration of the MQC sample. Therefore, the PIDs do not affect the analysis of Sorafenib. The lowest quantification for Sorafenib was established at the concentration of the LLOQ QC, which is 20 ng mL⁻¹. The precision and accuracy for Sorafenib at this concentration were determined to be 3.71% and 103.67%, respectively.

Goodness of fit

The data of four precision and accuracy batches were subjected to goodness of fit analysis after taking the back-calculated concentration of CC standards meeting the acceptance criterion using 1/x and 1/x² weighing. After performing the goodness of fit, 1/x² was found to be the best fit for regression.

Linearity

Linear calibration curves were plotted by using the partial least squares method, in which least square regression of quantities versus peak area ratio Sorafenib to Erlotinib with a weighting factor (1/x²) was utilized. Sorafenib was linear in the range of 20 to 8000ng mL⁻¹ with a coefficient of determination greater than 0.99 (Table 1).

Precision and accuracy

The precision of the assay was measured by the percentage coefficient of variation over the concentration range of LLOQC, LQC, MQC, and HQC samples, respectively, during validation. The intraday and inter-day precisions were less than 15.00% for each QC level of Sorafenib. The accuracy, determined from QC samples, was within 15.00% for each QC level. The results obtained are given in Table 2.

Recovery

Prepared 6 sets of recovery comparison samples by spiking 5 μ L dilution of quality control samples (LQC, MQC, HQC) of Sorafenib, 25 μ L of Erlotinib, and 995 μ L of Acetonitrile, representing 100% extraction and injected. The recovery comparison samples of Sorafenib were compared against the extracted samples of LQC, MQC, and HQC. The mean overall recovery of Sorafenib was 94.25%. The mean recovery of the internal standard was 93.55%.

Stabilities:

Refrigerated stock solution stability

Refrigerated stock solution stability was carried out for 10 days by making six injections of stability standard stock solution and the fresh standard (comparison stock) stock solution of Sorafenib and Erlotinib. The stability sample response was adjusted by multiplying it by a correction factor. The % change for Sorafenib was 1.59, and the % stability was 98.48%. The refrigerated stability of Erlotinib was carried out for 10 days, and the % change was 1.25%, and the % stability was 99.12%.

Standard stock solution stability at room temperature

Standard stock solution stability of Sorafenib and Erlotinib was carried out for 24 hours at room temperature by injecting six replicates of stock dilutions of stability standard stock solution and the fresh standard (comparison stock) stock solution. The response of the stability sample was corrected by multiplying by the correction factors. The standard stock solution % stability for Sorafenib was 97.12%, and for Erlotinib was 98.82%.

Short-Term room temperature stock dilution stability

Room temperature stability of stock dilution was carried out for 24 hours by injecting six injections of prepared stock dilutions of Sorafenib kept at room temperature against the dilution kept in the refrigerator. The percentage change for Sorafenib was 2.45%, and for Erlotinib was 2.12%, respectively.

Auto sampler stability

Six sets of quality control (QC) samples at low quality control (LQC) and high-quality control (HQC) levels were prepared and positioned in the auto sampler. These samples were injected after approximately 70 hours and quantified using freshly prepared calibration curve standards. The stability of the samples was confirmed over 70 hours. The nominal percentage at around 70 hours varied from 98.12% for HQC to 100.34% for LQC, respectively.

Freeze-Thaw stability

The stability of human plasma was assessed over five freeze-thaw cycles. Six injections of low-quality control (LQC) and high-quality control (HQC) samples were analyzed following these five freeze-thaw cycles. The quality control samples subjected to freeze-thaw were quantified using freshly spiked calibration curve standards. The percentage nominal values obtained ranged from 98.82% for HQC to 100.32% for LQC, respectively.

Long-Term stability of plasma sample below -70°C

The stability of Sorafenib for storage of plasma samples below -70°C was studied for 60 days by quantifying six sets each of LQC and HQC against the freshly spiked standards. The mean % nominal ranged from 95.76% (LQC) to 100.26% (HQC) for 60 days.

