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BIOANALYTICAL METHOD VALIDATION AND EFFECT OF GRADED DOSES OF PIPERINE ON PHARMACOKINETIC PARAMETERS OF POSACONAZOLE USING LCMS-MS METHOD

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1. INTRODUCTION

In recent years, the antifungal triazole, posaconazole has become increasingly important as a prophylatic and for treatment of systemic mycoses. Whether therapeutic drug monitoring can reduce the risk of treatment failures by avoiding sub-therapeutic plasma concentrations needs further examination. Based on the ability of posaconazole to inhibit cytochrome P450 3A4, several drug interactions can be expected, especially with agents that undergo extensive first-pass effect through the gut and the liver. However, more information is needed regarding dose modifications during concomitant administration of posaconazole with drugs in certain categories, such as vinca alkaloids and retinoids. In Europe, the drug has been approved for the treatment of invasive fungal infection (IFI), including second-line treatment of aspergillosis, fusariosis, chromoblastomycosis, mycetoma and coccidioidomycosis. Posaconazole is also approved for antifungal prophylaxis in neutropenic high-risk patients with acute myelogenous leukaemia (AML) or myelodysplastic syndrome (MDS) who are receiving cytotoxic chemotherapy and in immunosuppressed patients with graft-versus-host disease (GVHD) after peripheral blood stem cell transplant (PBSCT).[1] Posaconazole has enhanced activity against many old, new, and emerging fungal pathogens compared with the activities of other azoles and caspofungin (an echinocandin). Numerous in-vitro studies demonstrate that posaconazole has a broad spectrum of activity against majority of yeasts, filamentous fungi, and azole-resistant Candida species.[2,3] During the second wave of COVID-19, posaconazole was used to treat COVID-19 associated mucormycosis.[4]

Bioenhancers are the substances which increase the rate and extent of drug absorption as well as the capacity to deliver the active component to its action site in a substantial amount to evoke the desired pharmacokinetic profile.[5] Piperine is a major alkaloid of Pepper fruits belonging to family *Piperaceae* which acts as a bioenhancer to enhance the bioavailability of companion drugs either by inhibiting the drug metabolizing enzyme, cytochrome P450 or by transepithelial electrical resistance factor (TER) in controlling the permeability of intestinal mucosa.[6-8] Piperine enhances the bioavailability of structurally and therapeutically different drugs, either by increasing the absorption or by delaying the metabolism of the drug or by a combination of both processes.[9-10] After oral administration of posaconazole, absolute bioavailability has been estimated to range from 8% to 47%. The low bioavailability may be explained by the high lipophilicity of posaconazole, possibly some first-pass effect and special conditions of the patients (e.g. malabsorption based on cytotoxic chemotherapy and bone marrow transplantation). Important factors that contribute to enhanced bioavailability include administration with high-fat food, absence of severe diarrhoea or mucositis, constitutive low gastric pH values, divided daily dosing and absence of highly potent enzyme-inducing drugs.[11]

The mean t_{max} observed after administration of posaconazole suspension ranged from 5 to 6 hrs in healthy subjects. The mean elimination $t_{1/2}$ of posaconazole suspension is 25.1 to 29.2 hrs. Posaconazole is barely metabolized by the cytochrome P450 (CYP) pathway. About 17% is glucuronidated by UGT1A4 and the remainder is eliminated unchanged and 77% of the dose is excreted in feces, of which > 66% is unchanged, while unchanged 13% of the dose is eliminated in the urine.[12-13]

We have been working on ways to increase the bioavailability of drugs using piperine and its synthetic derivatives. Here we would like to report the effect of graded doses of piperine on the pharmacokinetic properties of posaconazole on oral co-administration in Wistar rats.

2. MATERIALS AND METHODS

2.1 Chemicals

The gift sample of piperine was purchased from Yucca labs, Mumbai and posaconzole was obtained from Sigma Aldrich Company. HPLC grade solvents including methanol (MeOH), acetonitrile (ACN) were also obtained from Sigma Aldrich Chemicals Pvt Ltd and were used without further purification.

