



# Effect of piperine and its analogs on pharmacokinetic properties of sorafenib tosylate: Bioanalytical method development and validation

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## ABSTRACT

Sorafenib tosylate (ST) is a drug of choice for the treatment of advanced hepatocellular carcinoma. The bioavailability of ST varies from 29% to 38% due to the presence of food, medication, and other factors. Moreover, the dose of ST (400 mg twice a day) is also higher due to its poor bioavailability. Piperine is known as a natural bioenhancer, and the use of a bioenhancer ultimately leads to a synergistic effect. In this study, the development and validation of a precise, accurate, and reproducible bioanalytical method was carried out using reversed-phase high-performance liquid chromatography (RP-HPLC). The objective of the study is to determine the effect of piperine and its analogs on the pharmacokinetics of ST using rats. The dose of ST alone and in combination with piperine and its analogs, given orally to male Wistar rats in suspension form, and the drug plasma concentration was examined using a validated RP-HPLC bioanalytical method. The separation of piperine and ST was achieved on the Chromatopak basic C18 HPLC column with the dimension of 250 mm × 4.6 mm × 5 μm, guarded along with the phenomenex guard column using ACN:Water:Formic Acid (70:29.5:0.5 v/v) as the mobile phase at 280 nm (isosbestic point of piperine and ST). The pharmacokinetic data of ST, along with piperine, exhibit a significant increase in area under the curve (AUC) of ST (1.66-fold), while the two derivatives of piperine (5a and 5d) exhibited almost double the increase in AUC compared to piperine (2.58 and 2.42-fold, respectively).

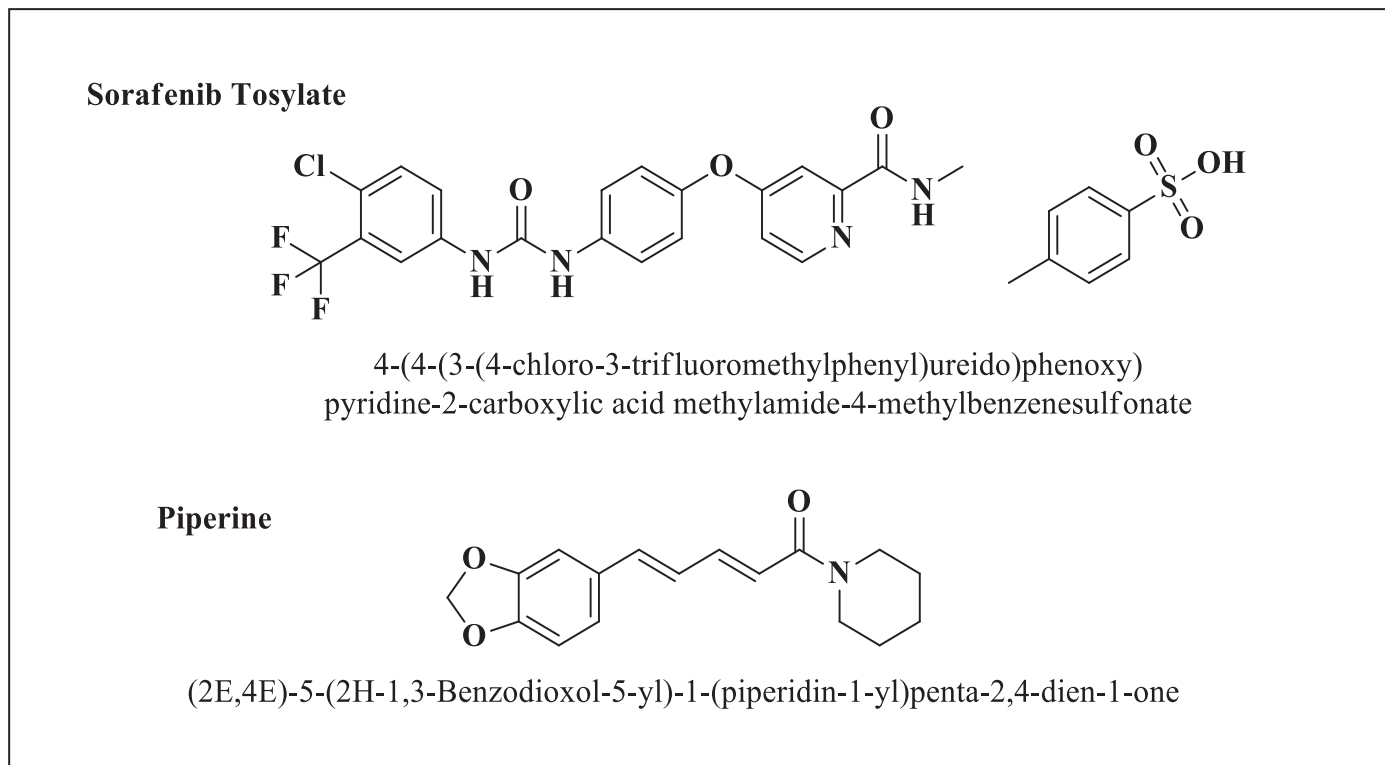
## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth most commonly occurring cancer mortalities worldwide. Hepatitis B and Hepatitis C (HBV and HCV) infections, chronic intake of alcohol, diabetes mellitus, and obesity are the major risk factors associated with HCC (Modi *et al.*, 2019; Thiel *et al.*, 2014). Among this, HBV and HCV were found to be the major causes for cancer-related deaths globally since they ultimately lead to the progression in the hepatic damage, resulting in liver cirrhosis and HCC. Males are more prone to develop liver cancer than women (Karaman *et al.*, 2014). Sorafenib tosylate (ST) was primarily approved to treat advanced HCC and renal cell carcinoma, the most commonly occurring cancer types in

African and Asian countries (Hsu *et al.*, 2009). Chemically, ST is 4-(4-{3-[4-chloro-3-(trifluoromethyl)phenyl]uredo}phenoxy)-N-2-methylpyridine-2-carboxamide 4-methyl benzenesulfonate, a multi-tyrosine kinase inhibitor given orally in the treatment of aberrant angiogenesis (Fig. 1). It bears a sturdy crystal lattice with low aqueous solubility and high lipophilicity (Log p = 3.8). The marketed oral formulation NEXAVAR®, containing ST as the active pharmaceutical ingredient (API), is given orally (dose: 200–400 mg twice a day) to humans. ST with oral bioavailability of 29%–38% comes under the BCS class III drugs. The interference of food and pH was found to have a significant effect on the bioavailability of the drug. A high-fat diet significantly reduces the absorption of the drug from the stomach and intestines. There are shreds of evidence which suggests that bioavailability could be decreased by treatment with antacids, specifically proton pump inhibitors (Herbrink, *et al.*, 2015; Kane *et al.*, 2006).

Piperine, a natural alkaloid obtained from pepper (Family: *Piperaceae*), is known for its bioenhancing properties. It is used as a kitchen spice and food additive worldwide. Piperine is a natural alkaloid well known for its bioenhancing property

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**Figure 1.** Chemical structure of ST and piperine.

when combined with several drugs with poor bioavailability, viz., isoniazid, rifampicin, and curcumin (Atal *et al.*, 1985; 2010). Various compositions of different classes of APIs contain piperine as a bioenhancer (EP0709098A1) (Patel *et al.*, 1997). Panacea Biotech Ltd. presented a report concerning the bioenhancing efficacy of piperine combined with nonsteroidal anti-inflammatory drugs as clinically significant at a dose of 5–20 mg/kg and later compared it with the available marketed products (US006017932A) (Singh *et al.*, 2000). Risorine® is a commercially available formulation marketed by Zydus Cadila, Ahmedabad, for the treatment of tuberculosis: isoniazid (200 mg), rifampicin (200 mg), and piperine (10 mg). The formulation consists of a 60% lesser dose due to the increased bioavailability and reduced resistance (Kang *et al.*, 2009; Shankaracharya *et al.*, 1997). Until today, no data are available in the literature concerning the combined efficacy of ST and piperine. In the present work, a validated bioanalytical method to determine ST in rat plasma was developed and was optimized to achieve robust, reproducible, accurate, stable, and precise results.

