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MULTIPLE-DOSE PHARMACOKINETICS OF A NOVEL, SYNTHETIC TRIOXANE-ANTIMALARIAL COMPOUND 97/78 IN RATS USING LC-MS/MS METHOD

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Abstract: Multiple dose pharmacokinetic study was studied in rats following oral dose administration at 47 mg/kg. The pharmacokinetic profile of 97/78 was investigated in form of its completely converted in-vivo active metabolite 97/63 after dose administration. Quantification of metabolite 97/63 in rat plasma was achieved on LC-MS/MS. Chromatographic run time was 4.0 min and the weighted (1/x2) calibration curves were linear over the range 1.56–200ng/ml. After oral administration of 97/78 in rats the peak plasma concentration was observed between 0.75 and 2 h. Compound was rapidly absorbed and declined within 24 h after each dosing. Multiple maximum concentrations were observed at different time points. Mean Residence Time of 97/63 was observed at 5.06±0.40 h. The terminal elimination half-lives did not significantly change for multiple doses (T1/2, 2.65±0.70 h) in comparison to single-dose (T1/2, 2.80±0.56 h) administration. Also there is no enhancement in AUC values during multiple dose administration, indicating no plasma accumulation of 97/63. There were no significant differences between peak and trough concentrations of 97/63 on days 1, 2, 3, 4 and 5. Trough plasma concentration states that the body systemic exposure is high. The average plasma concentration-time profile of 97/63 confirms no plasma accumulation of 97/63.

Key Words: Antimalarial, LC-MS/MS, Multiple-dose, 97/78, Pharmacokinetics

INTRODUCTION

Malaria is one of the most severe and devastating health problems worldwide. Malaria kills over a million each year and some 3.2 billion people living in 107 countries/territories are at risk. But malaria is a curable and preventable disease [1,2]. The increasing incidence of resistance in malaria prevalent areas against classical antimalarials has prompted worldwide research to design and develop new drugs, of ideally different molecular mechanism(s) of action from those against which the malarial parasites have developed resistance. Several semi-synthetic derivatives of artemisinin-the active ingredient of Chinese herb 'qinghao' (*Artemisia annua*) used traditionally for treating fevers-have been used increasingly over the past two decades. Artemisinin and its derivatives are considered to be the most rapidly acting antimalarials till date and are being used clinically worldwide. The endoperoxide sesquiterpene lactone moiety of this class of compounds is found to be indispensable for erythorocytic schizontocidal activity and reacts with intraparasitic heme forming free radicals. These free radicals appear to damage intracellular targets and perform their antimalarial activity [3-5].

Central Drug Research Institute (CDRI) has developed a promising antimalarial compound, 97/78 in its drug discovery program. 97/78 showed good potency against malarial parasites, *in-vitro* as well as *in-vivo* [6]. It posses a 1,2,4-trioxane nucleus similar to endoperoxide lactome of artemisinins, essential for antimalarial activity [7-9]. Initially 97/63 was synthesized as orally active antimalarial agent at CDRI. In preliminary studies it was observed that 97/63 is stable in rat plasma but shows low permeability and low oral bioavailability (unpublished data). This prompted to synthesize a prodrug 97/78 (Fig.1), a hemisuccinate derivative of 97/63, which was soluble in aqueous media and shows greater oral bioavailability than 97/63 (Fig.1). Since the compound 97/78 was spontaneously getting converted into its active metabolite 97/63 in *in-vivo* and *in-vitro* condition, hence it was not possible to estimate 97/78 in test samples and its pharmacokinetic profile of 97/78 was generated in terms of its metabolite 97/63. The present study shows pharmacokinetic behavior of 97/78 in the form of metabolite 97/63 and its plasma accumulation in male rats after multiple dose administration of 97/78 [10, 11]. The study samples were analyzed using Liquid chromatographic tandem mass spectrometric (LC-MS/MS) assay developed for quantification of metabolite 97/63 in rat plasma, having same mass spectrometric condition of monkey plasma [12]. Method validation parameters were determined for metabolite 97/63 but not for the parent 97/78, due to its instability in rat plasma.