2.2 Analytical instrument

Detection was performed using Agilent 6464 (Agilent Technologies, USA) triple quadrupole LC-MS/MS system; with Agilent 1260 series combined LC-system with software (Agilent Mass Hunter). The following settings were used: nozzle voltage at 500V, capillary voltage at 3500V, nebulizer with pressure of 55psi, gas temperature at 250° C, gas flow at 10 L/min and sheath gas flow at 11 L/min. Multiple reaction monitoring (MRM) mode was used to quantify posaconazole and piperine in positive ionization mode using transition of m/z 701.2→ 682.9 and 286.1→201.0 respectively. Data acquisition and processing were performed with software Agilent Mass Hunter.

2.3 LC-MS/MS conditions

Syncronis ^{TM} C18 column (150* 4.6 mm, 5 µm) was used for separation at 250° C. Ten microlitres sample were transferred to the column by partial-loop injections. The mobile phase A consist of ACN and mobile phase B was water containing 0.5% formic acid with gradient elution of time program 90:10, 90:10, 50:50, 10:90, 90:10 and 90:10 (A% : B%) at 0, 0.5, 3, 11, 13 and 15 mins.

2.4 Stock Solutions and Quality control samples

The stock solution of posaconazole and piperine (1mg/ml) was prepared in acetonitrile and further dilutions were done with acetonitrile (1-500 ng/ml for posaconazole and 1-50 ng/ml for piperine).

For quantification of posaconazole and piperine calibration standards were prepared at final concentration of 1, 10, 50, 100, 200, 300, 400, 500 ng/mL and 1, 5, 10, 20, 40, 60, 80, 100 ng/ml in rat plasma respectively. The eight non-zero calibrators were prepared at final concentration by using 100µl of plasma spiked with appropriate stock solution. QC samples with LLOQ, LQC, MQC, HQC for posaconazole with concentrations of 1, 10, 100 and 400 ng/ml and for piperine with concentration of 1, 5, 20 and 80 ng/ml were prepared in the same manner as were calibration standards.

2.5 Sample pretreatment

Posaconazole and piperine concentration in rat plasma was deterimined after samples had been subjected to protein precipitation. Plasma, 100µl, was spiked with required amount of drug stock solution. This mixture was vortexed for 2 mins. The volume was to get desired concentration by using ACN which also acts as precipitating solvent. Samples were centrifuged at 15,000 rpm for 20 min using an Eppendorf 5415D microcentrifuge and supernatant was loaded to the instrument for analysis. The injection volume was 10 µL for LC–MS/MS analysis.

2.6 Bioanalytical method validation

The LC–MS/MS method was validated based on FDA "Guidance for Industry".[14-16] Validation parameters included intra- and inter-day precision and accuracy, linearity, stability, carryover, and matrix effects. These parameters were fully characterized for posaconazole and piperine in rat plasma.

2.6.1 Linearity

The working solutions of posaconazole and piperine were diluted to 1, 10, 50, 100, 200, 300, 400, 500 ng/ml and 1, 5, 10, 20, 40, 60, 80, 100 ng/ml in 100 µl of rat plasma respectively. The calibration curve was obtained using the following criteria: (1) the mean value should be within ± 15 % of the theoretical value, except at the LLOQ, where it should not deviate by more than ± 20 %; (2) the correlation coefficients (r^2) of all calibration curves should be more than 0.980.

2.6.2 Specificity and Selectivity

Specificity is done to confirm that the method could specifically quantify posaconazole and piperine in the presence of rat plasma. The blank plasma sample and sample spiked with LLOQ was analyzed. Selectivity is done to quantify both the drugs even in the presence of other components, six individual plasma samples were used to prepare blank samples and spiked samples at the LLOQ level were analyzed.

2.6.3 Precision and Accuracy

The precision and accuracy of the developed method was evaluated using spiked four QC samples in three individual runs with LLOQ, LQC, MQC and HQC for posaconazole with concentrations of 1, 10, 100 and 400 ng/ml and for piperine with concentration of 1, 5, 20 and 80 ng/ml were prepared same as calibration standards. The intra- and inter accuracy expressed as the recovery (%) should not exceed 15% for all the QC levels (except for the LLOQ, for which it was set as ± 20% of the nominal values). Intra- and inter-precision were determined by the coefficient of variation of the mean determined concentration, which should be ≤ 15% at each level (≤ 20 % at LLOQ level).