## MATERIALS AND METHODS

### Material

Piperine (95% purity) was obtained from Aldrich Chemicals, United States. All the chemicals and reagents used in the reactions were purchased from different companies, like Loba Chemie, India; Alfa Aesar, United States of America; Aldrich, United States; and Merck India Ltd., India, and were employed without any further purification. The reactions were carried out using oven-dried borosilicate glassware and anhydrous solvents.

The reactions, as well as the final compounds, were checked using thin-layer chromatography (TLC). The stationary phase used was silica gel G-60 GF<sub>254</sub>. The plate was 0.22-mm thick. The plates were developed and then observed under ultraviolet light or by means of iodine vapors.

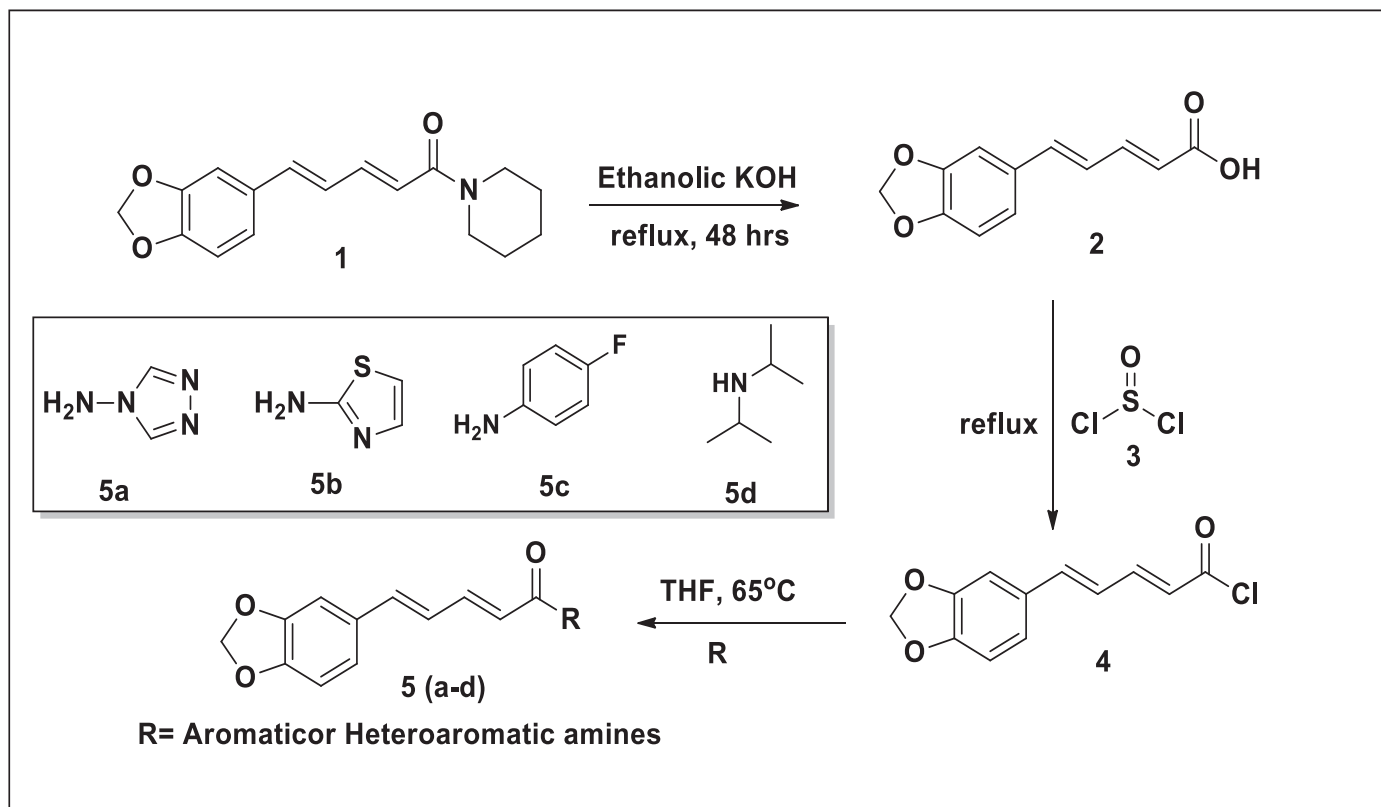
### Instruments

The melting points of the synthesized compounds were determined on Veego VMP-PM digital melting point apparatus and were uncorrected. <sup>1</sup>H NMR (Proton) and <sup>13</sup>C NMR (carbon-13) nuclear magnetic resonance (NMR) spectra were obtained on the Bruker Avance III (400 MHz) spectrometer using TMS: Tetramethylsilane as an internal standard. All the NMR spectra were obtained by dissolving the appropriate amounts of the analyte in dimethylsulfoxide (DMSO-D<sub>6</sub>). Chemical shifts (δ) and coupling constants (*J*-value) were represented in Hz. Final compounds were tested for purity using high-performance liquid chromatography (HPLC).

### General procedures

#### Preparation of piperic acid

To 40% alcoholic solution of KOH: Potassium Hydroxide, 30 ml piperine was added and refluxed for 48 hrs. The reaction was monitored by TLC. The mobile phase used was toluene:EtOAc (3:1 v/v). When the reaction was complete, the solvent was evaporated under reduced pressure. After this, water (50 mL) was added and it was filtered. Acidification of this was carried out with conc. HCl. This resulted in the formation of a yellow-colored compound of piperic acid. Filtration of this



**Figure 2.** Scheme for the synthesis of piperine derivatives.

suspension gave a crude product of piperic acid which was purified with the help of methanol to afford pure piperic acid. (Aziz *et al.*, 2015; Paula *et al.*, 2000)

#### General procedure for the synthesis of amides (5a–5d) from piperic acid

The pure acid (500 mg, 2.29 mmol) was taken in dry tetrahydrofuran (THF; 10 ml) (Fig. 2). The reaction mixture was further refluxed for 4–5 hrs. After 5 hrs, the excess amount of thionyl chloride ( $\text{SOCl}_2$ ) was decanted under reduced pressure, leaving behind a crude yellow-colored acid chloride residue. The resultant residue was further dissolved in a small amount of THF (2–4 mL), and the appropriate amine (2.3 mmol in 3 mL THF) was added, followed by 0.3 ml of triethylamine. The reaction mixture was heated, followed by stirring for 3–4 hrs. The reaction was monitored by TLC using toluene:methanol (3:1) as the mobile phase to afford the required amides (5a–5d). The crude compound was recrystallized using methanol to obtain pure yellow crystals. The FTIR spectra of synthesized derivatives are shown in Figure 8.

#### HPLC analysis

The analysis was carried out on a reversed-phase column (Chromatopak peerless basic C18 column of  $250 \times 4.6$  mm,  $5 \mu\text{m}$ ), protected with a Phenomenex C18 guard cartridge with the dimension of  $250 \times 4.6$  mm,  $5 \mu\text{m}$  at room temperature. The column was coupled with a Jasco reversed-phase HPLC system (autosampler and LC-Net II/ADC controller, pump PU-2089)

coupled with a UV (Jasco UV-2075 plus) detector, and Jasco–Borwin version 15 was used. The mobile phase ACN:Water:Formic Acid (70:29.5:0.5 v/v) was filtered using a  $0.25 \mu\text{m}$  membrane filter and degassed using ultrasonication. After sonication, at the flow rate of 1 mL/min, the mobile phase was pumped to the system, and the detection wavelength was 280 nm with 30 min run time (Table 1 and Fig. 2).

The stock solution of the drug was prepared by accurately dissolving 1 mg of ST into acetonitrile (1,000  $\mu\text{L}$ ). The stock solution was safely stored and protected from light, and further dilutions were prepared. An aliquot (100  $\mu\text{L}$ ) for each diluted solution was spiked with 100  $\mu\text{L}$  of blank plasma, yielding concentrations ranging from 5 to 50  $\mu\text{g}/\text{mL}$ , to obtain the calibration curve. Quality control (QC) samples were also prepared by following the same procedure mentioned earlier. All solutions were stored for 1 month at a temperature of  $4^\circ\text{C}$ .