Bench top stability

Short-term room temperature stability in plasma was determined for around 16 hours, using six sets each of LQC and HQC. The quality control samples were measured in relation to the newly spiked calibration curve standards. Sorafenib was found to be stable for around 16 hours. The percentage nominal ranged from 95.15% (HQC) to 99.46% (LQC).

The results of the stability studies are given in Table 3.

Application: Pharmacokinetic Study

The proposed validated bioanalytical method was successfully applied to determine Sorafenib concentration in plasma for a pharmacokinetic study in a healthy male volunteer, who was orally administered a tablet containing 400mg Sorafenib NEXAVAR (Sorafenib Tablet 400mg) administered under fasting conditions in 18 healthy adult male human subjects in a randomized two-way crossover study. Venous blood samples were collected into heparinized tubes, and blood samples were centrifuged (4000rpm for 10min); plasma was separated and stored at -70°C until assay. A total of 18 samples (including predose) will be taken per period. One before dosing at 0.00 and at 1, 2, 4, 6, 8, 10,

12, 18, 24, 30, 36, 42, 48, 72, 168, 336, and 504 hours post dose. The maximum plasma concentration was 3421.524ng mL⁻¹ for test and 3482.624 ng mL⁻¹for reference, which occurred at about 1 hour's post dosing. Overlay graphs of mean concentration versus time of the two formulations (test and reference) are shown in Figure 4. The area under the curve from 0 to 504h was determined with the help of the linear trapezoidal rule. The maximum concentration achieved (C_{MAX}) was obtained directly from the measured concentration without interpolation. The parametric point estimates for the mean of test medication/the mean of reference medication were found within the commonly accepted bioequivalence range of 0.8 to 1.25. Therefore, the results indicate that the proposed method is suitable for pharmacokinetic studies to determine the concentration of Sorafenib in human plasma. The research study was carried out in full compliance with the standards established by the International Conference on Harmonization and the US FDA. The pharmacokinetic data are tabulated in Table 4.

Conclusion

The developed method is simple, cost-effective, rugged, and a high-throughput method for the estimation of Sorafenib in human plasma. The method consists of a simple sample pretreatment by protein precipitation to give consistent and reproducible recoveries of Sorafenib. In the present work, a new LC-MS/MS method for the quantification of Sorafenib in human plasma with less processing volume, sensitivity, and shorter run time was developed for clinical study sample analysis. The proposed method is suitable for comparing the pharmacokinetic profiles of formulations with high sensitivity, selectivity, and accuracy.

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	0	5	4	5	3		
5	100.00	102.65	985.456	958.495	989.9720	33.96	99.00
6	200.00	201.96	204.39	198.47	201.40	28.56	100.88
7	400.00	412.56	384.65	394.53	397.13	141.80	99.31
8	600.00	625.15	614.86	608.64	616.83	84.64	102.69
9	800.00	834.45	814.48	805.35	818.27	151.00	102.29
Correlation coefficient (r)		0.999	0.996	0.994			

Table 2. Accuracy and Precision of the Determination

Table 1. Results of Linearity

S.No	STD	Ru n 1	Ru n 2	Ru n 3	Mean	Standard Deviation	%Accuracy
1	20.000	20.025	19.586	21.242	20.2843	0.86	101.42
2	40.000	42.649	38.354	40.574	40.5257	2.15	101.31
3	100.000	105.693	98.562	99.761	101.3387	3.82	101.34
4	400.000	385.93	392.85	402.97	393.921	8.57	98.48

S. No	QC Nominal Conc	Intra Day(n=6)			Inter Day(n=6)		
		Mean	SD	%CV	Mean	SD	%CV
LLQ	20.00	20.506	0.67	3.27	20.7348	0.771	3.67
LQC	60.00	60.7430	0.70	1.16	60.1735	1.244	2.05
MQC	1500.000	1488.9505	14.67	0.97	1456.73	37.96	2.60
HQC	7000.000	7014.4235	109.33	1.56	7018.55	155.06	2.21

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Table 3. Summarized results of stability studies