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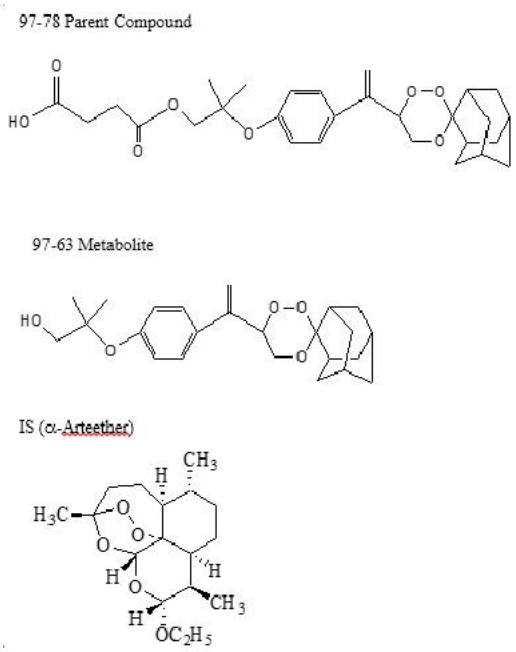
Figure 1: Chemical structure of parent compound 97/78, metabolite 97/63 and IS (α-Arteether).



MATERIALS AND METHODS

Chemicals and reagents

Pure (> 99%) reference standards of 97/78 and metabolite 97/63 were obtained from Medicinal Chemistry Division, CDRI, Lucknow, India. α-Arteether (**Fig. 1**) used as internal standard (IS) was obtained from Themis chemicals Limited (Mumbai, India). Acetonitrile, HPLC grade, was purchased from Thomas Baker (Chemicals) Limited (Mumbai, India). Ammonium acetate and glacial acetic acid AR were purchased from E Merck (India) Limited. Ultra-pure water of 18.2MΩ cm was obtained from a Milli-Q PLUS PF system. Heparin sodium injection I.P. (1000 IU/ml) was purchased from Biologicals E. Limited (Hyderabad, India). Drug free plasma was obtained from male rats obtained from Laboratory Animal Services Division of institute.

Study design

All experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Experimentation Ethics Committee. Studies were conducted in male *Sprague-Dawley* (*SD*) rats with oral administration of 97/78 with four animals. Sparse sampling approach was used for plasma samples in rats.

Dose administration

The oral dose was administered using a 2 ml syringe and feeding needle. Two samples were collected per rat, first by cardiac puncture and second (terminal sample) from inferior venacava [13,14]. After oral dose administration rats were also monitored for physiological behavior.

Formulations

The oral formulation for multiple-dose, 47 mg/kg was prepared in 1% gum acacia. The oral formulation was subjected to quality control (QC) checks to ensure strength and content uniformity prior to use. The strength of formulation for oral administration used was 25 mg/ml [15].

Animals

Young and healthy male *SD* rats weighing 250±25 g were housed in well ventilated cages and kept at room temperature 25±2°C on a regular 12 h light dark cycle. Animals were cared in accordance with the principles of guide for care and use of laboratory animals (Department of health education and welfare, number [NIH] 85-23, revised 1985). Animals were over-night fasted on first day before dose administration to minimize the effects of food on pharmacokinetics of 97/78. After first dose, food and water was given as well as formulation of 97/78 was also administered.

Sample Collection

Sparse sampling approach was used to collect blood samples. The total volume of blood withdrawn within 24 hours through cardiac puncture was less than 5% of blood volume. Blood samples were collected into heparinised tubes (22 IU Heparin per ml of blood) from 0-24 h (0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 18, 24 h) on first day, then after each dose administration and after last dose (5^{th} day) samples were collected up to 96 h post dose. Blood samples were stored on ice until centrifuged (within 30 min). Plasma was separated by centrifugation at 1500 × g for 10 min and stored at -60°C until analysis. Analyses were performed within 30 days of sample collection.

