Application of validated RP – HPLC method for estimation of pharmacokinetic parameters of novel satranidazole formulation

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ABSTRACT :

Satranidazole, a BCS class II antiprotozoal drug has poor solubility. *In vitro* dissolution studies have demonstrated enhanced solubility and improved dissolution rate of satranidazole solid dispersion. However *in vivo* studies are necessary to provide a proof of enhanced bioavailability and therapeutic efficacy of the formulated solid dispersion. Though bioanalytical methods for estimation of satranidazole have been reported, its application for bioequivalence studies of satranidazole solid dispersion is yet to be explored. The objective of the present study was to apply validated reversed phase high-performance liquid chromatography (RP-HPLC) method for *in vivo* estimation of satranidazole and compare various pharmacokinetic parameters of pure satranidazole and its solid dispersion. Satranidazole solid dispersion was formulated by solvent evaporation technique using Plasdone® S 630 as hydrophilic carrier. The validated assay procedure involved extraction of satranidazole and internal standard (IS) Tinidazole, from plasma by protein precipitation. The chromatographic method used Kromasil C_{18} (4.6 mm × 250 mm, 5µ) column. Mobile phase of phosphate buffer (0.01M, pH3.5): acetonitrile (65:35, v/v), was used at a flow rate of 1.0 mL/min. The eluate was monitored using a diode array detector set at 320 nm. Pure satranidazole and the formulated solid dispersion were administered orally (50 mg/kg) to male Wistar rats. Nominal retention times of IS and satranidazole were 5.5 and 7.7 min respectively. The method was found to be linear ($R^2 > 0.999$) in the concentration range 0.5 – 70 µg/mL. Absolute recovery was > 90% for both analyte and IS. The lower limit of quantification was 0.5 µg/mL. Analyte and IS were found to be stable during the freeze/thaw cycles, benchtop stability and long term stability. Satranidazole solid dispersion on oral administration to male Wistar rats demonstrated 1.85 folds increase in C_{max} and 164% increase in bioavailability as compared to pure satranidazole. However t_{max} values when compared using Wilcoxon rank sum test were found to be similar ($p > 0.05$). A specific, accurate, precise and reproducible RP-HPLC method was applied successfully for estimating the enhanced bioavailability and increase in C_{max} of satranidazole solid dispersion.

INTRODUCTION:

Satranidazole, chemically [1-methylsulfonyl - 3- (1-methyl-5-nitro - 2 - imidazolyl) -2- imidazolidinone] (Figure. 1(I)), is more active against aerobic, microaerophilic and anaerobic bacteria than metronidazole [1]. It is found to be significantly superior to metronidazole in treatment of caecal, hepatic amoebiasis, trichomoniasis and giardiasis [2]. Despite having lower incidence of side effects and significantly better tolerance than metronidazole [3], its oral bioavailability is limited by its poor solubility [4]. Several approaches such as cosolvency [4], hydrotropy, surface solid dispersions [5], solid dispersions [5,6] and complexation using β-cyclodextrins [7] have been explored successfully for solubility enhancement of satranidazole.

Satranidazole is not official in any pharmacopoeia. Spectrophotometric [8-12], HPLC [13-21], HPTLC [22,23] methods have been reported for individual and/or simultaneous estimation of satranidazole in pharmaceutical formulations. Bioanalytical methods for estimation of satranidazole in blood [24], rat plasma [25] and golden hamster [26] have been reported. However none of these methods have been applied for bioequivalence studies of pure satranidazole and its novel formulation after oral administration in male Wistar rats.

In the present study satranidazole solid dispersion was formulated by solvent evaporation technique using Plasdone® S 630 [27] as a hydrophilic carrier. The objective of the present study was to apply validated RP-HPLC method for *in vivo* estimation of satranidazole and compare the pharmacokinetic parameters of pure satranidazole and its novel solid dispersion formulation.

MATERIALS AND METHODS

Materials:

Satranidazole (Figure. 1(I)) was generously gifted by Alkem laboratories Ltd, Ankleshwar, India. Tinidazole (Figure.

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1(II)) and Plasdone®S 630 were received as gift samples from Aarti Drugs Ltd, Tarapur, India and Ashland Inc. USA, respectively. Acetonitrile (HPLC-grade) was purchased from Thomas Baker, Mumbai, India. All other chemicals and reagents were purchased from Research-Lab Fine Chem. Industries, Mumbai, India. The control rat plasma was obtained from Animal House, Sinhgad Institute of Pharmacy, Pune, India.