2.6.4 Carryover and Matrix effect

Carryover was assessed by using replicate QCs at LLOQ and HQC. The sequence of analysis was blank, LLOQ, HQC and blank. If carryover is observed it should be eliminated during method development. The impact of any carryover observed during method validation on the accuracy of the study sample concentrations should be assessed. Carryover is carried to ensure that the observed peak area in post blank injection was < 20% of the LLOQ. For rat plasma the matrix effect is assessed by the effect of ion suppression or enhancement for

posaconazole and piperine. The matrix effect was analyzed for six individual rat plasma sources. No matrix effect in the samples was expected if the precision from the six sources was ≤ 15%.

2.6.5 Recovery

Recoveries for extraction were investigated at three QC levels (LQC, MQC and HQC) in rat plasma. It was evaluated by standard addition method, in which definite amount of standard solution was added to extracted plasma samples of three QC levels of 80 %, 100 % and 120 % of posaconazole and piperine simultaneously.

2.6.6 Stability

The stability was performed for the QC samples at LQC and HQC concentraction. Benchtop stability was examined by keeping replicates of the low and high plasma quality control samples at room temperature for 9 hrs. While Freeze-thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2 – 3 hrs and refrozen for 12 hrs at -80° C. The stability was determined by calculating % accuracy at each level and it should be ± 15%.

2.7 Pharmacokinetics study

The aim of this study is to explore the effect on graded doses of herbal bioenhancer piperine on bioavaliabilty of posaconazole. 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/03/2021). The male Wistar rats of 9-12 weeks of age were used for the experimentation. The animals were quarantined at controlled environment with temperature of 22-25° C and relative humidity of 55-60 % and 12h light/12h dark cycle. Animals received a standard diet and water ad libitum. Before administration of drug the animals were grouped (n=6) and were fasted overnight. The single oral dose of posaconazole (40 mg/kg) with graded doses of piperine (graded doses = 2, 4, 6, 8 and 10 mg/kg) were given through oral gavage. At 2, 4, 6, 8, 10, 12, 24 and 36 hrs after oral administration blood (0.5ml) was collected by retro-orbital venous plexus method. The collected blood samples were stored in labeled tubes containing K-EDTA as an anticoagulant. Plasma was obtained by centrifugation of blood samples at 10,000 rpm for 10 minutes at 4° C and immediately stored at -70° C until analysis. For analysis the samples were thawed for about 60 mins. Concentrations of posaconazole in rat plasma of samples were found out by the validated LC-MS/MS bioanalytical method. The pharmacokinetic parameters for posaconazole were calculated using the validated WinNonlin 7.0 software. Pharmacokinetic parameters were determined using a noncompartmental approach with a log-linear terminal phase assumption.

3. RESULT AND DISCUSSION

3.1 Method development

Mass spectrometric detection optimization

Optimization of tandem mass parameters was carried out to obtain the maximum response for both the drugs. The MRM was selected based on the highest sensitivity. Detection of posaconazole and piperine was carried out using the following positive ionization mode using transition of m/z 701.2→682.9 and 286.1→201.0 respectively.

Chromatographic retention was optimized on the Syncronis ™ C18 column to obtain short runs but with sufficient retention to circumvent matrix effects. A product spectrum of posaconazole and piperine is shown in Figure no. 1 with the proposed fragmentation product. The combination of ACN and water acidified with formic acid was selected.

3.2 Plasma extraction optimization

The purification of sample from plasma components in bioanalytical method is essential to ensure maximum response. Plasma samples were extracted with different solvents like acetonitrile methanol, dichloromethane, diethyl ether and ethyl acetate. Acetonitrile resulted in high response and maximum recovery.

3.3 Validation

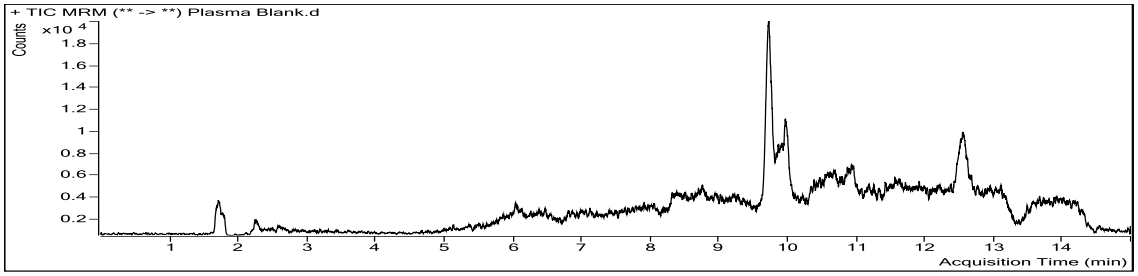
The calibration range (1–500 ng/ml) was selected based on the LC-MS/MS response and the average maximum concentration (2329.47 ng/ml).[17] The method was validated at the lower and in the higher concentration range possible (1–500 ng/ml) on the present instrument using current US-FDA guidelines. Representative blank and LLOQ (1 ng/ml) chromatograms of posaconazole and piperine is shown in fig 1.

