DETERMINATION OF 5*H*-BENZO[2,3][1,4]OXAZEPINO[5,6-*B*]INDOLES IN RAT PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC-ULTRAVIOLET METHOD: APPLICATION TO PHARMACOKINETIC STUDIES

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ABSTRACT

Objective: Recently, we reported newly synthesized 5H-benzo[2,3][1,4]oxazepino[5,6-b]indole) derivatives and proved their cytotoxicity against hepatocellular carcinoma specific Hep-G2 cell lines. We attempted herein to describe a reversed-phase high-performance liquid chromatographic method for the determination of three most active compounds 6a, 10a, and 15a in rat plasma to predict their pharmacokinetics parameters before *in vivo* study.

Methods: A rapid and sensitive reversed-phase high-performance liquid chromatographic was employed for the determination of 6a, 10a, and 15a in rat plasma. Each compound was separated by a gradient elution of acetonitrile and water with 1 mL/min flow rate. The detector was set at 270, 285, and 275 nm for 6a, 10a, and 15a and the recorded elution times were 2.00, 2.87, and 1.88 min, respectively.

Results: The calibration curve was linear with R^2 of 0.938, 0.875, and 0.923 over the concentration range of 0.1–50 μg/mL. The inter- and intra-day variations of the assay were lower than 12.26%; the average recovery of 6a, 10a, and 15a was 97.31, 92.56, and 95.23 % with relative standard deviation of 2.12%, 3.25%, and 2.28%, respectively. The C_{max} and T_{max} were ~ 46.34, 18.56, and 25.65 μg/mL and 2.0, 4.0, and 4.0 h for 6a, 10a, and 15a, respectively, which indicate a robust method of detection in the present experiment.

Conclusion: The study suggests that all of the three compounds have a lower rate of absorption, higher volume of distribution, and lower clearance rate, indicating good therapeutic response for *in vivo* activity.

Keywords: 5*H*-benzo[2,3][1,4]oxazepino[5,6-*b*]indoles, Reversed-phase high-performance liquid chromatographic, Pharmacokinetics, Rat plasma.

Graphical Abstract
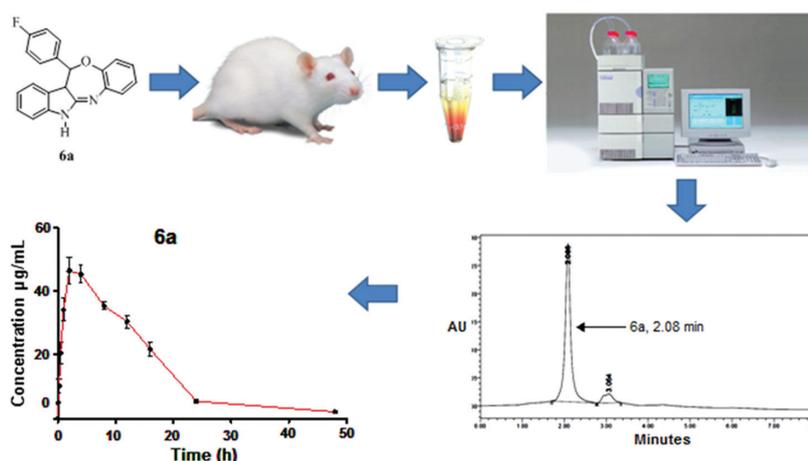
INTRODUCTION

Nitrogen-containing heterocyclic ring generally offers a polarized character and establishes a most efficient interaction with the receptor molecules to produce anticancer effects [1]. Among them, indole is one of the widely reputed scaffolds to identify new drug candidate as anticancer agents [2-4]. In addition, various azepines and analogs have also been subject of much investigation for cancer drug discovery [5-7]. Later, a fusion of two or more heterocyclic rings in a single molecular frame has become an appealing method of drug design to enhance the synergistic effects [8]. Owing to the importance of N-heterocycles, individual role of indole and azepine in cancer prevention, and ring fusion concepts, development of indole-fused azepines may be of critical importance toward the potential success in cancer drug discovery. In addition, considering potency and *in vitro* cell line selectivity, several indole-fused azepines including paullones [9-14] open a new venture for the discovery of novel potential anticancer agents. Although several

analogs of indole-fused azepines have been designed, synthesized, and screened for their cytotoxicity, there remain probably a space for the improvement in both efficacy and selectivity by the modification in ring structure.