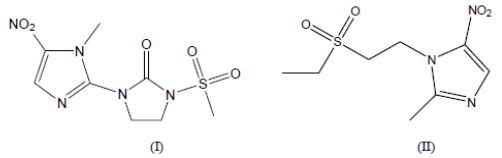

Figure 1 Structure of (I) Satranidazole and (II) Tinidazole (IS)

Chromatographic conditions:

The analysis was carried out on a Shimadzu HPLC system (Kyoto, Japan) which consisted of a binary gradient pump (model LC-20AD), a SPD-M20 diode array detector and a 7725i sample injector. The separation was performed on a Kromasil C_{18} (4.6 mm × 250 mm, 5µ) column. The isocratic mobile phase consisted of phosphate buffer (0.01M, pH 3.5): acetonitrile (65:35, v/v), filtered through a 0.45µm membrane filter and degassed prior to use. The mobile phase was delivered at a flow rate of 1.0 mL/min. The eluate was monitored at 320 nm, the maximal absorption for satranidazole and the same wavelength was found adequate for monitoring IS. The apparatus was interfaced to a DELL PC compatible computer using LC solution software [28].

Preparation of Standard and Quality Control samples:

Standard and quality control (QC) stock solutions of satranidazole and IS were prepared in acetonitrile. Appropriate dilutions of satranidazole were made in acetonitrile to produce working stock solutions in the range 0.5-70 µg/mL. Stock solutions were stored at approximately 4°C. Working stocks were used to prepare plasma calibration standards. A working IS solution (35 µg/mL) was prepared in acetonitrile. Calibration samples were prepared by spiking 200 µL of control rat plasma with appropriate amount of satranidazole and IS on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control rat plasma at appropriate concentrations (1, 25 and 65 µg/mL) and 100 µL aliquots were distributed into different tubes. The samples were stored at −20°C until analysis.

Sample preparation:

To deproteinize the biological sample, 400 µL of acetonitrile [29,30] containing 35µg/mL of Tinidazole (IS) was added to 200 µL of rat plasma sample. After centrifugation for 5 min at 3200 rpm and 4°C using refrigerated centrifuge, the supernatant was transferred to another eppendorf tube and a 20 µL aliquot was directly injected onto the HPLC column.

Validation Procedure:

The validation parameters studied were specificity, linearity, sensitivity, accuracy, precision, recovery and stability. The method was validated according to USFDA guidance for bioanalytical method validation [31].

Calibration curves

The calibration curves were constructed by least square linear regression analysis. The linearity was obtained by plotting the peak area ratio of satranidazole: IS against the nominal concentration of calibration standards.

Extraction recovery

Two sets of standards containing satranidazole and IS at three different QC concentrations (1, 25, and 65 µg/mL) were prepared one with and other without plasma as described above. The recovery was determined by comparing peak areas of spiked plasma extracts with those of unextracted samples prepared in acetonitrile and containing the same amount of analyte that was added to the extracted sample.

Precision and accuracy

The intra and inter precision and accuracy were estimated by analysing five replicates of spiked plasma samples with satranidazole at three different QC concentrations, i.e. 1, 25 and 65 µg/mL at three different times of the same day and three consecutive days respectively. Analytical precision was determined in terms of %RSD, while accuracy was evaluated by comparing the observed concentration with the true value of spiked concentration.

Stability

To ensure the stability of satranidazole in plasma samples during analysis and on storage, QC samples spiked with 1 and 65 µg/mL were subjected to freeze and thaw, short term, and long term stability studies. The samples were thawed by allowing them to stand at room temperature for approximately 2 h. The samples were stored at -20°C between freeze–thaw cycles. The stability of satranidazole was assessed after three freeze–thaw cycles. The short term (bench top) stability was determined by analyzing triplicates of QC samples at ambient temperature (25° ± 3°C) for 4 h. The long-term (freezer) stability of satranidazole in rat plasma was assessed by analyzing the QC samples stored at -20°C for 1 month. The stock solution stability of satranidazole (25 µg/mL) and IS (35 µg/mL) was determined for a period of 5 days on storage at 4° C followed by storage for 6 h at room temperature. The samples were processed and analyzed using the same procedure as described in the sample preparation section.