#### Method validation

The developed bioanalytical method was validated as per the guidelines provided by the United States Food and Drug Administration (USFDA) (Smolec *et al.*, 2005).

#### Selectivity

The selectivity of the developed HPLC method was carried out to investigate the interference of endogenous constituents present in the sample with the drug. The selectivity was carried out using six blank plasma (rat plasma) samples with the spiked samples (Fig. 4 and 5).

### Linearity, limit of detection (LOD), and lower limit of quantitation (LLOQ)

The linearity of analytes was determined by plotting the mean area under the curve (mAu) of ST versus the least square linear regression. The LLOQ and LOD were carried out based on the S/N ratio of 10:1 for LLOQ and 3:1 for LOD.

### Accuracy and precision

For evaluating interday and intraday precision and accuracy, six replicates of three different concentrations of QC samples were analyzed.

### Extraction recovery

The extraction recovery study was carried out by comparing the results of analytical QC samples of extracted samples and corresponding blank spiked samples with post extracted analytes. Six replicates of QC samples were estimated with the recovery of ST at three different concentration ranges. The recovery should not be more than 100% for the analytes.

### Stability

The stability of the solute is one of the critical parameters for the development of the bioanalytical method. There are various solutes available that readily degrade in the plasma before analysis, and multiple factors play an important role in stability studies of the analyte (e.g., sample preparation, method of extraction, storage, and cleanup). The stability studies for the analyte were estimated as:

**Short-term stability:** Storage of the analyte for 24 hrs at room temperature. Also, the analyte samples were stored at  $-20^{\circ}\text{C}$  for 24 hrs in the refrigerator.

**Long-term stability:** Storage of the analyte for 30 days at  $-80^{\circ}\text{C}$ .

**Freeze-thaw stability:** Prepared plasma samples were analyzed for freeze-thaw stability for three consecutive cycles from  $-80^{\circ}\text{C}$  to room temperature.

### In-vivo pharmacokinetics of ST

#### Animals

Male Wistar rats (weight  $180 \pm 30$  gm;  $n = 9$ ) were procured from Agharkar Research Institute, Pune. The protocol to carry out the pharmacokinetic study was approved by the

Institutional Animal Ethics Committee of Bharati Vidyapeeth (Deemed to be University), Poona College of Pharmacy, Pune (CPCSEA/PCH 01/2018-2019). The quarantine of animals was carried out in a controlled environment ( $22^{\circ}\text{C}$ – $25^{\circ}\text{C}$ , 55%–60% relative humidity, and 12 hrs dark/light cycles) for 1 week. Food pallets were provided to animals supplied by Navmaharashtra Chakan Oil Mill Ltd, Sangali, Maharashtra, India, and tap water was given ad libitum during the experimentation.

### Single oral administration

Before carrying out the study, the animals were fasted overnight and the animals were separated into seven groups ( $n = 9$ ; Table 2). They were administered with a single dose of ST (5 mg/kg; orally) and in combination with piperine and its synthetic derivatives at the dose of 5 mg/kg orally. The rat blood samples (0.5 mL per animal) were collected by retro-orbital venous plexus method at different time points, i.e., 0, 15, 30 minutes, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hrs. Food was provided to the animals after 4–5 hrs. Later, the collected blood samples were stored in labeled tubes containing Ca-EDTA as the anticoagulant and were shaken to prevent blood coagulation. The samples were centrifuged at 10,000 rpm for 2–5 min at  $4^{\circ}\text{C}$ . The plasma sample of 200  $\mu\text{L}$  was taken in a 2-mL Eppendorf tube, and the extracting solvent (100% acetonitrile, 200  $\mu\text{L}$ ) was added and vortexed for 5 min and were centrifuged at 15,000 rpm for 15 min at  $4^{\circ}\text{C}$ . After centrifugation, the supernatant was separated in different tubes and was reconstituted using 200  $\mu\text{L}$  of diluent and vortexed for 2 min, and was filtered using 0.22  $\mu\text{m}$  syringe filters. From this, 20  $\mu\text{L}$  of solution was injected onto the HPLC column (Fig. 4) (Di *et al.*, 2015; Singh *et al.*, 2018).

### Analysis of data

For estimating various pharmacokinetic parameters a non-compartmental analysis (NCA) model was used, and all the pharmacokinetic parameters were evaluated using PKSolver 2.0 USA software. The area under the plasma-drug concentration versus time curve ( $\text{AUC}_{0-t}$ ) was determined by trapezoidal rule.  $C_{max}$  and  $T_{max}$  were found from the above plotted curve. Rate of absorption ( $K_a$ ) and half-life ( $t_{1/2}$ ) were calculated, followed by the residual method and  $0.693/k_a$ . The rate of elimination ( $K_{el}$ ) and terminal half-life ( $t_{1/2}$ ) were calculated using equation  $-k/2.303$  and  $0.693/K_{el}$  respectively. the apparent volume of distribution ( $V_d$ ), and mean residence time (MRT). The following formula was used to calculate the relative bioavailability ( $F$ ):

$$F = \frac{\text{AUC of ST+Piperine or piperine derivative}}{\text{AUC of ST}} \times 100$$

**Table 1.** Instrumentation and chromatographic conditions.

Sr. No.	Test conditions	Results
1.	Elution	Isocratic
2.	Wavelength	280 nm
3.	Mobile phase	ACN:Water:Formic Acid (70:29.5:0.5 v/v)
4.	Column	Chromatopak peerless basic C18 column of 250 mm $\times$ 4.6 mm $\times$ 5 $\mu\text{m}$
5.	Guard column	Phenominex (C18 column of 250 mm $\times$ 4.6 mm $\times$ 5 $\mu\text{m}$ )
6.	Retention time (min.)	Piperine: 6.3 ST: 7.8
7.	Flow rate	1 mL/min
8.	Run time	20 min

**Table 2.** Grouping of animals.

No.	Groups ( $n = 9$ )
1.	Vehicle control
2.	ST
3.	ST + Piperine
4.	ST + 5a
5.	ST + 5b
6.	ST + 5c
7.	ST + 5d

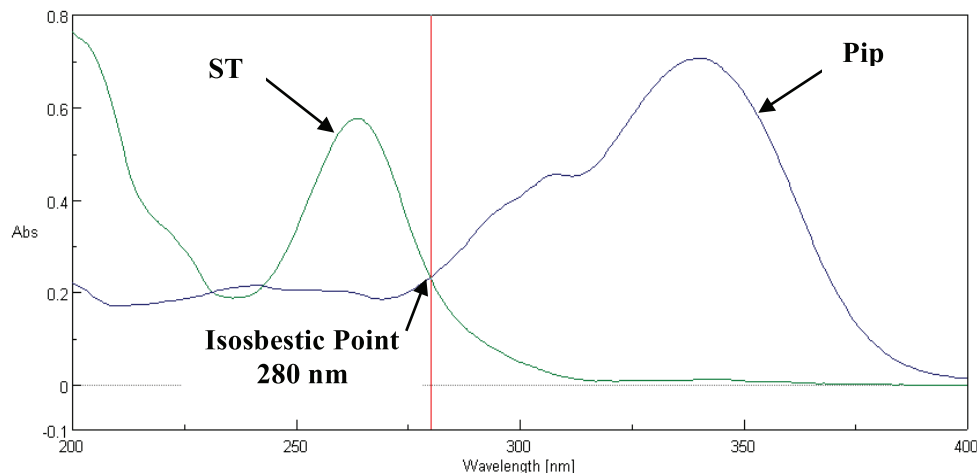


Figure 3. Selection of Wavelength using UV Spectrometer.