In view of the aforementioned facts, recently [15], we designed and synthesized a new structural class indole-fused benzooxazepines (IFBO), particularly 5H-benzo[2,3][1,4]oxazepino[5,6-b]indoles derivatives, on the basis of molecular modeling on various related targets and evaluated them for *in vitro* antitumor potency against hepatocellular carcinoma (HCC)-specific Hep-G2 cell lines. Three compounds such as 6a, 10a, and 15a (Fig. 1) potentially inhibited the growth of tumor cells with GI_{50} <10 μg/mL. Among these three derivatives, 4-fluoro derivative of 5*H*-benzo[2,3][1,4] oxazepino[5,6-*b*] indole (6a) demonstrated the most pronounced effect on % control growth of tumor cells.

A quantitative determination of newly synthesized compounds in rat plasma is a prerequisite criterion before *in vivo* experiment [16,17]. Thus, in continuation to our previous study, we described herein the pharmacokinetic studies of all the three compounds in rat plasma with an oral administration of 10 mg/kg dose. The study was performed using reverse-phase high-performance liquid chromatography (RP-HPLC) attached with photodiode array detector (PDA). The intra- and inter-day variations were analyzed to find the precision of the developed method. In addition, to evaluate the accuracy of the method, a recovery test was performed by spiking analytes into plasma with various standard solvents. The optimized method is simple and robust for detection of 5H-benzo[2,3][1,4]oxazepino[5,6-*b*]indole derivatives in rat plasma. All the three active compounds 6a, 10a, and 15a are shown in Fig. 1. The structures of these synthesized compounds were drawn in ChemDraw Ultra version 12 (Cambridge Soft).

METHODS

Chemicals
HPLC grade acetonitrile (ACN), methanol (CH_3OH), and water (H_2O) were purchased from the Himedia Laboratories, Mumbai, India. All the compounds 6a, 10a, and 15a were synthesized previously in our laboratory using the chemicals provided by Sigma-Aldrich and Himedia [15]. All other reagents used were of analytical grade.

Animals and plasma samples collection
Healthy Wister albino male rats (120–140 g) were purchased from the Experimental Animal Center, Lucknow, and the animal ethical committee already approved the study protocol (Approval No. SDCOP&VS/AH/CPCSEA/01/0038/R2). The rats were caged standard laboratory conditions (temperature 25±5°C and light/dark cycle of 12 h) and kept for 1 week for acclimatization. They maintained with free access to a commercial pellet diet and water *ad libitum*. The animal was fasted overnight before starting the experiment.

All the compounds (6a, 10a, and 15a) by dissolving into 0.25% carboxymethyl cellulose (CMC) were administered orally, and blood was collected at 0.00, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 16, 24, and 48 h time points from retro-orbital plexus (n=3). Blood samples were collected into ethylenediaminetetraacetic acid containing 2 mL Eppendorf tube and centrifuged at 10,000 rpm for 10 min to separate the plasma and stored at –80°C until analysis. HPLC analysis was carried out within 20 days of plasma samples collection.

Chromatographic system and analytical conditions
Chromatography separation was performed using the LC-10AD chromatograph (Shimadzu, Japan) with double pump, vacuum degasser, and 20-μL loop manual sample injector, interfaced with PDA detector (Shimadzu, Japan). All are controlled by the Shimadzu Lab Solutions CS software (version 5.3, Alltech, USA). The separation was performed using a Shimadzu RP-C_{18} column (5.0 μ particle size, 4.6 mm internal diameter, and 250 mm length). An isocratic elution system was employed with a mobile phase consisted of ACN and water in ratios of 9:1, 7:3, and 9.5:0.5 v/v for 6a, 10a, and 15a, respectively, which was freshly prepared and degassed by ultrasonication. The flow rate was set at 1 mL/min. During the analysis, the mobile phase was not allowed to recirculate during the analysis. The temperature was maintained at 40°C throughout the experiment, and the total run time was 6 min. The column was washed after every run in an elution solvent ratio (50:50) with the injection of blank CAN. Chromatograms were monitored by UV detection in the range of 200–400 nm at a single wavelength, i.e., 270, 285, and 275 nm for 6a, 10a, and 15a, respectively.

