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DEVELOPMENT OF A RAPID AND SENSITIVE LC-MS/MS ASSAY FOR THE DETERMINATION OF SORAFENIB IN HUMAN PLASMA

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Abstract:

A highly efficient liquid chromatography/tandem mass spectrometric method was devised to quantify Sorafenib in human plasma with rapidity and sensitivity. The samples underwent extraction using the solid-phase extraction technique, employing a pretreatment solution of 100mM ammonium formate along with Sorafenib 13CD3 as the internal standard. Separation was carried out on a Phenomenex C18 column (50X4.6mm, 5µm) using a mobile phase composed of 10mM ammonium formate, methanol, and acetonitrile in the ratio of 20:5:75 (v/v/v) at a flow rate of 0.8 ml/min. Mass detection utilized the Turbo ion spray interface in positive ion multiple reaction monitoring mode (MRM), monitoring the ion transitions from m/z 465.1→252.1 (sorafenib) and 469.1→256.1 (sorafenib 13CD3, ISD). The calibration curves exhibited linearity within the range of 5-5000 ng/ml. The method demonstrated acceptable results for linearity, accuracy, precision, recovery, and matrix effect, with accuracy falling between 99.1% and 108.9%. The method precision showed a coefficient of variation (% CV) less than 2.4%.

Keywords: Sorafenib, LC-MS, Human plasma.

Introduction [1-2]

Bio-analytical method validation (BMV) is the procedure employed to ensure the suitability of a quantitative analytical method for biochemical applications. Bioanalysis involves the quantitative measurement of substances like drugs and their metabolites, as well as biological

molecules found in unusual locations or concentrations. This field also encompasses the analysis of macromolecules and proteins.

LC-MS is commonly employed in laboratories for both quantitative and qualitative analysis of drug substances, drug products, and biological samples. Its crucial role lies in the evaluation and interpretation of data related to bioavailability, bioequivalence, and pharmacokinetics. Throughout the entire process of drug research and quality control, LC-MS is utilized to analyze biological samples. Key parameters for validating bioanalytical methods include precision, accuracy, and sensitivity.

Sorafenib, an antineoplastic agent, is used to treat advanced stages of kidney cancer (advanced renal carcinoma) and liver cancer (hepatocellular carcinoma). It functions as a protein kinase inhibitor, targeting several protein kinases, particularly C-RAF. Sorafenib induces autophagy, potentially inhibiting tumor growth. In this study, we present a sensitive, rapid, and specific LC-MS/MS technique for quantifying Sorafenib in human plasma, offering the advantage of a shorter runtime (3 minutes).

Methods:[3-20]

Materials and Reagents:

Sorafenib and Sorafenib 13CD3 internal standards were provided by Clear Synth Ltd., Hyderabad. Methanol, Acetonitrile(Gradient grade) were purchased from (Thermo Fisher Scientific India private limited, Mumbai, India) and Ammonium formate (AR grade) from Merck specialitiespvt.limited, Mumbai, India.

Drug free potassium EDTA plasma was obtained from MS Doctors pathological Lab, Hyderabad, India.

Stock solutions and Standards:

A stock solution of Sorafenib was created by dissolving the drug in methanol, resulting in a concentration of 1 mg/ml, and then stored at 2-8°C. Working dilutions were made from this stock solution to prepare calibration and quality control (QC) samples. An internal standard stock solution (Sorafenib 13CD3, 1000 µg/ml) was prepared by dissolving it in methanol to achieve a working dilution concentration of 2000 ng/ml each.

Calibration standards were established within the range of 5-5000 ng/ml for Sorafenib. QC samples were prepared by adding plasma to the appropriate amount of working solution, resulting in concentrations of 14.99 ng/ml (LQC), 2025.30 ng/ml (MQC), and 3755.2 ng/ml (HQC). Both calibration standards and QC samples were spiked with their respective working dilutions to reach the final concentration. These spiked samples were then placed in polypropylene tubes and stored at -70°C±15°C.