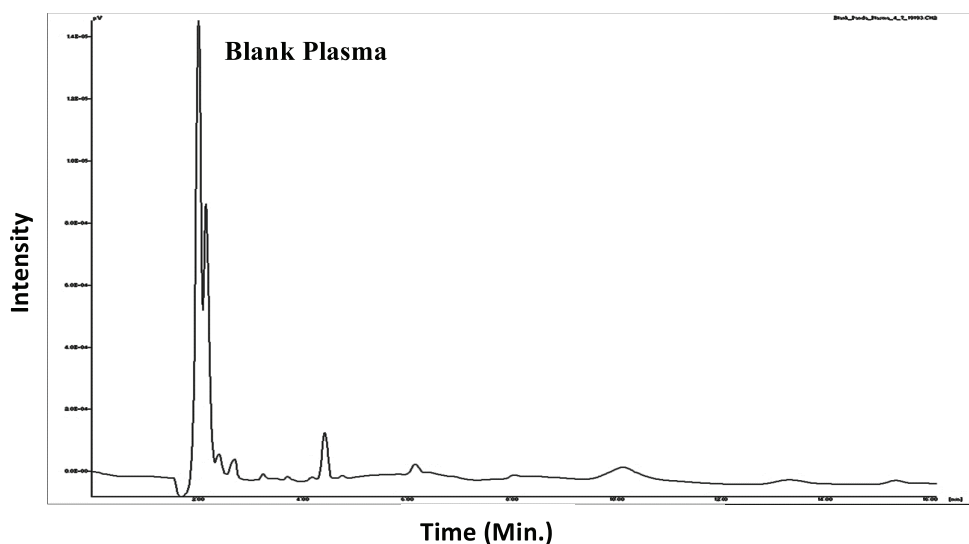


Figure 4. Chromatogram of Blank Plasma.

## RESULTS

### Characterization of the synthesized compounds

*(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-N-(1H-1,2,4-triazol-1-yl)penta-2,4-dienamide (5a)*

Yield: 85.7%; melting point: 230–231 °C; FTIR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 3,443.28 (-NH), 3,018.09 (aromatic-CH), 2,911.02 (O-CH<sub>2</sub>-O), 1,694.16 (-C=O), 1,602.56 (methylene dioxy group), 1,602.66 (diene), 1,494.56 (C=C). <sup>1</sup>H NMR (400 MHz, DMSO-D<sub>6</sub>)  $\delta$ : 5.93 (d,  $J$  = 15.7 Hz, 1H), 6.15 (d,  $J$  = 10.8 Hz, 2H), 6.87–7.03 (2H), 6.90 (dd,  $J$  = 8.4, 0.4 Hz), 6.95 (dd,  $J$  = 17.7, 10.2 Hz), 7.14 (d,  $J$  = 17.7 Hz, 1H), 7.25 (dd,  $J$  = 1.9, 0.4 Hz, 1H), 7.39 (dd,  $J$  = 15.7, 10.2 Hz, 1H), 7.58 (dd,  $J$  = 8.4, 1.9 Hz, 1H), 8.15 (s,  $J$  = 1.7 Hz, 2H). <sup>13</sup>C NMR (DMSO)  $\delta$ : 165.51, 157.75, 149.05, 148.48, 144.48, 142.25, 141.92, 130.93, 125.46, 124.41, 123.11, 109.09, 106.34, 101.87. Mass spectrum ESI (-ve) averaged mode:  $m/z$  283.9 ( $M^+$  C<sub>14</sub>H<sub>12</sub>N<sub>4</sub>O<sub>3</sub>).

*(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-N-(thiazol-2-yl)penta-2,4-dienamide (5b)*

Pale yellow powder, yield: 92.7%;  $R_f$  value: 0.51 [toluene: methanol (3:1)]; melting point: 178–180 °C; FTIR KBr ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 3,160 (-NH stretch), 2,882.09 (-CH<sub>2</sub>), 1,673.91 (C=O), 1,609.31 (methylene dioxy group), 1,609.90 (diene), 1,490.70 (C=C). <sup>1</sup>H NMR (400 MHz, DMSO-D<sub>6</sub>)  $\delta$ : 12.24 (s, 1H), 7.53–7.34 (m, 2H), 7.29 (s, 2H), 7.16 (d,  $J$  = 19.3 Hz, 1H), 6.87 (dd,  $J$  = 56.6, 31.0 Hz, 5H), 6.35 (d,  $J$  = 14.5 Hz, 1H), 6.02 (s, 2H), 5.94 (d,  $J$  = 12.7 Hz, 1H). <sup>13</sup>C NMR (101 MHz DMSO)  $\delta$ : 163.85, 158.62, 148.58, 148.41, 143.59, 140.84, 138.26, 130.99, 125.26, 123.74, 121.83, 114.06, 108.91, 106.28, 101.78. Mass spectrum ESI (-ve) averaged mode:  $m/z$  301.06 ( $M^+$  C<sub>15</sub>H<sub>12</sub>N<sub>2</sub>O<sub>3</sub>S).

*(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-N-(4-fluorophenyl)penta-2,4-dienamide (5c)*

Colorless crystals, yield 82 %;  $R_f$ : 0.651 [mobile phase: *n*-hexane: acetone (2:3 v/v)]; melting point 157–158 °C; FTIR

(KBr) ( $\text{cm}^{-1}$ ): 3,224.52 (-NH), 1,699.94 (-C=O), methylene dioxy group (1,617.02), C=C (1,488);  $^1\text{H}$  NMR (DMSO- $\text{D}_6$ )  $\delta$ : 9.901 (s, NH exchangeable), 8.036 (s, 1H), 7.327 (d,  $J = 14.4\text{Hz}$ , 1H), 7.263 (d,  $J = 9.2\text{Hz}$ , 1H), 7.17 (d,  $J = 14.8\text{Hz}$ , 1H), 7.003–6.91 (m, 4H), 6.503 (dd,  $J = 16\text{Hz}$ , 1H), 6.062 (s, 1H), 5.946 (dd,  $J = 16\text{Hz}$ , 1H).  $^{13}\text{C}$  NMR (DMSO)  $\delta$ : 79.11, 79.44, 79.77, 101.83, 106.22, 108.94, 108.98, 115.79, 115.98, 121.58, 123.50, 123.58, 124.18, 124.79, 125.31, 125.44, 126.82, 126.93, 131.17, 139.72, 140.27, 142.03, 145.10, 148.45, 148.58, 164.69, 168. Mass spectrum:  $m/z$  312.10 ( $\text{M}^+ \text{C}_{18}\text{H}_{14}\text{FNO}_3$ ).

(2*E*,4*E*)-5-(benzo[d][1,3]dioxol-5-yl)-*N,N*-diisopropylpenta-2,4-dienamide (5*d*)

Yield 79%;  $R_f$ : 0.681 [mobile phase:  $n$ -hexane:ethyl acetate (7:3)  $v/v$ ]; melting point: 196–198 °C; FTIR (anhydrous KBr  $\nu_{\text{max}}$   $\text{cm}^{-1}$ ): 3,036 aromatic C-H; 2,547 O-CH<sub>2</sub>-O; 1,675 C=C (diene); 1,601–1,447 C=C (benzene ring). 1,047 (C=N).  $^1\text{H}$  NMR (DMSO- $\text{D}_6$ )  $\delta$ : 1.21 (d,  $J = 6.8$  Hz, 12H), 4.16 (sept,  $J = 6.8$  Hz, 2H), 5.96 (d,  $J = 15.8$  Hz), 6.14 (2H, d,  $J = 10.8$  Hz), 6.86–7.01 (2H, 6.90 (dd,  $J = 8.4, 0.4$  Hz), 6.94 (dd,  $J = 17.7, 10.2$  Hz, 1H), 7.12 (d,  $J = 17.7$  Hz, 1H), 7.24 (dd,  $J = 1.9, 0.4$  Hz, 1H), 7.35 (dd,  $J = 15.8, 10.2$  Hz, 1H), 7.58 (dd,  $J = 8.4, 1.9$  Hz, 1H).  $^{13}\text{C}$  NMR (DMSO)  $\delta$ : 168.08, 148.57, 148.45, 145.07, 140.25, 125.32, 123.55, 121.60, 114.27, 108.99, 106.18, 101.90, 101.83, 48.76, 46.56, 19.46, 18.98, 11.50, 10.57.  $m/z$  300.99 ( $\text{C}_{18}\text{H}_{23}\text{NO}_3$ , 301.17).