Preparation of standard solutions
Stock solutions of 1 mg/mL for all the three compounds were prepared in ACN. Plasma stock solution of 50 μg/mL was prepared by spiking 10 mL of the stock solution into 190 mL of rat plasma. Later, standard solutions for calibration standards were prepared at a concentration of 0.1, 0.5, 1, 2, 5, 10, 25, and 50 μg/mL (n=3) by serial dilution. Quality control (QC) samples were prepared in triplicate at the concentrations of 0.4, 2, and 20 μg/mL, representing low, medium, and high concentration QC samples, respectively [18].

Sample extraction, optimization, and preparation of calibrants and test samples
The extraction efficiency of all the compounds was evaluated from rat plasma using various solvents, i.e., ACN, ACN: H_2O (1:1), CH_3OH and CH_3OH: H_2O (1:1). The comparison of liquid-liquid extraction (LLE) was carried out using 100 μL of 50 μg/mL concentration and 100 μL of extracting solvent into a 2 mL Eppendorf tube. Then, the tubes were vortexed for 30 min and centrifuged at 10000 rpm for 10 min. Later, the supernatant was decanted out into another tube and dried in an oven at 40°C. The tubes were reconstituted with 50 μL of various mobile phases, vortexed for 10 min, and 20 μL was injected for HPLC-UV analysis. Area under curve (AUC) data were collected where ACN was found to be the best extracting solvent for all the compounds analyzed (Fig. 2). The figure was drawn on GraphPad Prism 5 trial version.
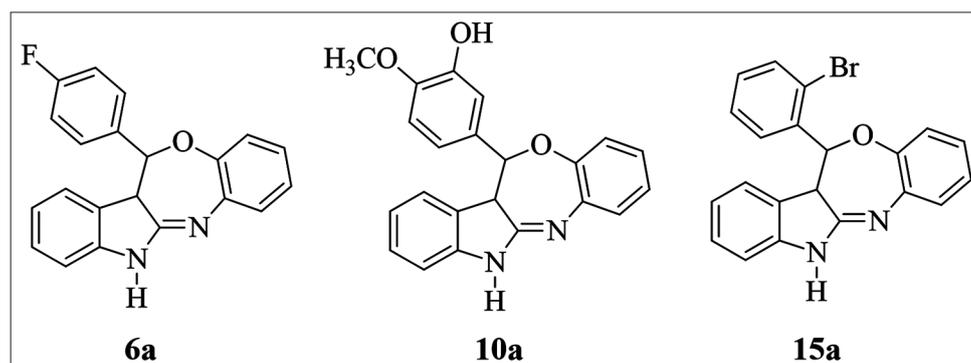