Sample Preparation:

Spiked samples, taken from the freezer, were thawed to room temperature. To prepare the sample, 300 µg/ml of blank human plasma was spiked with 50 µl of internal standard solution and 0.4 ml of 100mM ammonium formate pretreatment solution in a polypropylene microcentrifuge tube. The resulting mixture was loaded onto a Strata-X cartridge (300mg/1cc) preconditioned with methanol and milli-Q water. After loading, the cartridges were washed with milli-Q water and 10% methanol, followed by a 3-minute drying period.

The compounds were eluted from the cartridges using 1 ml of methanol solution. Subsequently, the methanol elution solution was evaporated to dryness and reconstituted with 500 µl of the mobile phase. The prepared sample solution was then transferred into 1 ml vials, and 10 µl was injected into the chromatographic system.

Optimized LC - MS/MS conditions:

Chromatographic separation was carried out using the Shimadzu Prominence series system from Japan. A [C18, (50X4.6mm, 5 µm)] column was utilized and maintained at 35 ℃. The autosampler temperature was kept at 8±1℃. Samples were eluted with a mobile phase consisting of 10mM ammonium formate, methanol, and acetonitrile (20:5:75, v/v/v) at a flow rate of 0.8 ml/min, with a total runtime of 3.0 minutes.

The HPLC system was coupled with a MDS, Sciex API-2000 mass spectrometer, equipped with a Turbo ionspray interface in negative ionization mode. Analysis was conducted in multiple reaction monitoring (MRM) mode, monitoring ion transitions from m/z 465.1→252.1 (sorafenib) and 469.1→256.1 (sorafenib 13CD3, ISD). The MS/MS conditions were as follows: the ion spray voltage was set at 5000V, and the ion source gas, curtain gas, and collision gas were maintained at 20, 15, and 15 psi respectively. Compound-dependent parameters included declustering potential (DP) of 100V, entrance potential (EP) of 10V, collision energy (CE) of 50V, and collision cell exit potential (CXP) of 15V.

Validation Procedure:

The validation process was conducted over six different days, adhering to the FDA guidelines for bioanalytical method validation. On each day of analysis, calibration standards were meticulously prepared in duplicate at concentrations of 5, 10, 25, 250, 1000, 2000, 4000, and 5000 ng/ml. QC samples were independently prepared in batches before validation, at concentrations of 14.99 ng/ml (LQC), 2025.30 ng/ml (MQC), and 3755.2 ng/ml (HQC). During the validation, in addition to the calibrators, five QC samples at each concentration were thawed and analyzed daily. Each validation run included two blank samples (zero concentration) (Fig-2) and two samples containing only the internal standard (IS), alongside the calibrators and QC samples.

1. Selectivity, sensitivity and suitability of assay:

The assessment of interference caused by plasma constituents at the retention time of the internal standard involved processing ten sets of blank plasma, along with two sets of haemolytic and lipemic plasma each (Fig-1). Comparing responses in the blank chromatograms with the extracted lower limit of quantification (LLOQ) samples confirmed the absence of direct interference. No significant interference was detected in drug-free plasma at the retention times of sorafenib, ensuring precise and accurate quantification. At the LLOQ level, the precision and accuracy were determined to be 2.3% and 101.2% respectively, demonstrating the reliability of the assay. Additionally, an investigation into assay suitability revealed no cross talk between analytes and the internal standard (Sorafenib 13CD3).

2. Extraction efficiency:

Protein precipitation (PP) and liquid-liquid extraction (LLE) were initially attempted due to their simplicity and cost-effectiveness. However, it was observed that LLE resulted in lower extraction recovery, and PP yielded inconsistent recovery outcomes. Solid phase extraction (SPE) was identified as the optimal method, providing consistent and stable recoveries for both analytes and the internal standard (IS). The Strata-X cartridges were preconditioned with methanol and water. A plasma sample was introduced, followed by the addition of 0.4 ml of 100 mM ammonium formate as a pretreatment solution. The cartridges were washed sequentially with milli-Q water and 10% methanol. Subsequently, elution was performed with 1 ml of methanol solution. Recovery results for analytes and IS obtained through elution with 1 ml of 10% methanol are summarized in Table 1.