Pharmacokinetic study:

The experimental protocol was approved by the Institutional Animals Ethics Committee and the study was performed in male Wistar rats (250 – 300 g) according to the guidelines of Committee for The Purpose of Control and Supervision on Experiments on Animals (CPCSEA) for experimental animal care (Protocol approval no. SIOP/IAEC/2012/34) [28]. Rats were housed in standard wire mesh plastic cages in a room maintained at 22 ± 0.5° C and 12h light and 12h dark cycle. Animals were given standard pellet food and water *ad libitum*. The rats were acclimated to the facilities for three weeks and fasted with free access to water for 14h prior to experiment. During experimentation, the rats were divided in three groups with six rats in each group. The control group received orally 1% sodium carboxymethyl cellulose, the vehicle used for satranidazole solid dispersion. The second group was administered pure satranidazole and the third group was administered satranidazole solid dispersion. Pure satranidazole and its solid dispersion were delivered orally as suspension in 1% sodium carboxymethyl cellulose. The suspensions were made homogeneous by sonicating for 5min just before administration. About 0.5ml blood samples were collected via retro-orbital venous plexus of rats in microcentrifuge tubes containing 10 µL of EDTA at designated time points (1, 2, 3, 4, 6, 8, 16, 24h) after administration of drug. The blood samples were transferred to micro-centrifuge tube

and then centrifuged (4500 rpm, 15min at 4°C) to obtain plasma as supernatant layer. 200 µL of plasma was isolated in separate micro-centrifuge tubes without disturbing the buff layer and was refrigerated at -20°C until further analysis. Plasma (200 µL) samples were spiked with 400 µL of IS in acetonitrile (35 µg/mL) and processed as described earlier. The resultant supernatant (20 µL) was injected to HPLC system.

Pharmacokinetic Analysis:

The total area under the plasma concentration – time curve from time zero to the last quantifiable time point (AUC_{0-t}) was calculated using the trapezoidal rule. The area from the last datum point to time infinity ($AUC_{t-\infty}$) was calculated by dividing the last measured plasma concentration by the terminal-phase rate constant (K_{el}). K_{el} was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase. The half-life ($t_{1/2}$) of the terminal elimination phase was obtained using the relationship $t_{1/2} = 0.693/ K_{el}$. The peak plasma concentration (C_{max}) and the corresponding time (t_{max}) were directly read from the experimental data. C_{max} and $AUC_{0-\infty}$ were compared using two tailed unpaired student's *t test* and $p<0.05$ was considered as statistically significant. t_{max} was expressed as median range and comparison between t_{max} of pure satranidazole and solid dispersion was made using Wilcoxon rank sum test.

RESULTS AND DISCUSSION

Specificity and chromatography:

A typical overlaid chromatogram for the control rat plasma (free of analyte and IS, Figure 2a), rat plasma spiked with satranidazole (70 µg/mL) and IS (35 µg/mL, Figure 2b) and *in vivo* plasma sample obtained after oral administration of satranidazole at a dose of 50 mg/kg (Figure.2c) showed specificity, since IS and satranidazole were well resolved and no interfering peaks from endogenous components of normal plasma were observed. The peaks were sharp and symmetrical with good baseline resolution and minimal tailings. The theoretical plates for satranidazole and IS were 3717 and 2712 respectively, tailing factor was found to be 1.82 and resolution between satranidazole and IS was 3.64.The values obtained for these parameters indicated suitability of the system for the proposed study.

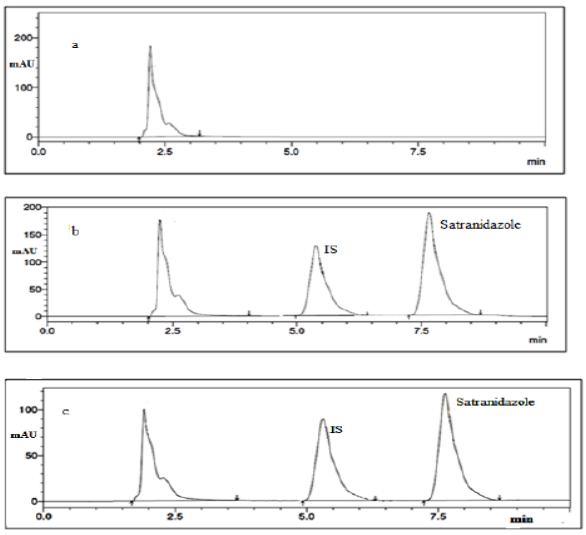

Figure 2 HPLC Chromatograms of 20µL injection of a. blank plasma; b. blank plasma spiked with satranidazole (70µg/mL) and IS (35µg/mL); c. plasma sample at 3h after oral administration of satranidazole at a dose of 50 mg/kg to rats. The retention time of satranidazole and IS were 7.7 min and 5.5min respectively.