Fig. 1: Chemical structure of 6a (12-(4-fluorophenyl)-12,12a-dihydro-5H-benzo[2,3][1,4]oxazepino[5,6-b]indole), 10a (5-(12,12a-dihydro-5H-benzo[2,3] [1,4]oxazepino[5,6-b]indol-12-yl)-2-methoxyphenol), and 15a (12-(2-bromophenyl)-12,12a-dihydro-5H-benzo[2,3][1,4] oxazepino[5,6-b]indole)
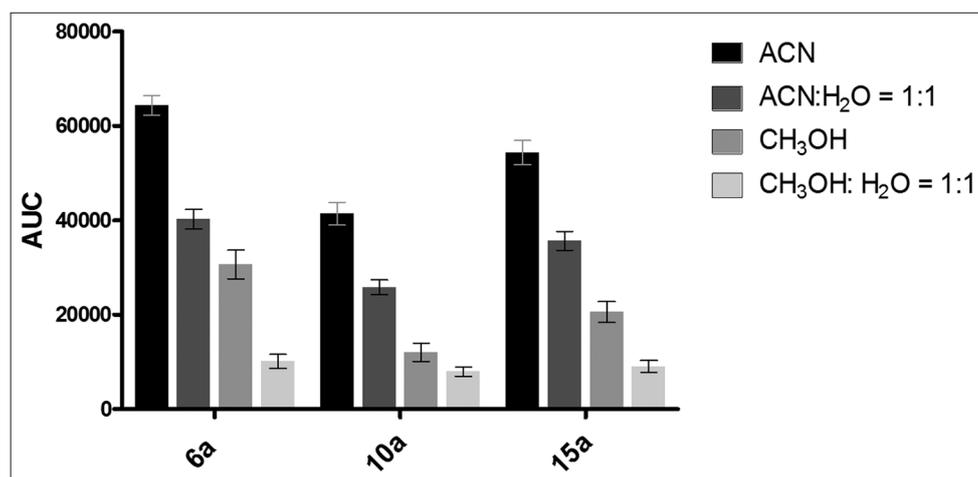
Fig. 2: Effects of solvents on area under curve of compounds 6a, 10a, and 15a in rat plasma. ACN: Actonitrile; H_2O: Water; CH_3OH: Methanol; all the three compounds 6a, 10a, and 15a are IFBO derivatives. Data are given in mean±SD (n=3)

For the preparation of calibration curve, the above-mentioned calibrants (100 μL) were taken into Eppendorf tube, and 100 μL blank ACN was added to it. The remaining procedure was similar to a previously described method. For test compounds, 100 μL of various time point samples were taken into Eppendorf tube, and 100 μL of blank ACN was added to it. Other procedures are same as previous one.

Stability studies
Long-term stability of 6a, 10a, and 15a was performed by preparing QC samples at three concentration ranges in three replicates such as low (0.1 μg/mL), medium (5 μg/mL), and high (50 μg/mL) for four dissimilar days and stored at 4°C. The analysis of all these samples was performed after 0, 7, 15, and 30 days of storage, and the data of samples were calculated by the particular calibration standard curve. The results revealed as % deviation from 0 days concentration. Moreover, the short-term stability of all the compounds was also performed at room temperature over 8 h by analyzing replicates (n=3) at three QC levels.

Method validation
The developed method was validated in terms of selectivity, linearity, accuracy (% recovery), and precision (intra- and inter-day variability with % relative standard deviation [RSD]), lower limits of quantification (LLOQ) according to the accepted guidelines [19,20]. Linearity was assessed over a concentration range of 0.1–50 μg/mL at seven different concentration levels. The intra- and inter-day variability was confirmed by calculating % bias from the theoretical concentration using equation: % bias = (observed concentration – nominal concentration)/nominal concentration × 100) [21]. The intra- and inter-day precisions were calculated by subjecting the data to the one-way analysis of variance respective to their % RSD.

Pharmacokinetic study
The developed method was utilized in evaluations of pharmacokinetic parameters of all the compounds in rat plasma. All the pharmacokinetic parameters such as maximum plasma concentration (C_{max}), time of maximum concentration (T_{max}), AUC at infinite time ($AUC_{0-\infty}$), mean residential time (MRT), and clearance rate (CL) were calculated using WinNonlins (5.1 software, trial version).

RESULTS AND DISCUSSION

In our recent investigation, three compounds 6a, 10a, and 15a derived from a novel structural class 5H-benzo[2,3][1,4]oxazepino[5,6-b] indoles exhibited a remarkable cytotoxicity profile against HCC-specific Hep-G2 cell line. In continuation to this finding and before performing *in vivo* animal study, we wished to assure whether these drugs have a satisfactory absorption, distribution, and clearance rate that would

serve a considerable therapeutic response. To accomplish the goal, we investigated the oral pharmacokinetics properties of all the three active compounds in rat plasma with an oral administration of 10 mg/kg dose. The study was conducted using RP-HPLC method for the determination of 5H-benzo[2,3][1,4]oxazepino[5,6-b]indoles in rat plasma, and pharmacokinetic parameters were calculated by software using the obtained data. All plasma samples were analyzed successfully using HPLC to achieve better selectivity, sensitivity, and reproducibility of 6a, 10a, and 15a in rat plasma.