Table 1 : Recovery results of Sorafenib and ISTD

Sample name	**% Mean recovery Absolute**	**% Mean recovery relative**
	Sorafenib	**Sorafenib**
LQC	85.3	85.3
MQC	84.3	84.2
HQC	86.1	86.7
Sorafenib 13CD3 at MQC level	86.4	85.1

3. Matrix effect:

Across all processed lots, including haemolytic and lipemic plasma lots at both lower (LQC) and higher (HQC) concentration levels, no significant matrix effect (ion suppression or enhancement) was detected. The analyte and internal standard (IS) exhibited comparable ionization behavior and extraction efficiency under the specified method conditions. The precision for the IS-normalized matrix factor for Sorafenib at LQC and HQC levels was determined to be 2.1% and 3%, respectively, indicating the suitability of the IS for the quantification assay. Detailed matrix effect results are presented in Table 2.

Table 2: Matrix factor

ISNMF-Internal standard normalized matrix factor		
Blank Plasma Lots	**Sorafenib**	
	LQC	**HQC**
LOT-1	1.00	1.05
LOT-2	1.04	1.08

LOT-3	1.01	1.01
LOT-4	1.00	1.02
LOT-5	1.02	1.02
LOT-6	1.01	1.01
LOT-7 hemolytic	1.03	1.04
LOT-8 hemolytic	0.99	1.05
LOT-9 lipemic	0.96	0.96
LOT-10 lipemic	1.00	1.02
Mean	1.006	1.026
SD	0.0211	0.0304
%CV	2.1	3.0

Matrix effect assessment was conducted using a direct quantification approach. Three replicates of lower (LQC) (Fig-3)and higher (HQC) (Fig-4)quality control samples from six plasma lots, along with two lots each of haemolytic and lipemic plasma, were examined. The results revealed that over 67% of the processed QC samples were accepted across all lots, indicating no substantial impact from plasma constituents (matrix) on the quantification method.

4. Linearity:

The calibration curve was constructed by plotting the peak area ratio on Y-axis and concentration on X-axis.

The correlation coefficient of the calibration curve was ≥ 0.998.

5. Method accuracy and precision (Inter and Intraday):

Accuracy and precision were assessed through multiple analytical runs conducted over six consecutive days. Each analytical run included a calibration curve and six replicates of quality control samples at four concentration levels. For Sorafenib, the intraday precision ranged from 0.8% to 2.1%, while the interday precision was between 1.3% and 1.8%.

The accuracy for both intraday and interday analyses fell within the range of 98.5% to 108.9% and 95.4% to 104.2% for Sorafenib, respectively. These results indicate that the method was accurate, robust, and consistently reproducible across the calibration range for both analytes. Detailed accuracy and precision outcomes are presented in Table 3.

Table: 3 Accuracy and Precision

Sorafenib				
QCname/Nominal Concentration	**Intra batch (n=6)**		**Inter batch (n=12)**	
	% Accuracy	**% CV**	**% Accuracy**	**% CV**
LLOQQC/50 ng/mL	99.1	1.2	96.1	1.5
LQC/ 150 ng/mL	101.2	1.0	95.4	1.3
MQC/6300 ng/mL	98.5	0.8	102.1	1.8
HQC/11450 ng/mL	108.9	2.1	104.2	1.6

5. Stability evaluation:

Various stability experiments were conducted based on the extraction method conditions, including autosampler stability (41 hours), freeze and thaw stability (7 cycles), dry extract stability (46 hours), room temperature stability (20 hours), long-term stability (85 days), and total blood stability (3.5 hours). The mean percentage stability of the analyte was determined to be within ±15% at both lower (LQC)and higher (HQC) concentration levels. Additionally, stock solution stability and working dilution stability were within ±10%. A detailed presentation of the stability conditions and results can be found in Table 4.