S. No.	Stability Parameter	Stability Duration
1	Refrigerated Stock Solution Stability	10 Days
2	Room temperature Stock Dilution Stability	24 Hours
3	Autosampler Stability	70 Hours
4	Bench Top Stability	16 Hours
5	Freeze Thaw Stability	5 cycles
6	Long Term Plasma Stability at below -70°C	60 Days

Nominal Conc		0		0
1	62.566	60.57 4	6268.512	6493.66 4
2	60.210	61.12 6	6850.512	6771.59 6
3	59.242	59.27 6	6886.248	6888.85 0
4	61.842	60.89 6	7052.452	6597.54 4
5	63.090	65.55 8	6867.850	6492.84 8
6	61.426	62.12 2	6928.282	7125.52 2
Mean	61.3960	61.59 20	6808.976 0	6728.33 73
% Stability	100.32		98.82	

Table 3.1: Bench Top Stability (BTS)

S.No	Comparis on LQC	BTS LQC	Comparis on HQC	BTS HQC
QC Nominal Conc	60.000	60.00 0	7000.000	7000.00 0
1	63.566	63.57 4	6244.450	6485.90 4
2	61.210	62.12 6	6890.572	6571.45 6
3	57.242	58.27 6	6846.248	6565.97 0
4	62.842	61.89 6	7102.652	6451.53 2
5	66.910	64.90 6	7047.050	6504.84 8
6	62.142	61.12 2	7288.282	6829.58 2
Mean	62.3187	61.98 33	6903.209 0	6568.21 53
% Stability	99.46		95.15	

Table 3.3: Long-Term Stability in human plasma at -80.0°C (LTS)

S.No	Comparis on LQC	BTS LQC	Comparis on HQC	BTS HQC
QC Nominal Conc	60.000	60.00 0	7000.000	7000.00 0
1	60.566	60.57 4	6912.512	6490.68 4
2	62.210	60.12 6	6882.512	7113.51 6
3	60.242	58.27 6	6648.248	7048.85 0
4	60.842	58.89 6	6872.452	6899.94 4
5	67.178	57.70 4	6907.850	6715.64 8
6	60.426	60.12 2	6868.282	6929.92 2
Mean	61.9107	59.28 30	6848.642 7	6866.42 73
% Stability	95.76		100.26	

Table 3.2: Freeze and Thaw Stability in human plasma at -80.0°C (FTS) (5th cycle)

S.No	Comparis on LQC	BTS LQC	Comparis on HQC	BTS HQC
QC	60.000	60.00	7000.000	7000.00

Table 4. Summarized results of mean pharmacokinetic parameters

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Pharmacokinetic Parameters	Test (Mean±S.D)	Reference (Mean±S.D)
C _{max}	3421.524±241.3 25 ng mL ⁻¹	3482.624±282.5 12 ng mL ⁻¹
AUC _{0-t}	12546.2±142.24 5 ng.h/mL	12823.5±126.24 2 ng.h/mL
AUC _{0-inf}	12695.6±425.34 2 ng.h/mL	12985.4±125.33 6 ng.h/mL
T _{1/2}	30.0±0.36 hrs	30.0±0.42 hrs
T _{max}	1.0±0.05 hrs	1.2±0.06 hrs

S.D, standard deviation; C_{max}, maximum plasma concentration; AUC_{0-t}, area under the plasma concentration time curve from zero hour to 504 h; AUC_{0-inf}, area under the plasma concentration time curve from zero hour to infinity; T_{max}, time point of maximum plasma concentration; T_{1/2}, half-life of drug elimination during the terminal phase.