#### Validation of the developed HPLC method

The developed bioanalytical method was validated by following the guidelines provided by the USFDA.

#### Selectivity

The selectivity of the method was carried out to verify the interference of analytes (piperine and ST) during the method development. The selectivity was demonstrated by analyzing the chromatograms obtained by six blank plasma samples with the corresponding spiked blank plasma. There was no interference

obtained at the retention time of 6.3 min for piperine and 7.83 min for ST (Fig. 5).

#### Linearity

The linear range for ST was from 5 to 50  $\mu\text{g/mL}$  in the rat plasma. Calibration curve data were used to find out the correlation coefficient ( $r^2$ ), slope, and intercept of the regression equation. For ST, the regression equation was found to be  $y = 5,107.7(x) - 478.76$  ( $r^2 = 0.999$ ). The values for LOD and lower limit of quantitation (LLOQ) were 1.63 and 4.96  $\mu\text{g/ml}$ , respectively (Table 3 and Fig. 6).

#### Accuracy and precision

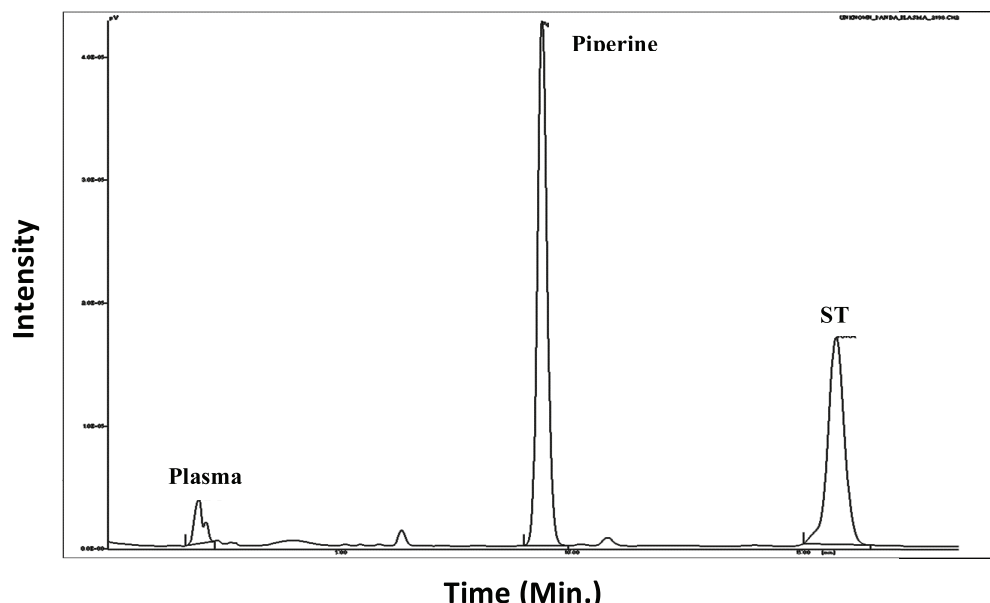
The interday and intraday precision studies were carried out using rat plasma at concentrations of 5, 25, and 45  $\mu\text{g/ml}$ . At all the

**Table 3.** Linearity data of ST.

Sr. No.	Validation parameters	ST
1.	Linearity range	5–50 $\mu\text{g/mL}$
2.	Correlation coefficient ( $r^2$ )	0.999
3.	Intercept	478.76
4.	Slope	5,107.7
5.	$S_{yx}^b$	2,533.722
6.	Regression equation	$y = 5,107.x - 478.76$
7.	LOD	1.636 $\mu\text{g/mL}$
8.	LLOQ	4.960 $\mu\text{g/mL}$
Precision		
9.	Interday (%RSD)	Precise
	Intraday (%RSD)	Precise
10.	Confidence limit of slope <sup>a</sup>	4,979.031
		5,236.338
11.	Confidence limit of intercept <sup>a</sup>	-4,470.13
		3,512.13

<sup>a</sup>95% confidence limit.

<sup>b</sup>Standard deviation of residual from line.



**Figure 5.** Chromatograms of ST and piperine in rat plasma.

selected levels, the percentage of relative standard deviation (%RSD) was found to be below the range. This showed that the method was reproducible and precise for ST in the biological matrix (Table 4).

**Stability**

The decomposition of solutes has been observed in the plasma prior to chromatographic analysis, such as sample preparation, extraction procedure, clean-up, storage conditions of the sample vials, etc. The stability profile of the prepared samples/analyte was evaluated under short-term and intermediate conditions (Table 5 and 6). Short-term stability was carried out by storing the samples at -20°C for 24 hrs; long-term stability studies were carried out by keeping the sample at -80 °C. The RSD value for change in the concentration related to the nominal concentration was found to be less than 20%.

**Extraction recovery**

The recovery study of ST was carried out using low, medium, and high concentration ranges. The recovery of ST

was found to be 93%–97%, with %RSD of 6%–8%. Studying the recovery revealed that the pre-treatment of samples used in the study was found reproducible for ST. The results of recovery studies of ST are given in Table 8.

**Robustness**

Some deliberate changes were made in the flow rate (1 ± 0.2 mL) and wavelength (280 ± 2 nm) in order to study the robustness of the developed method. Three quality control sample (5, 25, and 45 µg/mL) responses were recorded by altering individual parameters at a time. The method was considered robust if %RSD fell under the range of ±15% deviation (Table 7).

**In vivo pharmacokinetic study of ST**

Here, we report successful development of a validated bioanalytical HPLC method of analysis. It was applied to study the effects on various pharmacokinetic parameters of ST alone, along with piperine, and some of its synthetic derivatives.

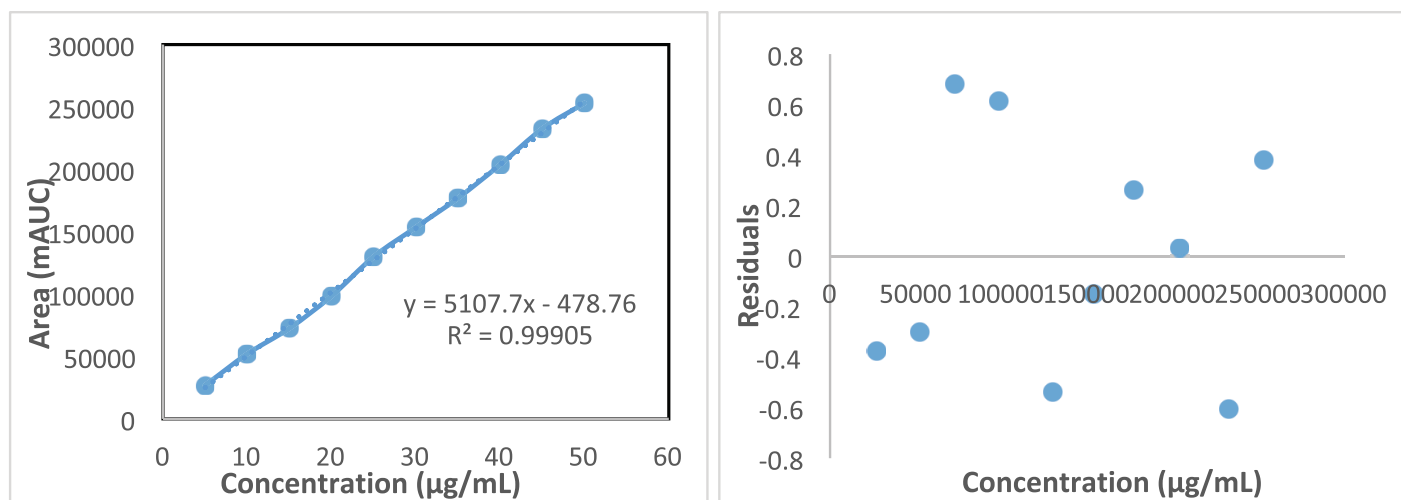


Figure 6. Linearity data and concentration versus residual plot for ST for rat plasma.