Table 4: Stability Evaluation

Stability Experiment	**stability condition**	**%Mean stability**	
		Sorafenib	
		LQC	**HQC**
Autosampler stability	41 hrs at 10° C	96.8	91.7
Free and thaw stability	7 cycles at -70° C ± 15°C	101.2	95.6
Dry extract stability	46 hrs at 2-8° C	102.1	98.4
Room temperature stability	20 hrs at Room temperature at 25° C ± 5°C	101.2	106.3

Long term stability	85 days at -70° C ± 15°C	99.2	101.8
Stability in blood	3.5 h Room temperature at 25° C ± 5°C	94.5	95.6

CONCLUSION

A novel LC-MS/MS procedure was developed for the precise estimation of Sorafenib utilizing the positive electrospray ionization technique. This method proved to be high throughput, sensitive, and selective, with a brief runtime of 3.0 minutes. Validation was carried out following the current guidelines set by USFDA, ANVISA, and EMA. Individual Q1 mass spectra of Sorafenib and the internal standard (ISTD) were obtained, and the most abundant product ions were selected for multiple reaction monitoring (MRM) analysis to ensure high signal intensity. MRM transitions employed were m/z 465.1/252.1 (Sorafenib) and m/z 469.1/256.1 (Sorafenib -13CD3, ISTD) with a dwell time of 200 ms per transition. Optimized MS/MS conditions for each transition were utilized to maximize signal intensity while minimizing background noise. The analytes were extracted using solid-phase extraction (SPE) and then chromatographed on a Phenomenex C18 analytical column (50mm, 4.6 mm, 5 µm). No significant interferences were detected at the retention times of Sorafenib and the ISTD in blank plasma samples, including those with hemolytic and lipemic properties.

Having a straightforward, rapid, and robust LC-MS/MS method for determining Sorafenib concentration in clinical samples is crucial. This method is applicable for evaluating pharmacokinetics in bioequivalence/bioavailability studies and therapeutic drug monitoring in both healthy volunteers and patients.