Optimization of plasma sample pre-treatment conditions
LLE is a well-established technique for the extraction of concentrated, high-purity compounds from a broad range of biological samples. An appropriate LLE solvent is often used to eliminate the sample medium and take out the analyte since matrix effect occurs due to the presence of other ions in the biological sample [22,23]. A number of common organic solvents such as dichloromethane, chloroform, ACN, and methanol were used to extract 6a, 10a, and 15a from spiked blood plasma. Among them, ACN was found the most efficient solvent for the recovery of all the three compounds under reduced milieu effects. It has also been observed that better peak shapes and shorter retention times (RT) were obtained with ACN as compared to other solvents as an organic phase. The signal intensities for these compounds were steadily increased on increasing the percentage of ACN from 10% to 90% and slowly decreased from 90%. LLE followed by HPLC analysis revealed that all the compounds 6a, 10a, and 15a were of non-polar nature. Meanwhile, we played with an eluting solvent in different ratios of ACN: H_2O to separate out 6a in HPLC column. It was observed that the ratio 9:1 v/v allowed the highest AUC with a RT of 2.08 min (Fig. 3a). Thus, we decided to elute 6a using ACN and H_2O in a ratio of 9:1. Similarly, as per the better AUC obtained, separation of 10a and 15a was performed using an eluting solvent in the ratios of 7:3 and 9.5:0.5 v/v with RT of 2.87 and 1.88 min, respectively (Fig. 3b and c).

Fig. 3 shows a typical HPLC chromatogram obtained after the injection of a standard solution of 6a, 10a, and 15a where no interfering peak was observed that suggests the specificity of the method and its suitability for the routine QC analyses. Later, this optimized method was adopted to run the calibrants, QC, and test samples. Finally, data were calculated through WinNonlin 5.1 trial version.

Method validation
The developed method was used to validate in term of specificity, linearity, LLOQ, accuracy (% recovery), and precision (intra- and inter-day variability with % RSD). The proposed method was fully validated according to international guidelines [19,20,24]. To verify the specificity of method, blank natural plasma samples aspirated from rats and the rat plasma spiked with 6a, 10a, and 15a were analyzed,

and possible interferences with the analytes were observed by visual comparison of chromatograms with those obtained from the respective samples. To evaluate the linearity of the HPLC method, calibration curves were constructed and linearity was established by the least-squares linear regression. Best linearity was obtained between the analyte peak area and the corresponding concentrations over a wide range of 0.1–50 µg/mL with R^2 of 0.938, 0.875, and 0.923 for 6a, 10a, and 15a, respectively. The LLOQ was found to be 0.01µg/mL, which makes the optimized method useful for a wide range of derivatives and exhibits a high sensitivity for drug monitoring and pharmacokinetic studies. The precision was determined by calculating RSD values of six consecutive injections of the working standard solutions of 6a, 10a, and 15a in three replicates. The % RSD was observed within the limit of

±20% for all the three compounds which are acceptable for the routine measurement (Table 1).