Table 4. Intraday and interday precision and accuracy of ST.

Analyte	Spiked (µg/mL)	<sup>a</sup> Mean ± SD	% RSD	<sup>a</sup> Measured Conc.	% Accuracy	
ST	Intraday	5	26,782.53 ± 332.22	1.24	5.32 ± 0.11	106.4
		25	127,920.36 ± 652.70	0.51	25.14 ± 0.76	100.56
		45	233,566.44 ± 822.11	0.351	46.82 ± 0.16	104.04
	Interday	5	26,105.56 ± 127.85	0.48	5.62 ± 0.05	112.4
		25	128,809 ± 161.37	0.125	25.67 ± 0.10	102.48
		45	233,855.98 ± 822.11	0.150	45.93 ± 0.31	108.73

<sup>a</sup>Average of three (n = 3); Concentration (µg/mL).

Table 5. Short-term stability of ST.

Analyte	Conc. (µg/mL)	<sup>a</sup> Bench-top stability			<sup>b</sup> Freeze–thaw stability		
		Area ± SD	% RSD	% Accuracy	Area ± SD	% RSD	% Accuracy
ST	5	26,902.83 ± 89.95	1.07	119.62	26,501.89 ± 643.45	2.394	105.66
	25	128,505.5 ± 134.90	0.10	101.02	128,611.04 ± 241.38	0.18	101.10
	45	232,523 ± 115.60	0.04	101.50	226,788.64 ± 2,551.33	1.12	100.34

<sup>a</sup>Bench-top stability represents the percentage stability of samples in 24 hrs.

<sup>b</sup>Freeze–thaw stability represents the percentage stability in 24 hrs.

**Table 6.** Long-term stability of ST.

Analyte	Conc. ( $\mu\text{g/mL}$ )	<sup>a</sup> Bench-top stability			<sup>b</sup> Freeze-thaw stability		
		Area $\pm$ SD	% RSD	% Accuracy	Area $\pm$ SD	% RSD	% Accuracy
ST	5	23,912.42 $\pm$ 1,045.58	4.37	95.50	24,380.05 $\pm$ 613.73	2.394	105.66
	25	126,473.7 $\pm$ 2,216.20	1.75	99.42	126,282.37 $\pm$ 1,970.9	0.18	101.10
	45	222,405.5 $\pm$ 3,079.76	1.38	96.97	223,228.36 $\pm$ 1,537.2	1.12	100.34

<sup>a</sup>Bench-top stability represents the percentage stability of samples in 30 days.

<sup>b</sup>Freeze-thaw stability represents the percentage stability in 30 days.

**Table 7.** Robustness of ST.

Wavelength (nm)	Conc $\mu\text{g/mL}$	<sup>a</sup> Mean area $\pm$ SD	% RSD	Flow rate (mL/min)	Conc ( $\mu\text{g/mL}$ )	Mean area $\pm$ SD	% RSD
278	5	26,398.89 $\pm$ 360.24	1.36	0.8 (mL/min)	5	26,242.28 $\pm$ 482.44	1.63
	25	128,640 $\pm$ 1,066.92	0.82		25	128,329.5 $\pm$ 1,130.0	0.88
	45	232,933.8 $\pm$ 1,423.22	0.611		45	231,624 $\pm$ 1,150.44	0.49
280	5	26,403.92 $\pm$ 448.44	1.84	1.0 (mL/min)	5	26,171.7 $\pm$ 266.44	1.01
	25	128,555.3 $\pm$ 1,285.65	1.00		25	128,450.7 $\pm$ 906.40	0.70
	45	232,218.2 $\pm$ 1,221.66	0.52		45	233,024.3 $\pm$ 1,266.3	0.54
282	5	26,644.33 $\pm$ 481.44	1.80	1.2 (mL/min)	5	27,242.41 $\pm$ 537.56	1.97
	25	129,122 $\pm$ 743.05	0.57		25	127,075 $\pm$ 969.86	0.76
	45	232,567.6 $\pm$ 796.28	0.34		45	234,877.4 $\pm$ 1,063.3	0.45

<sup>a</sup>Average of three ( $n = 3$ ).

**Table 8.** Extraction recovery of ST.

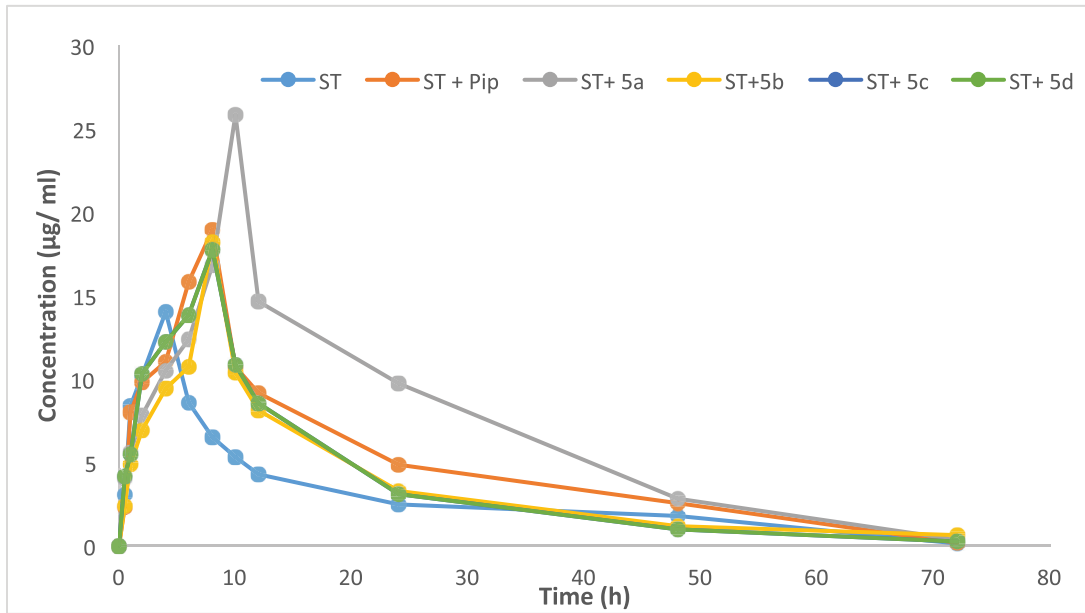
Analyte	Conc	% Amount Added	Total Amount	Area (mAu) $\pm$ SD	% RSD	Amount recovered	% Recovery
ST	LQC 5 $\mu\text{g/mL}$	80	4	18,666.66 $\pm$ 1,237.067	6.627	3.748	93.70
		100	5	24,029.22 $\pm$ 1,277.49	5.316	4.798	95.96
		120	6	28,654.50 $\pm$ 1,195.18	4.171	5.703	95.06
	MQC 25 $\mu\text{g/mL}$	80	20	96,905.93 $\pm$ 2,520.16	2.600	19.066	95.33
		100	25	122,293.60 $\pm$ 2,921.83	2.389	24.036	96.14
		120	30	140,220.90 $\pm$ 612.01	0.436	27.546	91.82
HQC 45 $\mu\text{g/mL}$	80	36	174,175.59 $\pm$ 10,026.46	5.756	34.194	94.98	
	100	45	221,350.94 $\pm$ 8,511.98	3.845	43.430	96.51	
		120	54	268,236.51 $\pm$ 7,413.31	2.820	51.55	95.46