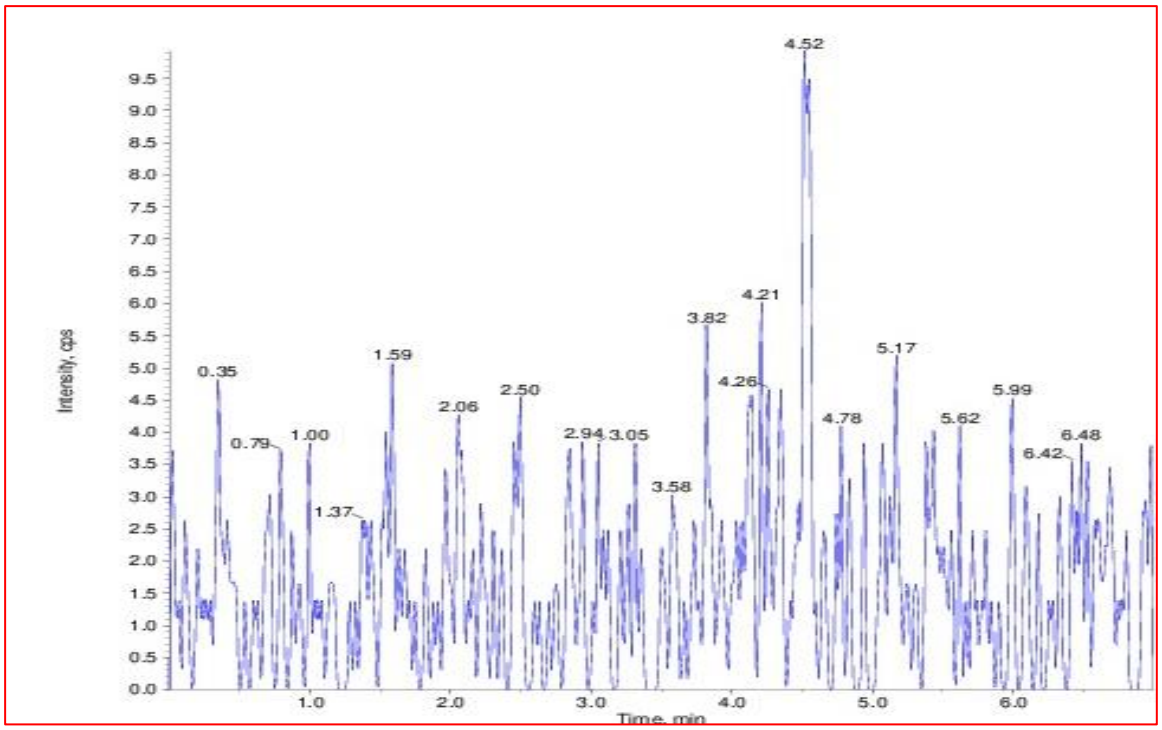
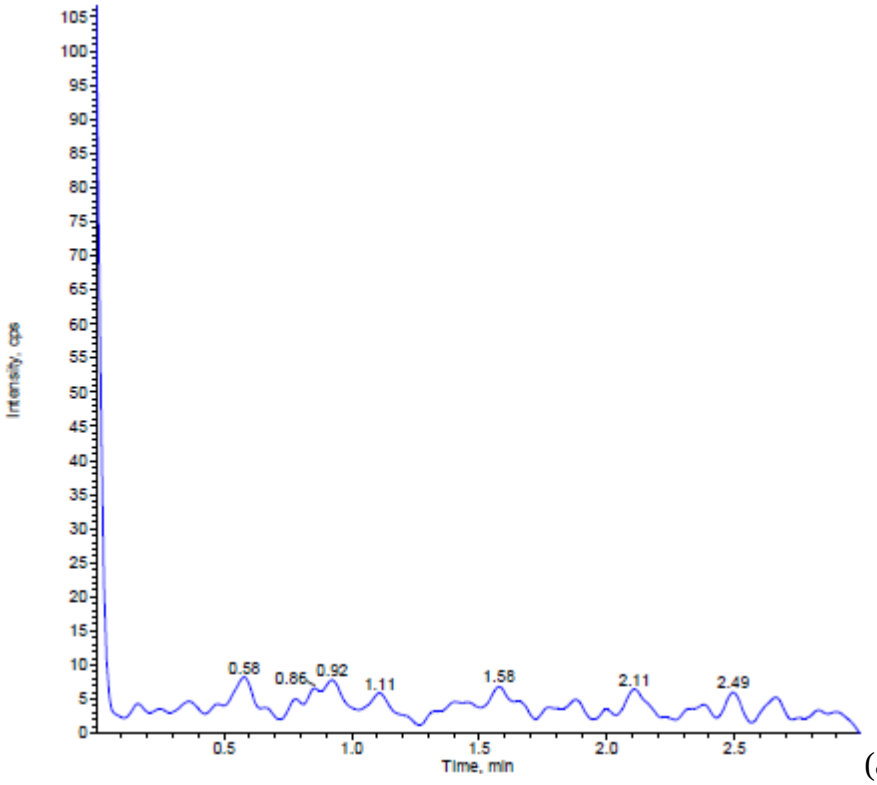
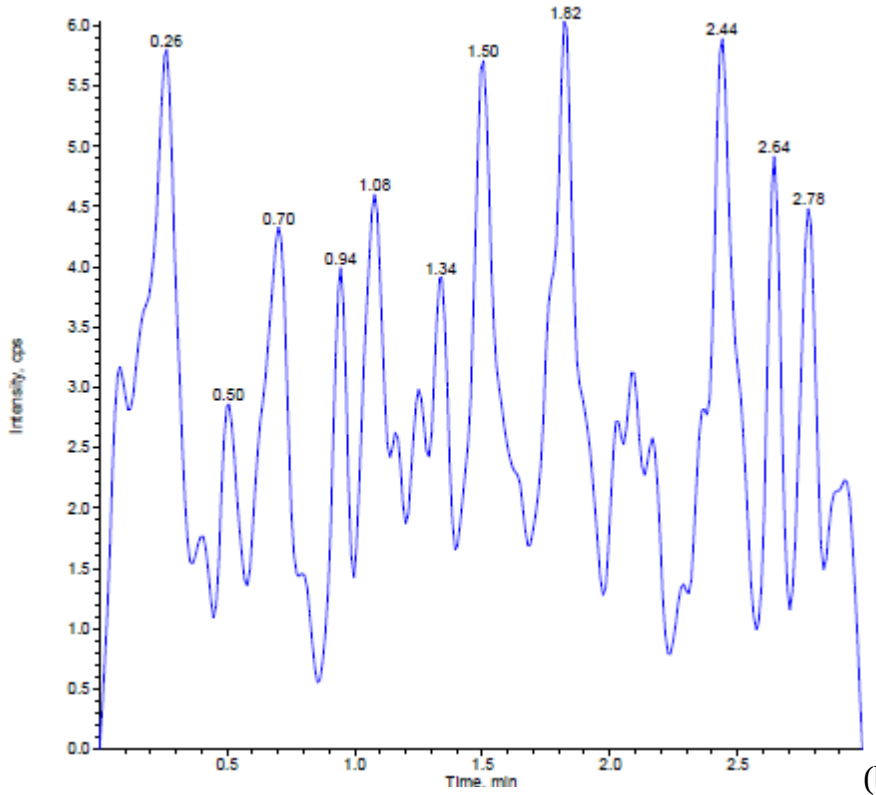