LC–MS/MS instrumentation and assay validation

LC-MS/MS API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Canada) with Analyst 1.4.1 software was used for sample analysis. The mass spectrometer was operated at electrospray ion source (ESI) positive ion mode and the analytes were quantified using multiple reactions monitoring (MRM) mode. Optimized precursor (ammonium adducts of analytes, $M+NH_4^+$) to product ion transitions monitored, *m/z* 518.4→ 173.1; 418→119.1 and 330 → 267 were used for quantification of 97/78, metabolite 97/63 and IS respectively. The Declustering potential (DP), collision energy (CE) were optimized for individual analytes. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada).

HPLC system consisted of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin-Elmer instruments, Norwalk, CT, USA). Mobile phase was delivered isocratically at flow rate 0.45 ml/min. Chromatographic separation was achieved on Columbus C_{18} (50 mm×2 mm i.d., 5 µm particle size) column with guard, (Phenomenax USA). Elution was carried out using acetonitrile: ammonium acetate buffer (pH 4, 10mM) (80:20 %v/v) as mobile phase. The chromatographic run time was 4.0min and was optimized injection volume was 20µL. The analysis was carried out at ambient temperature and pressure of chromatographic system was ~ 600-800 psi.

Calibration standards, quality control samples and test samples were prepared by a simple one step protein precipitation process with acetonitrile. The processing volume of plasma was 100 µL and to it 10µL IS was added to each sample to get final concentration of 4ng/ml followed by vortex mixing for 15 seconds. Protein precipitation of plasma samples were carried out by addition of acetonitrile in order to make the final volume 0.5 ml. The extraction tubes were then vortex mixed for 60 seconds and centrifuged at 12000 × g for 5 min. The clear 200 µL supernatant was transferred to auto-injector vials and injected for analysis.

The assay was validated in rat plasma in terms of accuracy, precision, linearity, sensitivity and selectivity as key parameters. The assay validation was performed for three days by selecting three concentration levels (as low, medium and high) covering the entire expected linearity range in rat plasma.

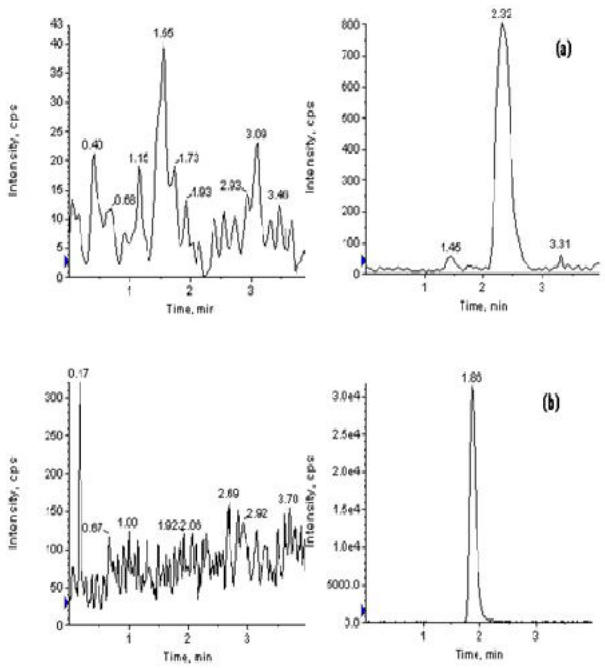
Pharmacokinetics and statistical analysis