**Table 9.** Pharmacokinetic profile of sorafenib in the presence of piperine and its synthetic derivatives.

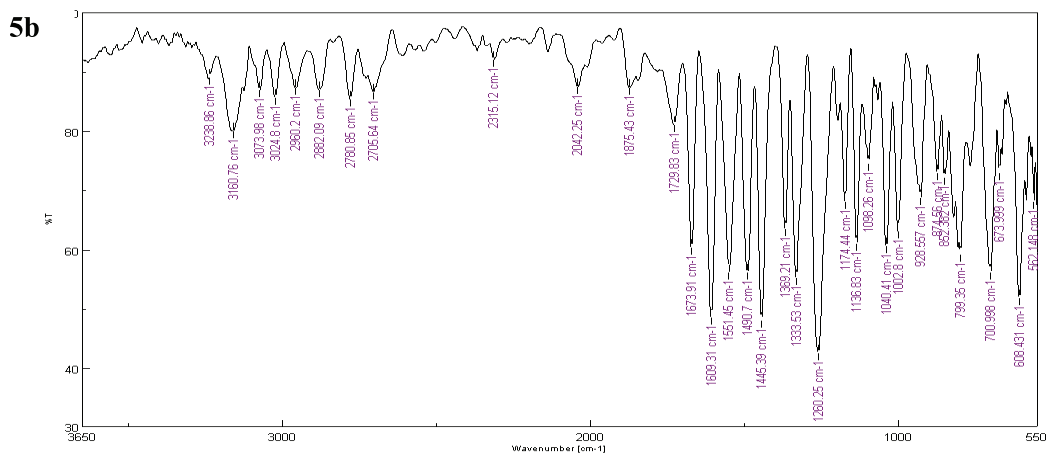
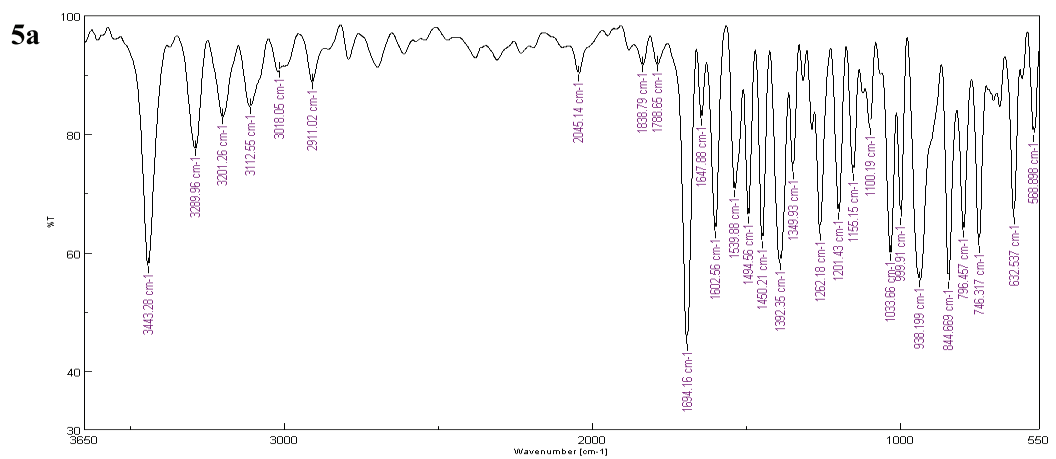
Parameter	Units	Value					
		ST	ST + Pip	ST + 5a	ST + 5b	ST + 5c	ST + 5d
$T_{1/2}$	Hrs	12.21 $\pm$ 0.27	11.16 $\pm$ 0.07	10.3 $\pm$ 0.13	18.3 $\pm$ 2.60	13.3 $\pm$ 0.5	11.1 $\pm$ 0.22
$T_{\text{max}}$	Hrs	4	8	10	8	8	10
$C_{\text{max}}$	$\mu\text{g/ml}$	14.00 $\pm$ 0.15	19.13 $\pm$ 0.07	25.96 $\pm$ 0.61	18.22 $\pm$ 0.97	17.76 $\pm$ 0.2	23.02 $\pm$ 0.09
$\text{AUC}_{0-4}$	$\mu\text{g/ml}^{\circ}\text{Hrs}$	210.32 $\pm$ 7.8	350.52 $\pm$ 1.9	515.96 $\pm$ 22.7	264.90 $\pm$ 7.6	273.21 $\pm$ 4.6	502.4 $\pm$ 3.61
$\text{AUC}_{0-\infty \text{ obs}}$	$\mu\text{g/ml}^{\circ}\text{Hrs}$	212.59 $\pm$ 8.1	353.7 $\pm$ 1.99	521.17 $\pm$ 22.4	282.22 $\pm$ 9.0	284.19 $\pm$ 4.1	509.46 $\pm$ 4.03
$\text{AUMC}_{0-\infty \text{ obs}}$	$\mu\text{g/ml}^{\circ}\text{Hrs}$	4,249.58	7,001.19	10,533.00	6,693.38	4,661.61	10,239 $\pm$ 270
$\text{MRT}_{0-\infty \text{ obs}}$	$\mu\text{g/ml}^{\circ}\text{Hrs}^2$	19.98	19.87	20.89	23.56	16.75	20.09
$K_{\text{el}}$	Hrs	0.05 $\pm$ 0.01	0.04 $\pm$ 0.02	0.03 $\pm$ 0.01	0.04 $\pm$ 0.02	0.04 $\pm$ 0.02	0.032 $\pm$ 0.02
$V_{\text{d}}$	L	357.1429	261.2585	192.5506	274.3935	281.6936	215.4963
Fold increased in bioavailability when compared to ST alone	–	–	1.66	2.58	1.26	1.34	2.42

<sup>a</sup>AUC = Area under the curve;  $T_{1/2}$  = Half-life;  $C_{\text{max}}$  = Maximal observed concentration;  $T_{\text{max}}$  = Maximum observed time; MRT = Mean residence time;  $K_{\text{el}}$  = Rate of elimination;  $V_{\text{d}}$  = Volume of distribution.