Figure 1: Chromatogram of phospholipid elution and long run

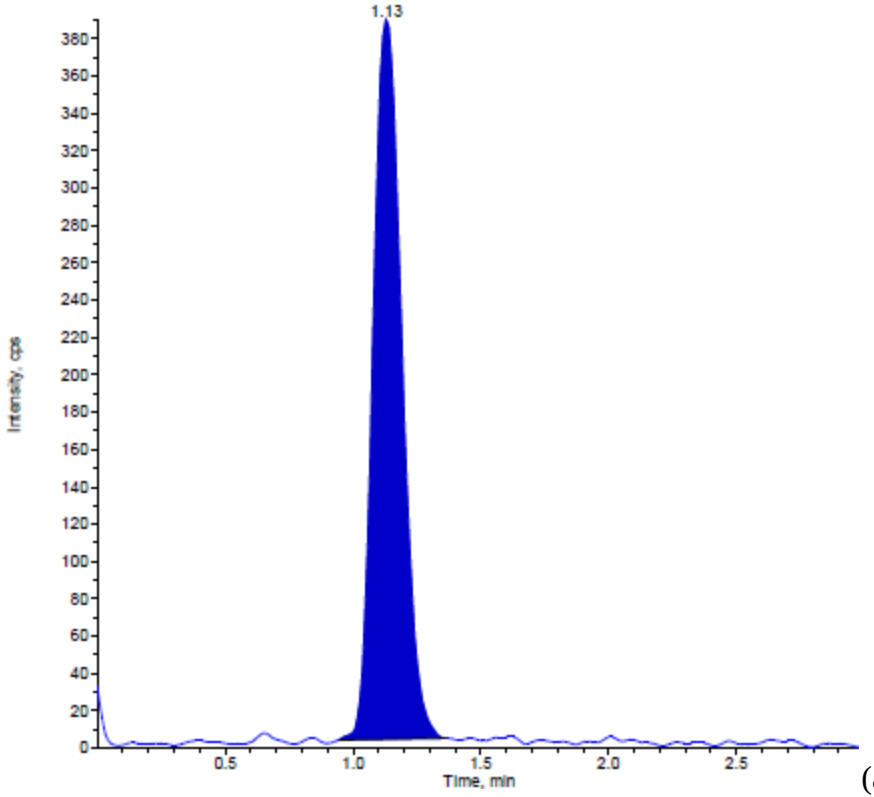


(a)