The accuracy of method was determined by analyzing the percentage recovery of 6a, 10a, and 15a in plasma samples. We assessed the accuracy of the method by repeated analytical recovery of three known concentrations (0.1, 0.5, and 1 µg/mL) for 6a, 10a, and 15a, respectively, in three replicates. The average recovery of 6a, 10a, and 15a was 97.31%, 92.56%, and 95.23% with RSD of 2.12%, 3.25%, and 2.28%, respectively, which showed the accuracy of the method. Stability of compounds in rat plasma is another important parameter often considered for every pharmacokinetic study. All the three compounds were found to be stable over 30 days in normal plasma when stored
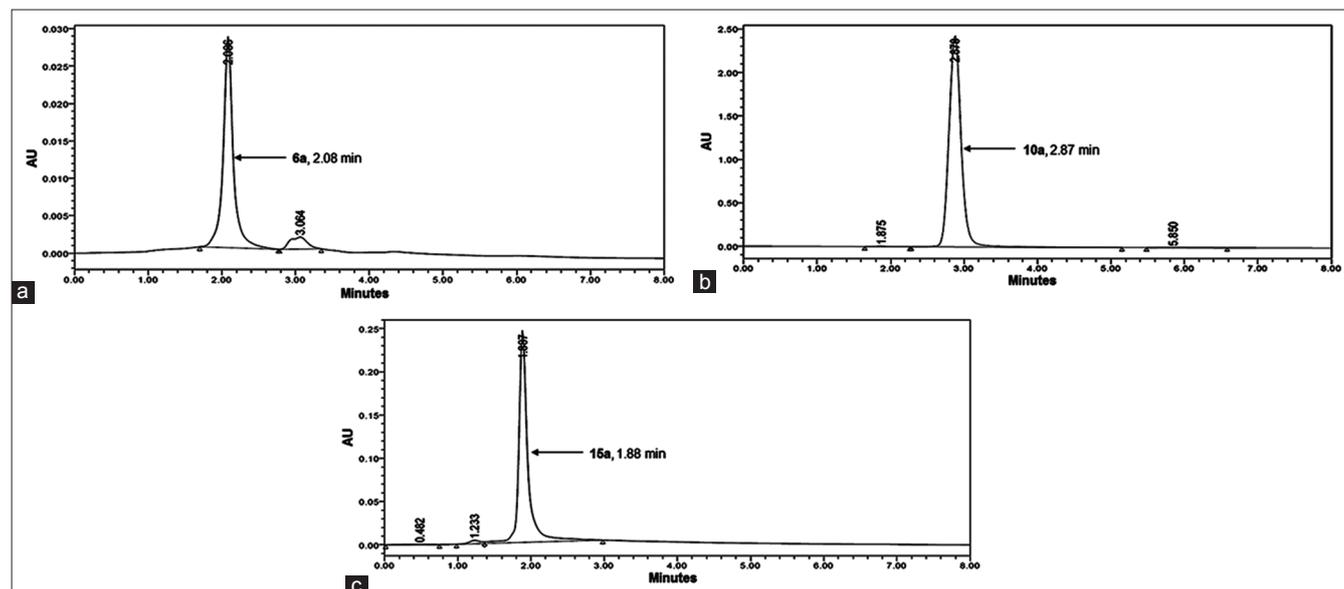

Fig. 3: High-performance liquid chromatography of (a) 6a, (b) 10a, and (c) 15a. The observed retention time was 2.00, 2.87, and 1.88 min for compounds 6a, 10a, and 15a, respectively

Table 1: The intra- and inter-day precision of 6a, 10a, and 15a in rat plasma

Compounds	Spiked concentration(µg/mL)	Intra-day precision (n=3)		Inter-day precision (n=3)	
		Mean±SD (µg/mL)	RSD (%)	Mean±SD (µg/mL)	RSD (%)
6a	0.1	0.08±0.002	2.91	0.09±0.002	2.34
	0.5	0.51±0.05	12.26	0.58±0.02	7.85
	1	0.89±0.07	6.27	0.82±0.09	9.29
	2	1.95±0.25	9.71	1.89±0.26	10.90
	5	5.04±0.48	8.85	5.55±0.31	5.85
	10	10.49±0.51	6.06	10.77±0.38	6.59
	25	24.46±0.88	4.78	26.79±0.97	5.20
	50	48.67±1.21	3.76	50.66±1.36	4.14
10a	0.1	0.1±0.001	1.38	0.2±0.002	1.47
	0.5	0.44±0.05	10.36	0.52±0.04	5.88
	1	1.14±0.06	4.85	1.35±0.06	4.07
	2	2.24±0.12	7.01	2.44±0.18	5.89
	5	4.79±0.22	6.85	4.64±0.19	6.74
	10	10.24±0.35	4.09	10.67±0.28	4.98
	25	25.67±0.76	3.28	25.98±0.68	3.82
	50	49.76±1.06	2.16	50.43±1.34	2.74
15a	0.1	0.4±0.001	2.56	0.3±0.003	2.58
	0.5	0.48±0.06	11.33	0.58±0.05	6.80
	1	1.05±0.07	4.85	1.19±0.08	5.85
	2	1.84±0.22	8.68	1.99±0.19	7.16
	5	4.99±0.32	6.85	5.14±0.28	6.14
	10	10.34±0.41	4.89	10.17±0.38	4.98
	25	24.97±0.94	2.98	25.48±0.84	3.82
	50	50.66±1.56	1.76	50.81±1.73	1.87