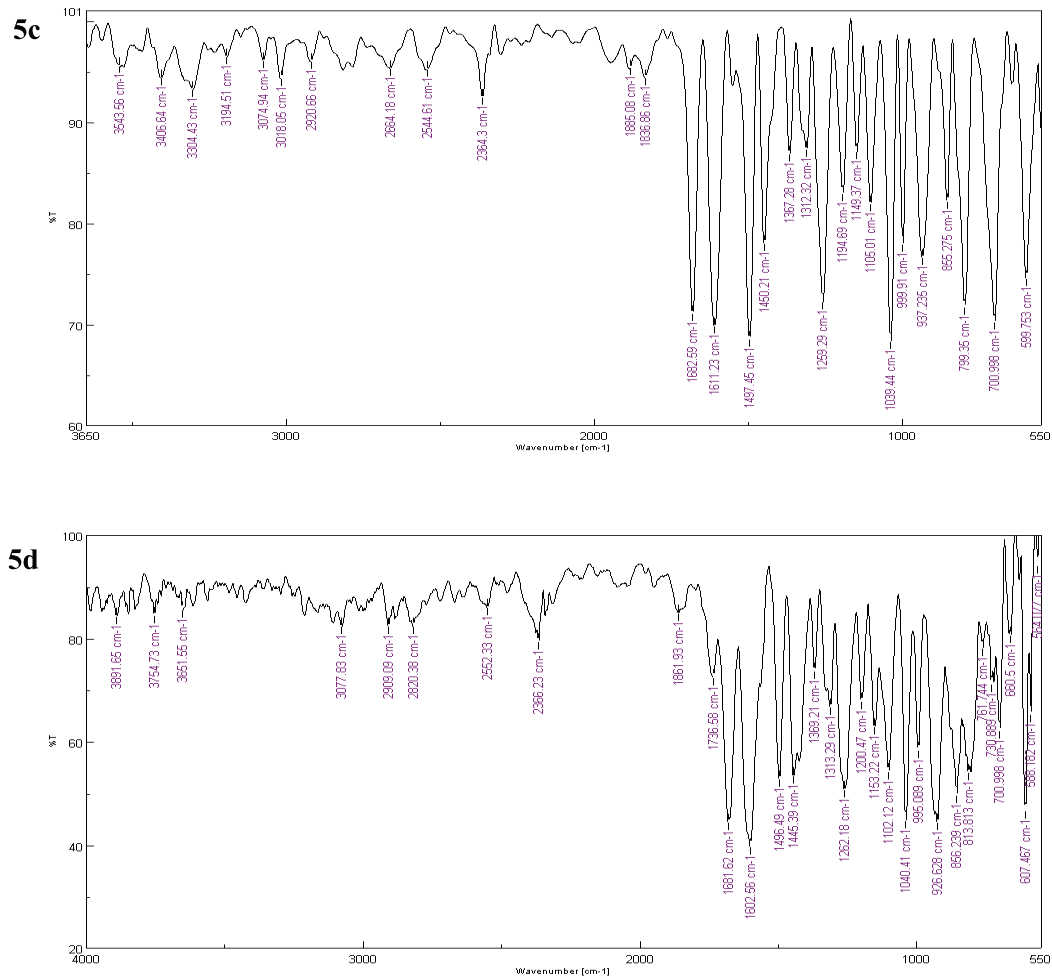




**Figure 7.** Mean plasma concentration time profiles of ST alone or in combination with piperine and its synthetic derivatives through the oral route (dose of 5 mg/kg body weight).



Continued



**Figure 8.** FTIR spectra of synthesized piperine analogs (5a–5d).

The pharmacokinetic results are shown in Table 9. *In-vivo* oral pharmacokinetics was conducted using male Wistar rats for a period of 72 hrs. The plasma drug concentration-time profiles are shown in Figure 7. Our study revealed that there is a dramatic change in the pharmacokinetics of ST when combined with piperine. Nevertheless, one of the analogs of piperine (**5a**) shows a 2.58-fold increase in the bioavailability, while with piperine it shows a 1.66 fold increase in the bioavailability compared to ST alone. Here, the piperine analog (**5a**) alters the pharmacokinetics of ST by increasing the maximum concentration ( $C_{max}$ ) and systemic exposure (AUC), as well as by reducing the rate of elimination ( $K_{el}$ ). The results of the study show that the presence of **5a** increased the AUC from  $210.32 \pm 7.8$  to  $515.91 \pm 22.77$ , along with the enhancement of  $C_{max}$  from  $14.0 \mu\text{g/mL}$  for ST to  $25.46 \mu\text{g/mL}$ , and reduction in the  $K_{el}$  from  $0.05 \pm 0.01$  to  $0.04 \pm 0.02$  fraction/hour. In terms of the above-mentioned results, AUC and  $C_{max}$  were notably elevated in the presence of **5a** combined with ST (Table 9). At the same time,  $K_{el}$  was decreased, which suggests that **5a** has a significant impact on the pharmacokinetics of ST.

#### Statistical analysis

The plasma concentrations are represented as mean  $\pm$  SD. Different parameters to study pharmacokinetics were evaluated

via NCA in PKsolver software 2.0 USA. All the parameters were compared using Student's *t*-test. Statistically significant results were obtained from the data in this study ( $p > 0.05$ ).

#### DISCUSSION

The pilot study provided shreds of evidence for the enhancement of the bioavailability of ST through the simultaneous administration of piperine (known as a versatile bioenhancer) and some of its synthetic analogs in systemic circulation, when orally administered to male Wistar rats devoid of side effects. However, the validated method was subsequently used for the assessment of the effect of piperine and piperine derivatives (5a–5d), suggesting the complete but slow absorption of ST from the gastrointestinal tract (GIT) with variations in  $C_{max}$ . The values of  $AUC_{0-t}$ ,  $AUC_{0-\infty}$ ,  $C_{max}$ , and  $T_{max}$  suggest the delayed absorption of the drug in the GIT compared to ST alone. The term ‘bioavailability enhancer’ was first invented by Atal *et al.* (1985), an Indian scientist at the Regional Research Laboratory (Jammu) in the year 1985 (Kesarwani and Gupta, 2013). Piperine, a principal ingredient obtained from the *Piper* species (*Piper longum* and *Piper nigrum*; Family: *Piperaceae*), is known to enhance the bioavailability of various xenobiotic agents of different categories of various human diseases (Derosa *et al.*, 2016). Significant differences in the pharmacokinetic parameters of ST observed in the

presence of piperine and its analogs led to the significant alterations in the dose of ST by delaying/slowing down the absorption from the GIT. Many nutrient/drug interactions could be explained, and they act by inhibiting the p-glycoprotein or CYP3A4; increase in the plasma drug concentration-time profile of a xenobiotic agent through the combination of these substances which exhibits the dual mechanism over absorption and metabolism (Lin, 2003). The possible mechanism of piperine acting as a bioenhancer may be due to the increase in absorption and inhibiting the rate of elimination of the drug from the body. The current research suggests that the significant increase in  $C_{max}$  may lead to a reduction in the dose of ST in patients with HCC.

In the present research work, derivatization of piperine was achieved by following the previously prescribed methods with some modifications for the synthesis (Mu *et al.*, 2012). The synthesized derivatives were confirmed using various spectroscopic methods such as FTIR,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR, and mass spectroscopy. NMR spectroscopy signifies that the desired product was successively achieved. The difference between piperine and its derivatives is mainly the piperidine ring; for example, in the compounds 5a–5d the piperidine ring was replaced with heteroaromatic ring (i.e., triazole and thiazole), aromatic ring (i.e., 4-fluorophenyl), and aliphatic chain (i.e., diisopropyl amine). Another major difference was piperine derivatives present in secondary amide. In  $^1\text{H}$  NMR data, this difference can be precisely identified. In piperine, the signals for piperidine ring protons were observed at  $\delta$  value of 3.37 (d,  $J = 11.1$  Hz) and 1.58–1.80 (m, 2H) ppm. In 5a the peak of amide proton was observed at 8.9 ppm and the peak of the triazole ring at 8.15 ppm; in 5b the peak of amide proton was observed at 12.24 ppm and peak of 7.29 ppm; in 5c, amide proton was observed at 9.901 ppm and aromatic proton was observed in the range of 7.0–7.32 ppm; lastly, in 5d the peak of aliphatic proton of diisopropylamine was observed at 4.16 ppm and 1.21 ppm, respectively. The characterization data obtained from the NMR spectroscopy confirmed the final product.

## CONCLUSION

Although our results are limited to the preclinical study, it provides an initial insight to shift the focus from developing novel molecules that presents various adverse effects to the healthy tissues to the combination therapy of nutrients/less toxic molecules to provide better targeted effects and lesser toxic effects. In conclusion, the current results indicate an insight that the co-administration of piperine and its synthetic analogs exhibits significant changes in the pharmacokinetics of ST, which may be by inhibiting the p-glycoproteins or by inhibiting the CYP3A4 metabolic enzyme present in enterocytes. This could be of great interest due to the co-administration of piperine and its analogs which can be a better alternative that enhances the bioavailability of the drug, including the reduction in the burden of the medication or simply by reducing the dose of the medications given to the patients for a longer time.

## ABBREVIATIONS

LOQ: Limit of quantitation; HOQ: High QC level; MQC: Middle QC level; ST: Sorafenib tosylate; GIT: Gastrointestinal tract; HCC: Hepatocellular carcinoma; NCA: non-compartmental analysis; RSD: Relative standard deviation; TLC: Thin-layer chromatography, AUC: Area under the curve BCS: Biopharmaceutical Classification, TMS: Tetramethylsilane, KOH: Potassium Hydroxide, THF: Tetrahydrofuran, FTIR: Fourier Transform Infrared Spectroscopy, EDTA: Ethylenediaminetetra

Acetic Acid, ESI: Electrospray ionization, GIT: Gastrointestinal Tract, LQC: Lower QC level.

## AUTHORS' CONTRIBUTIONS

AT contributed to carrying out the analysis, representing the results, and prepared the manuscript. KRM provided the technical assistance in preparing the manuscript. SYG provided the correspondence to the manuscript and helped revising the manuscript.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest concerning this manuscript.

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