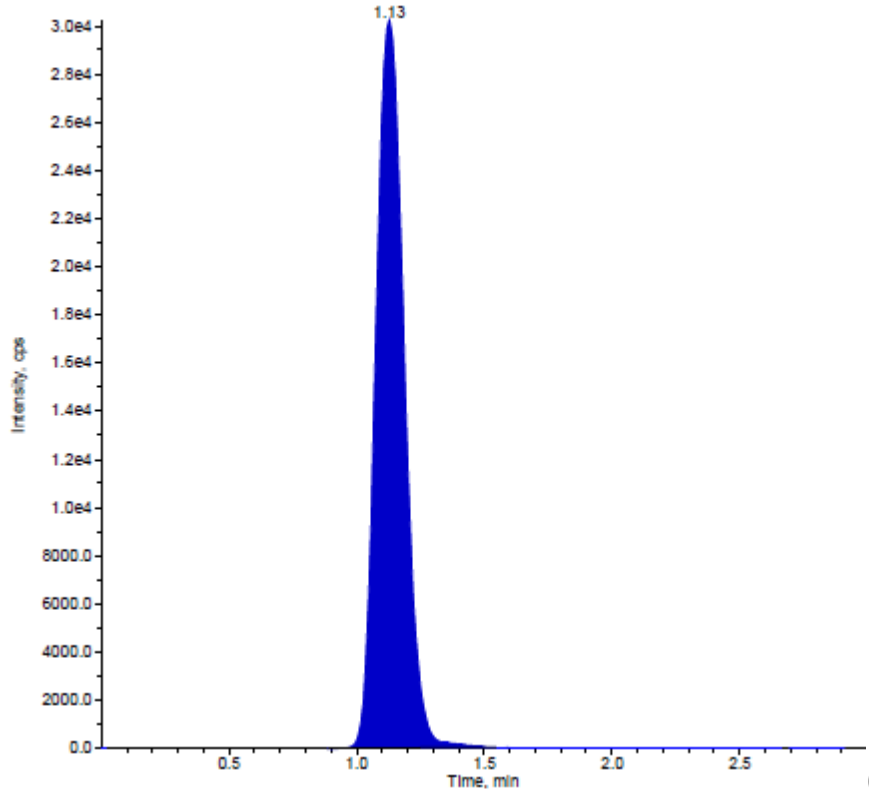


(b)

Figure 2: Blank plasma chromatograms of Sorafenib (a) and Sorafenib -13CD3 (b)