Pharmacokinetic parameters included are the maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (T_{max}), terminal elimination half-life ($T_{1/2}$ β), mean residence time (MRT) and area under curve ($AUC_{0-\infty}$). The area under the plasma concentration versus time curve (AUC) for metabolite 97/63 was calculated by the linear trapezoidal rule. The area from the last concentration point (C_{last}) to infinity was calculated as C_{last}/β, where β is the terminal elimination rate constant calculated by regression taking at least three data points in the terminal elimination phase. The terminal elimination half-life ($T_{1/2}$ β) was calculated by 0.693/β. Maximum plasma concentrations (C_{max}) and the time for maximal concentration to be reached (T_{max}) were derived from graphical analysis of plasma concentration versus time. Statistical comparisons of the pharmacokinetic parameters of the metabolite 97/63 in male rats were assessed utilizing ANOVA for paired data. In all tests a probability level of significance was pre-set at α =0.05. Results are expressed as mean ± S.D.

RESULTS

Selectivity, sensitivity, linearity, accuracy, precision were measured and used as the parameters to validate and assess the bioanalytical assay performance [12, 16]. The peak area ratios of analytes with IS in rat plasma were linear with the analyte concentration over range 1.56–200ng/ml. The coefficient of correlation (r^2) for metabolite 97/63 was more than 0.995 over the concentration range used. LC–MS/MS analysis of blank rat plasma samples showed no interference with the quantification of analytes and IS. Representative chromatograms of extracted blank plasma and blank plasma spiked with 97/63 and the IS indicating the specificity and selectivity of the method, are shown in fig. 2.

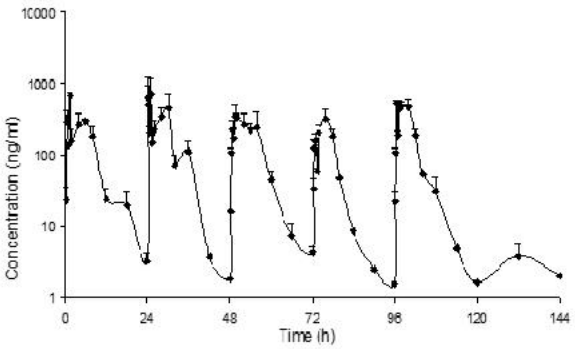
Figure 2: Representative blank and spiked (at 1.56 ng/ml) chromatogram of (a) 97/63 and (b) IS (α-Arteether) at concentration 4 ng/ml in rat plasma.



Accuracy (% bias) and precision (% relative standard deviation) for both intra- and inter-day calculated with five determinations per concentration level for three days (five replicates of low, medium and high QC samples) in rat plasma were within the acceptance limits of ±20% for LLOQ and ±15% at all other concentration levels.

The present study describes the multiple oral doses pharmacokinetics of 97/78 at 47mg/kg. The time course of mean plasma concentrations of 97/78 after multiple dose oral administrations in rats is shown in Fig.3. Given the variability associated with plasma concentrations, only mean values along with their SEM obtained at each sampling time (N= 3) are plotted.

Figure 3: Mean plasma concentration versus time profile of 97/63 following multiple oral administrations of 97/78 in male rats (47mg/kg/day × 5 days)



The validated assay for rat plasma was successfully applied for multiple oral dose pharmacokinetic study in rats. The average plasma-concentration versus time profile of 97/63 after multiple oral-dose administration of 97/78 is illustrated in Fig. 3. After oral administration of 97/78 in rats the peak plasma concentration was observed between 0.75 and 2 h. Concentration-time profile after oral dose shows multiple peaks. The oral concentration time profile in rats at all dose level studied was characterized by presence of multiple peaks. The irregular concentration-time profile after oral dose could not be explained by any standard compartment models. Hence non-compartmental approach was used to fit the data. Multiple peaks after oral administration may be due to rapid *in-vivo* conversion to metabolite and also with varying rate of conversion in individual rats. The compound was rapidly absorbed and declined up to 24 h after each dosing. Multiple T_{max} appeared at 1.1±0.57, 24.75±0.57, 50.75±1.76, 75.33±1.15 and 97.12±0.53 h and their corresponding C_{max} values were 660.3±142.1, 704.67±457.9, 532.3±141.9, 363.0±161.18 and 636.3±97.16ng/ml respectively after each dosing. Terminal (elimination) half-lives ($T_{1/2}$) and MRT of 97/63 were observed at 2.65±0.70 h and 5.06±0.40 h respectively after last dose administration. The AUC_{0-t} values of 97/63, after each dosing are shown in Table 1.