3.3.1 Linearity

The concentrations of calibration standards used were (for posaconazole) 1-500 ng/ml and (for piperine) 1-50 ng/ml. Eight non-zero calibration standards were prepared for both the drugs in three separate validation runs. Linear regression was carried out on the analyte peak area versus concentration of analyate. The results were evaluated by linear regression analysis. The correlation coefficients of posaconazole and piperine calibration curves were found to be 0.9967 and 0.9982 (in positive ionization mode) respectively.

3.3.2 Specificity and Selectivity

In the selectivity study, the remnant blank plasma sample was analyzed and no interfering peak was found at retention time of 8.32 and 9.53 mins corresponding to posaconazole and piperine in the chromatogram of the blank (Fig.1). As part of the specificity study, an interference study was carried out. Responses of posaconazole and piperine in blank samples shows no interference at retention time (Fig. 1).



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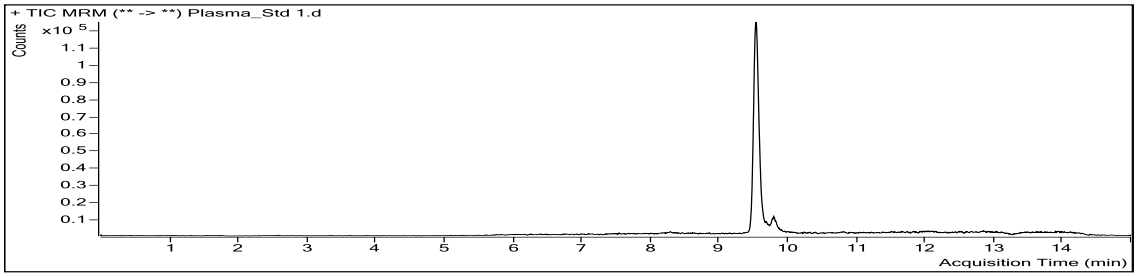

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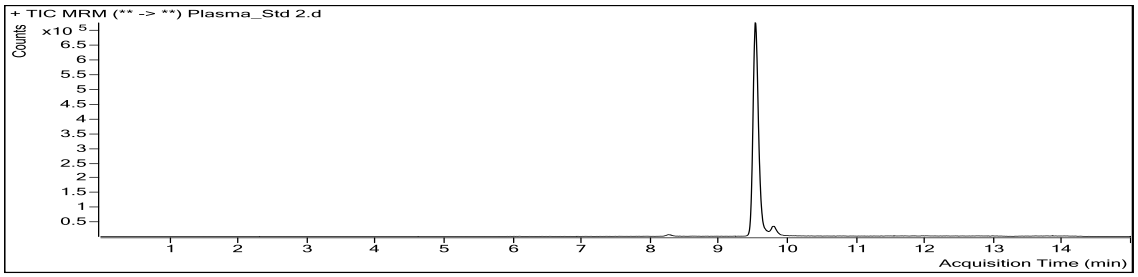

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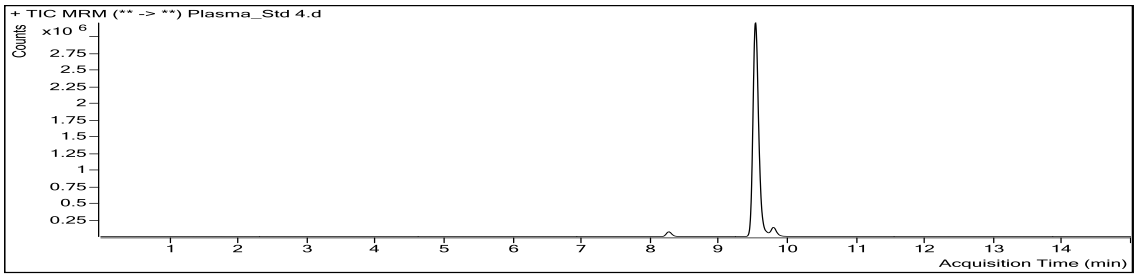