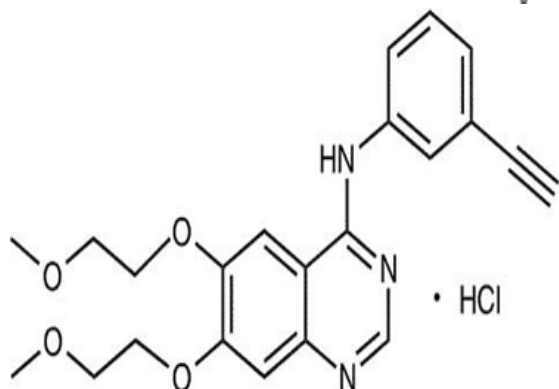
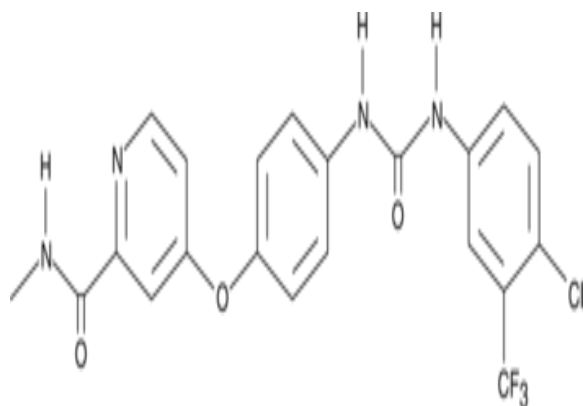


Figure 1. Sorafenib
Figure 2. Erlotinib Hydrochloride

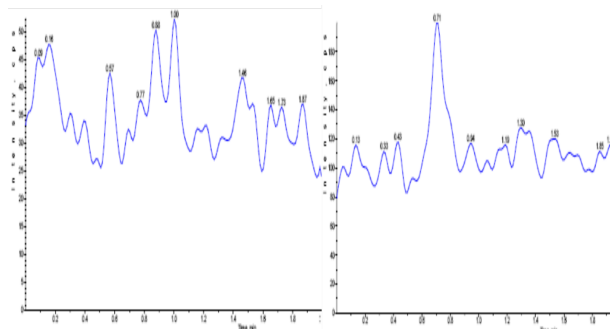


Figure 3 (A). Drug-free blank plasma

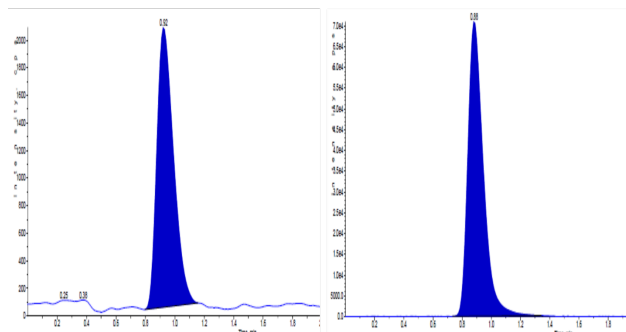


Figure 3 (B). LLOQ QC Sample with IS

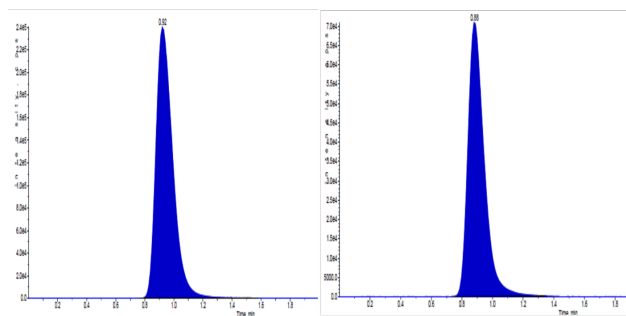


Figure 3 (C). HQC Sample with IS

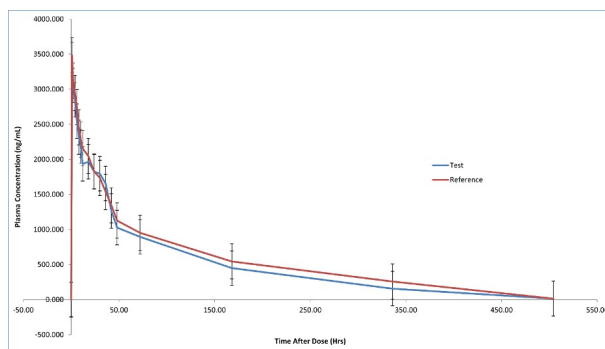


Figure 4. Mean plasma concentration-time profile of Sorafenib after oral administration of 400mg Sorafenib test and reference tablet formulation to 18 healthy male volunteers