DISCUSSION

Validated LC-MS/MS method for simultaneous quantitation of both 97/78 (parent) and 97/63 (metabolite) over the concentration range 1.56–200ng/ml was considered but since 97/78 was found to be unstable in rat plasma, so validation parameters were determined for metabolite 97/63 only [12]. Validated method was applied for quantitation of 97/63 in plasma samples after multiple dose administration of 97/78. Parent 97/78 was completely converted to metabolite 97/63 after oral dose administration.

The average plasma concentration versus time profile of 97/63 in male rats after multiple dose administration (Fig.3) confirms no plasma accumulation of 97/63. The compound 97/78 was rapidly absorbed after each dosing which gets converted into metabolite 97/63 and was eliminated from body up to next 24 h. The terminal half-lives did not significantly change upon repetitive dosing in case of multiple doses ($T_{1/2}$, 2.65±0.70 h) and with single-dose ($T_{1/2}$, 2.80±0.56 h) administration. Also there is no enhancement in AUC values during multiple dose administration, indicating no plasma accumulation of 97/63. There were no significant differences between peak and trough concentrations of 97/63 on days 1, 2, 3, 4 and 5 (Fig.3 and Table 1). In all the cases percentage extrapolation from the last measured time point to infinity (i.e. percent extrapolation between AUC_{0-t} and $AUC_{0-\infty}$) was less than 5%.

Table 1: Comparative Pharmacokinetic parameter for 97/63 after multiple and single oral administration of 97/78 at 47 mg/kg dose to male rats (mean ± SD) N=3.

PK Parameter	Days	Multiple-Dose	*Single-Dose*
C_{max} (ng/ml)	D1	660.33 ± 142.1	*1986.6±525.4*
	D2	704.67 ± 457.9	**
	D3	532.33 ± 141.9	**
	D4	363.00±161.18	**
	D5	636.33±97.16	**
T_{max} (h)	D1	1.1±0.57	*0.92±0.52*
	D2	24.75±0.57	**
	D3	50.75±1.76	**
	D4	75.33±1.15	**
	D5	97.12±0.53	**
$T_{1/2}$ el (h)	#	2.65±0.70	*2.80±0.56*
$AUC_{0-\infty}$ (ng h/ml)	#	12801.33±2314.73	*4669.98±59.3*
AUC_{0-24} (ng h/ml)	D1	2660.03±863.2	*4652.76±68.4*
AUC_{24-48} (ng h/ml)	D2	3378.50±356.5	**
AUC_{48-72} (ng h/ml)	D3	2726.48±377.61	**
AUC_{72-96} (ng h/ml)	D4	1608.77±309.28	**
AUC_{96-120} (ng h/ml)	D5	2825.82±805.5	**
MRT (h)	#	5.06±0.40	*4.2±0.63*

after last dose administration

** not applicable

The presence of more than one plasma peak (multiple peaks) indicates that enterohepatic circulation may play an important role in the pharmacokinetics of 97/78 in rats. Generally the mechanisms proposed to explain multiple peak phenomena include enterohepatic recycling (EHC), selective and differential absorption from gastrointestinal tract (GIT) and variations in gastrointestinal motility and gastric emptying [17,18,19].

CONCLUSION

Pharmacokinetic estimation in terms of elimination half-life ($T_{1/2}$), C_{max} and AUC after multiple oral administrations were found to be significantly different in male rats. No relevant accumulation of

metabolite 97/63 was demonstrated during once daily multiple dosing. These results are in agreement with the terminal half-life of approximately 3 h in multiple-doses (47 mg/kg) of 97/78 in present study.

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