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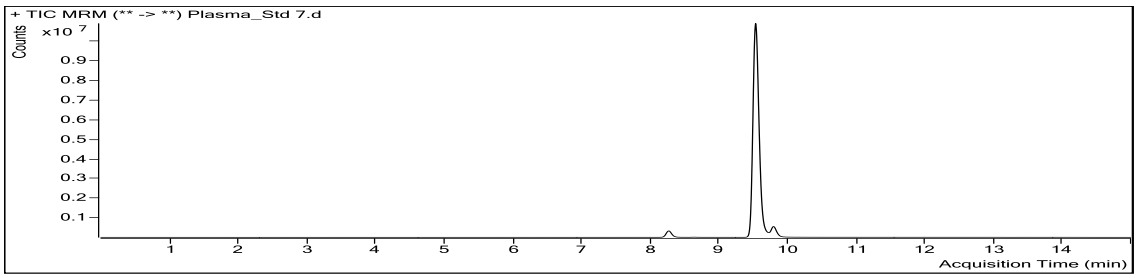

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Fig. 1: Representative MRM chromatogram for (A) blank plasma, (B) LLOQ (C) LQC (D) MQC (E) HQC for posaconazole and piperine of retention time at 8.32 and 9.53 mins.

3.3.3 Precision and Accuracy

Intra- and inter-day precision and accuracy for posaconazole and piperine were determined following the recommendations of the FDA guidelines. Parameters for four QC samples are reported in table no.1. Precision and accuracy values were within ± 15 % deviation and 15 % at each concentration level (100 ± 20 % at LLOQ level) respectively as required by the guidelines.

Table 1: Intra- and inter-day accuracy and precision for Posaconazole and Piperine in rat plasma (Results expressed as % accuracy and % CV for precision).

QC levels	Posaconzole		Piperine	
Intraday Accuracy and Precision				
	Accuracy (%)	Precision (% CV)	Accuracy (%)	Precision (% CV)
LLOQ	98	0.1201	98.82	0.218
LQC	105.75	1.827	94.906	4.660
MQC	102.29	10.52	98.36	2.855
HQC	100.22	4.17	100.82	1.304
Interday accuracy and precision				
	Accuracy (%)	Precision (% CV)	Accuracy (%)	Precision (% CV)
LLOQ	96.32	0.137	108.32	0.334
LQC	107.907	2.119	99.50	2.151
MQC	103.907	3.052	98.36	9.502
HQC	100.655	2.505	99.08	3.865

3.3.4 Carryover and matrix effect

Carry-over was evaluated by injecting blank sample after LLOQ and HQC of calibration standards. The peak areas at the retention times of the analyte in the blank samples were compared to the mean area of the analyte. No carryover was observed for retention times of both the drugs. As a result, the carry-over fulfilled the acceptance criteria. Matrix effect at high and low QC level was absent which was analyzed for six individual sources. These results also facilitate the accurate assessment of the posaconazole and piperine levels in all matrices by using plasma calibration only.

3.3.5 Recovery

The recovery at three QC levels was evaluated by comparing concentrations of extracted samples at QC versus extract of blank spiked with the analyte post extraction. The recovery study was done by standard addition method. The results of extraction are shown in table 2.

Table 2: Summary of extraction recovery of QC samples. Result expressed as % recovery.

QC level	% Drug	Total amount (ng/ml)	Amount recovered (ng/ml)	% Recovery	% Drug	Total amount (ng/ml)	Amount recovered (ng/ml)	% Recovery
	Posaconazole				**Piperine**			
LQC	80	8	7.583	94.788	80	4	4.056	101.3934
	100	10	9.228	92.282	100	5	5.353	107.0579
	120	12	11.508	95.901	120	6	5.628	93.79643
MQC	80	80	77.855	97.319	80	14	14.030	100.2143
	100	100	97.341	97.341	100	20	19.950	99.75
	120	120	116.959	97.466	120	26	25.548	106.4498
HQC	80	320	318.666	99.583	80	64	65.258	101.9657
	100	400	403.005	100.751	100	80	80.069	100.0862
	120	480	479.636	99.924	120	96	99.242	103.3766

3.3.6 Stability

Stabilitiy studies were conducted to evaluate the stability of posaconzole and piperine in rat plasma. The stability in each matrix was evaluated for freeze thaw and benchtop stability as shown in table no.3. The stability QC samples should be compared to freshly prepared calibration curves and QCs. The accuracy at each level was within the acceptance criteria i.e. ± 15 % of nominal concentration.