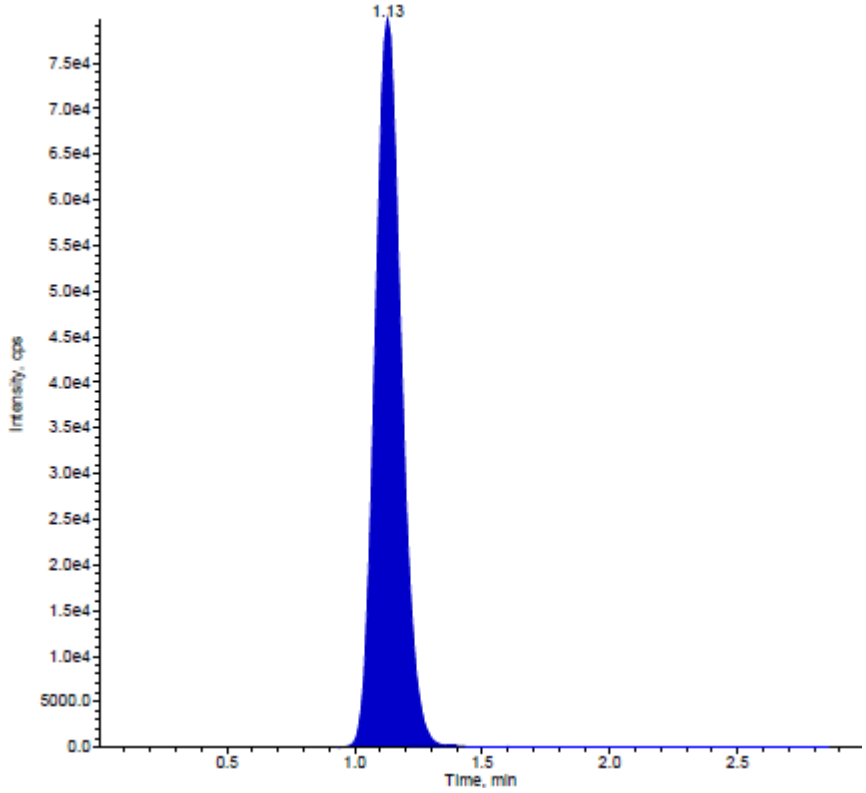


(a)

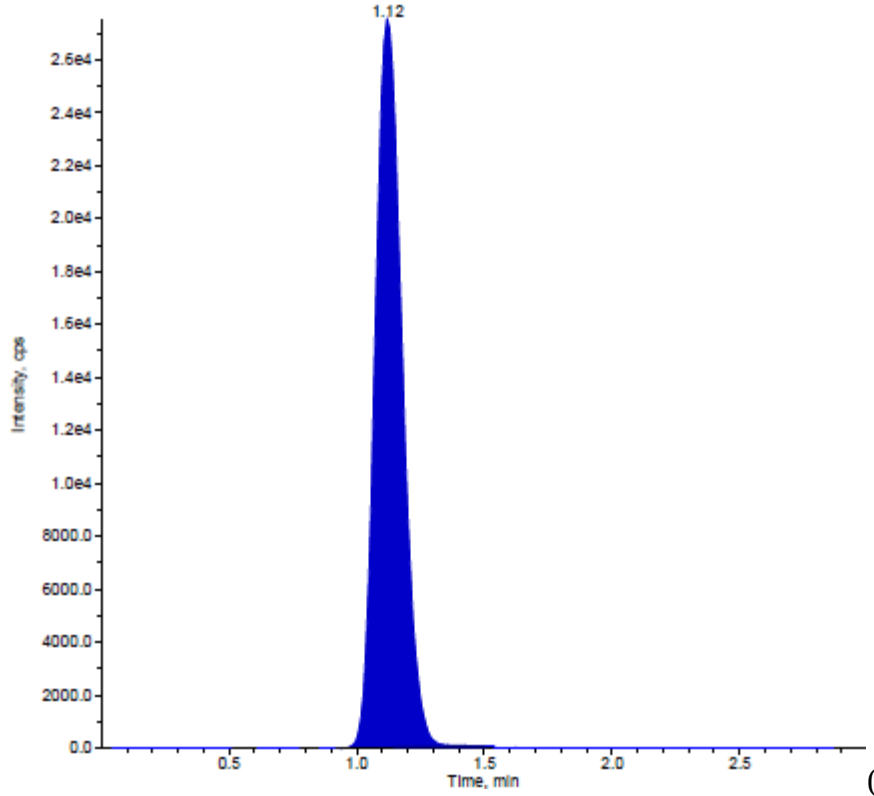


(b)

Figure 3: LLOQ level chromatograms of Sorafenib (a) and Sorafenib -13cd3 (b)



(a)



(b)

Figure 4: HQC level chromatograms of Sorafenib (a) and Sorafenib -13CD3 (b)

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