Calibration curve:

The calibration ranges were selected based on the expected concentration in study samples to which the method was to be applied. The peak-area ratio (satranidazole : IS) versus satranidazole concentration was found to be linear over the concentration range 0.5 – 70 µg/mL. The linear regression equation of the calibration curve was $y = 0.073x + 0.018$ ($R^2 > 0.999$). The standard curve had a reliable reproducibility over the standard concentrations of the analyte across the calibration range. The lowest concentration with RSD < 20% was taken as LLOQ and was found to be 0.5µg/mL. The RSD at LLOQ was found to be 10.66%.

Extraction recovery:

The extraction recovery of satranidazole at concentrations of 1, 25 and 65 µg/mL is presented (Table 1) The extraction recovery at all the concentrations exceeded 90% indicating high recovery. The RSD values of recovery study were less than 15% indicating consistent, precise and reproducible recovery over the ranges studied.

Table 1 Extraction recovery of satranidazole from rat plasma

Spiked concentration (µg/mL)	Extraction recovery (%)[a]	RSD %[b]
1	95.09 ± 1.00	2.54
25	90.86 ± 2.65	2.01
65	95.43 ± 1.56	2.08

[a] *Values are mean ± S.D (n = 3)*

[b] *RSD% = SD/mean x 100.*

Accuracy and precision:

Accuracy and precision data for intra- and inter-day plasma test samples is presented (Table 2).

Table 2 Intra and inter day precision and accuracy of determination of satranidazole in rat plasma

Spiked Concentration (µg/ml)		Measured concentration (µg/mL)			
	Run	**Mean**	**SD**	**RSD %[a]**	**Accuracy %[b]**
Intra-day variation (three replicates at each concentration)					
1	1	0.97	0.031	3.13	97.86
	2	1.06	0.075	7.06	106.66
	3	1.11	0.107	9.59	111.80
25	1	23.40	0.292	1.25	93.58
	2	25.02	0.558	2.22	100.09
	3	24.91	0.672	2.69	99.66
65	1	64.21	0.891	1.39	98.79
	2	65.32	0.726	0.70	100.50
	3	66.25	0.834	1.27	100.39
Inter-day variation (five replicates at each concentration)					
1		1.02	0.101	9.879	102.08
25		24.84	0.703	2.831	99.37
65		64.23	0.616	0.958	98.83

[a] *RSD= SD/mean x 100*

[b] *Accuracy = Measured concentration /actual concentration x 100*

The intra-day precision (% RSD) was found to be between

3.13- 9.59 at 1µg/mL, between 1.25 –
2.69 at 25 µg/mL and 0.70 – 1.39 %, at 65 µg/mL. The inter-day precision (% RSD) was found to be 9.879, 2.831 and 0.958 % at 1, 25 and 65 µg/mL respectively. The intra-day accuracy (%) ranged from 97.86 – 111.80 % at 1 µg/mL, 93.58 – 100.09% at 25 µg/mL and 98.79 – 100.50 at 65µg/mL. The inter day accuracy (%) was 102.08, 99.37 and 98.83 at 1, 25 and 65µg/mL respectively. The method precision (%RSD) and accuracy at LLOQ were 5.42% and 96.08% respectively (intra day) and 6.49%, %RSD and accuracy of 95.40 % (inter day).

Stability:

The results for freeze and thaw, short term (bench-top), long term (freezer) are shown (Table 3).

The QC samples were found to be stable for 4 h at room temperature and at -20° C for one month. The samples were also found to be stable when subjected to three freeze thaw cycles. The stock solutions of satranidazole (25 µg/mL) and IS (35 µg/mL) were found to be stable when stored for a period of 5 days in refrigerator at 4° C followed by 6 h at room temperature and showed 95.82 % and 92.37 % mean stability for satranidazole (25 µg/mL) and IS (35 µg/mL) respectively. The results for various types of stabilities were found to be within the acceptance limit of 85.00 - 115.00 %. Both accuracy and precision of QC samples during this evaluation were within the assay variability of ± 15%. The proposed method allows determination of satranidazole at concentrations that are good enough for the given dose, with a simple, rapid and efficient sample pre-treatment and short elution time, thus fulfilling all the required criteria for the plasma assay to be considered functional in pharmacokinetic studies.