Table 3: Stability under different conditions (Freeze Thaw and Benchtop) at Low and High level of QC samples is expressed as % accuracy.

	QC levels	Posaconazole (% Accuracy)	Piperine (% Accuracy)
Freeze thaw stability (3 cycles)	LQC	99.55	98.31
	HQC	101.821	95.41
Benchtop stability	LQC	103.913	101.08
	HQC	98.65	98.09

3.4 Pharmacokinetic study

Pharmacokinetic parameters were calculated for the drug after oral administration in rat by LCMS-MS method. Rat plasma concentration was determined in positive ionization mode at different time points. The dose was administered for six groups (Posaconazole + Piperine dose in mg/kg for Group A= 40, Group B= 40+2, Group C= 40+4, Group D= 40+6, Group D= 40+8, Group E= 40+10) and concentration was determined. Major PK parameters obtained from this study included maximum concentration (C_{max}), time to reach the maximum concentration (T_{max}), elimination half life ($t_{½}$), area under the curve from 0 to last quantifiable sample ($AUC_{0\text{-}last}$), area under the curve from 0 to infinity ($AUC_{0\text{-}\infty}$)apparent volume of distribution (Vz/F), and apparent total clearance (CL/F).

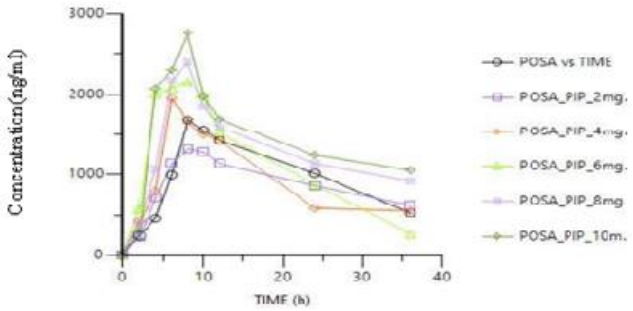

Fig. 2: The plasma concentration time profile of posaconazole for (A to F) six groups.

The pharmacokinetics studies shows increase in AUC as gradual increase in dose of piperine from 2 mg to 10 mg. AUC of single posaconazole was found to be 35123.49 h*ng/mL and that of AUC of posaconazole along with coadministration of 10 mg piperine was found to be 51848.98 h*ng/mL. The 1.476 fold increase in bioavailability of posaconazole is observed. The gradual increase in AUC as increase in dose of piperine is shown in table.4 and fig.2. The data shows wide range of $T_{1/2}$ from 9.47 h (group D) to 34.87 h (group F).

Table 4: Pharmacokinetic parameters of posaconazole in rats plasma following graded doses of piperine on oral administration.

	Group A	Group B	Group C	Group D	Group E	Group F
AUC_{last} (h*ng/mL)	35123.49	31259.08	32907.31	40360.41	46042.13	51848.98
T_{max} (h)	8	8	6	8	8	8
C_{max} (ng/mL)	1686.9	1317.79	1958.28	2160.04	2406.5	2766.15
Vz_F (mL/kg)	20211.87	28413.708	20173.078	12319.847	20508.92	19463.23
Cl_F (mL/h/kg)	819.3306	719.65443	878.19876	901.02118	468.6546	386.85184
MRT last (h)	17.04434	17.354799	15.015594	13.742144	16.711355	16.48037
$T_{1/2}$ (h)	17.099	27.36713	15.9222	9.47754	30.333	34.873
K_{el} (1/ h)	0.040537	0.0253277	0.0435332	0.0731357	0.0228512	0.01987

Table 5: Summary of dose dependent increase in AUC of posaconazole.

Groups	AUC(h*ng/mL)	Fold increase
Group A	35123.49	1
Group B	31259.08	0.8899
Group C	32907.31	0.9369
Group D	40360.41	1.1491
Group E	46042.13	1.3108
Group F	51848.98	1.4761

4. CONCLUSION

A rugged LC–MS/MS method for the quantification of posaconazole and piperine in male Wistar rat plasma has been developed and validated. Piperine enhances the bioavailability of posaconazole in a dose dependent manner.

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