Mean: Average of number of experiment, SD: Standard deviation, RSD: Relative standard deviation. Data are given in mean±SD (n=3)

at 4°C. The short-term stability was carried out at room temperature over 8.0 h and long-term also performed after 30 days preservation using three replicates at three concentration levels. The percent deviation was calculated and found within acceptable range (Table 2). The calculated stability of spiked plasma indicated that 6a, 10a. and 15a had no considerable degradation under the above-mentioned environmental condition.

Pharmacokinetic study

The newly developed and validated method was utilized in the pharmacokinetic study of 5H-benzo[2,3][1,4]oxazepino[5,6-b]indole derivatives after the single oral administration of 10 mg/kg dose. The mean plasma concentration-time profile of compounds 6a, 10a, and 15a in male Wistar rats is shown in Fig. 4. The figure was drawn on GraphPad Prism 5 trial version.

The calculated non-compartment model parameters of pharmacokinetic study are summarized in Table 3 where the maximum plasma concentration (C_{max}), time of maximum exposure (T_{max}), and AUC at infinite time ($AUC_{0-\infty}$) were found to be 46.34±0.92 μg/mL, 2.0 h, 747.18±18.31 μg.h/mL for 6a, 18.56±0.75 μg/mL, 4.0 h, 256.78±10.21

μg.h/mL for 10a, and 25.65±0.83 μg/mL, 4.0 h, 376.22±12.21 μg.h/mL for 15a, respectively.

The data represented in Fig. 4 clearly explained that all the three compounds have slow absorption rate which is useful for liver cancer treatment as they remain in gastrointestinal tract in higher amount for long time. Although all the three compounds exhibit good pharmacokinetic property, compound 6a showed promising pharmacokinetic behavior among all the three compounds as it shows maximum concentration within optimum time duration. The MRT was found as 11.64±0.28, 12.70±1.31, and 11.58±1.20 h for 6a, 10a, and 15a, respectively, indicating that all the three compounds have higher volume of distribution. Compound 6a has higher clearance rate (CL) than those of compounds 10a and 15a again signified its better effectiveness.

CONCLUSION

Our recently reported compounds 5H-benzo[2,3][1,4]oxazepino[5,6-b] indoles hold a promise to be a potential drug candidate for liver cancer treatment through *in vitro* cell line study. Before performing the *in vivo*
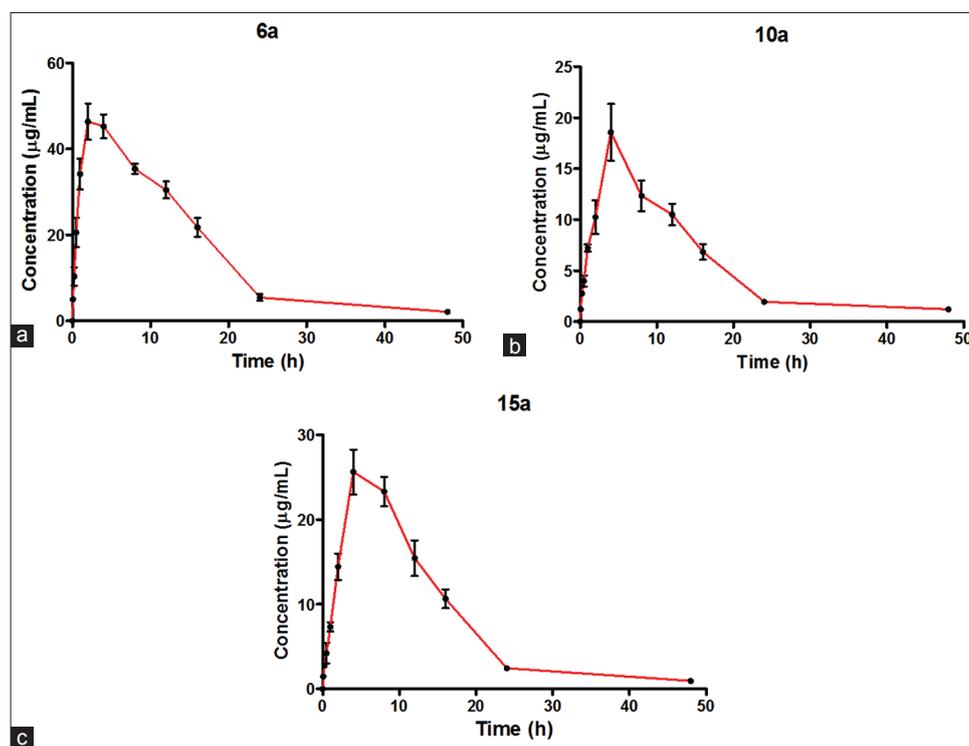