Application of the analytical method in Pharmacokinetic studies:

After single oral administration of pure satranidazole and satranidazole solid dispersion equivalent to 50 mg/kg satranidazole to male Wistar rats, the plasma concentrations of

Sample Concentration (µg/mL)	Stability condition	Mean ± SD (µg/mL) n=3	Accuracy [a] (%)	Precision[d] (% CV)
1 (LQC)[b]	3 F/T	1.082 ± 0.084	108.17	7.74
	4h(BT)	1.024 ± 0.096	102.43	9.35
	30 days at -20°C	1.076 ± 0.091	107.63	8.47
65(HQC)[c]	3 F/T	65.05 ± 0.160	100.07	0.25
	4h(BT)	64.96 ± 0.388	99.95	0.59
	30 days at -20°C	65.03 ± 0.105	100.05	0.16

Table 3 Stability of satranidazole in rat plasma

[a] Accuracy = mean assayed concentration/actual spiked concentration x 100

[b]Low quality control; [c]High quality control, %CV= Coefficient of variation; F/T: freeze–thaw; BT: benchtop, [d] % CV = SD/mean*100

satranidazole were determined by the described method. The mean plasma concentration versus time profiles for satranidazole (Figure 3) reveals that the newly developed analytical method has the required sensitivity to characterize the absorption, distribution and elimination phases of satranidazole following oral dosing. The pharmacokinetic parameters were calculated using compartmental analysis (table 4). The data revealed significant increase ($p < 0.05$) in C_{max} and $AUC_{0-\infty}$ of satranidazole solid dispersion (17.58 ± 1.66 µg/mL, 145.71 mg-h/L) in comparison to that of pure satranidazole suspension (9.47 ± 1.23 µg/mL, 88.23 mg-h/L). The median t_{max} for pure satranidazole and satranidazole solid dispersion was found to be 3 h and 2.5 h respectively.

Parameters	Pure Satranidazole	Satranidazole solid dispersion
C_{max} [a] (µg/ml)	9.47 ± 1.23	17.58 ± 1.66
$AUC_{0-\infty}$ [b] (mg-h/L)	88.23	145.17
t_{max} [c] (h)	3.0 (2-6)	2.5 (1-4)
K_{el} [d] (h^{-1})	0.08	0.07
$t_{½}$ [e] (h)	8.74	9.55

Table 4 Pharmacokinetic parameters of pure satranidazole and its solid dispersion btained after a single oral administration in rats (50 mg/kg)

[a]C_{max}: Peak plasma concentration, [b]$AUC_{0-\infty}$: area under the curve from the last datum point to time infinity, [c] t_{max} : time required to achieve peak plasma concentration, [d] K_{el} : terminal-phase rate constant, [e] $t_{1/2}$: half-life. C_{max} is expressed as mean ± SD and t_{max} is expressed as median value. The figure in parantheses represents range of values.

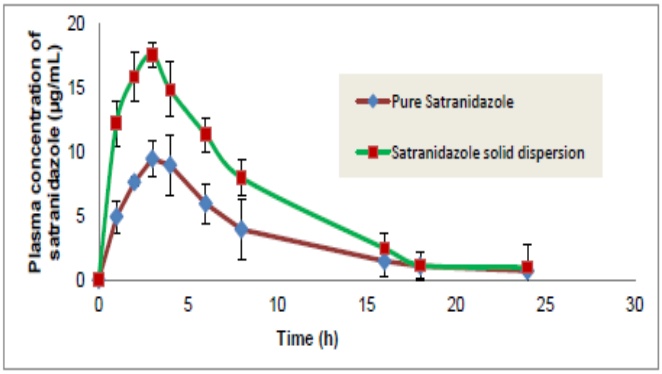

Figure 3 Plasma concentration vs time profiles of pure satranidazole and satranidazole solid dispersion after single oral dose of 50mg/kg in male Wistar rat. Error bars represent SEM of six determination.

CONCLUSION:

Short elution time, baselines with low background and good separation between satranidazole and IS and endogenous substances were accomplished. The developed method is simple, specific, precise, and accurate and is suitable for quantification of satranidazole in plasma samples from bioavailability and pharmacokinetic studies in rats. The method was successful for estimating the increase in the relative bioavailability (164%) and the C_{max} (1.85 folds) of satranidazole solid dispersion as compared to its pure form. The t_{max} values when compared using Wilcoxon rank sum test were found to be similar ($p > 0.05$). Statistical analysis using Students *t-test* ($p< 0.05$) showed significant difference between C_{max} and $AUC_{0-\infty}$ values of satranidazole solid dispersion and pure satranidazole. The method was successfully applied to provide a proof of enhanced bioavailability of satranidazole solid dispersion.

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