Fig. 4: (a-c) Plasma drug concentrations after single oral administration of compounds 6a, 10a, and 15a at 25 mg/kg. Data are given in mean±SD (n=3)

Table 2: The short- and long-term stability of 6a, 10a. and 15a in rat plasma

Compounds	Spiked concentration (μg/mL)	Short-term stability		Long-term stability	
		Mean±SD (μg/mL)	RSD (%)	Mean±SD (μg/mL)	RSD (%)
6a	0.1	0.08±0.002	2.05	0.07±0.001	2.25
	5	5.46±0.27	3.08	5.18±0.44	3.36
	50	50.65±2.38	2.80	50.78±2.32	2.15
10a	0.1	0.15±0.002	1.63	0.10±0.001	1.21
	5	5.35±0.81	4.17	5.48±0.24	5.12
	50	50.43±2.84	2.80	50.70±2.30	2.15
15a	0.1	0.11±0.002	1.53	0.17±0.001	0.98
	5	5.05±0.74	4.98	5.48±0.45	4.47
	50	50.23±2.13	2.27	50.85±2.36	2.32

Mean: Average of number of experiment, SD: Standard deviation, RSD: Relative standard deviation. Data are given in mean±SD (n=3)

Table 3: Pharmacokinetic parameters of 6a, 10a, and 15a after oral dose administration

Parameters	6a	10a	15a
C_{max} (µg/mL)	46.34±0.92	18.56±0.75	25.65±0.83
T_{max} (h)	2.00	4.00	4.00
$AUC_{0-\infty}$ (µg.h/mL)	747.18±18.31	256.78±10.21	376.22±12.21
AUMC (µg.h^2/mL)	8697.57	3261.16	4358.09
MRT (h)	11.64±0.28	12.70±1.31	11.58±1.20
CL (h^{-1})	0.280	0.008	0.065

C_{max}: Maximum drug concentration in plasma, T_{max}: Time at which the C_{max} observed, AUC: Area under curve, AUMC: Area under the first movement curve, MRT: Mean residual time, CL: Clearance rate. Data are given in mean±SD (n=3)

animal study, it was necessary to explore the pharmacokinetic profiles to assure the real fate of these newly synthesized compounds in rat plasma. We, therefore, developed and validated a RP-HPLC method using a LLE technique for the quantification of our recently synthesized compounds 6a, 10a, and 15a in rat plasma. Results obtained from our experiment clearly exhibited that all the three compounds have a lower rate of absorption, higher volume of distribution, and lower clearance rate that particularly indicate a good pharmacological response. The newly optimized methods for 6a, 10a, and 15a may be more robust with respect to accuracy and precision. Thus, all the three newly synthesized compounds are suitable for further *in vivo* study using chemically induced HCC rat model.

ACKNOWLEDGMENT

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