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Hepatic Metabolic Studies of 1, 2, 4-Trioxanes Derivative: A New Antimalarial

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Abstract

Trioxanes are the new antimalarial series of potent compound having the capability to harness the over growing menace of malaria and its acquisition of resistance. In this manuscript the authors have presented results of the metabolic studies of a potent trioxane series of antimalarial compound, CDRI-97/78, developed at Central Drug Research Institute. About 75% of the CDRI-97/78 was metabolised to CDRI-97/63. Further metabolites of CDRI-97/63 were not noticed. The metabolism of CDRI compound 97/78 and 97/63 was mainly in the microsomal fraction only a small fraction can be attributed towards the cytosolic fraction, thus reflecting the prominence of Phase 1 reactions during metabolism. The metabolic reaction as depicted by the kinetic parameters show that the clearance of CDRI- 97/78 is about twice more in S9 fraction as compared to the microsomal fraction. Thus pointing towards further metabolism of the CDRI- 97/63, also the nonsignificant change observed in case of the V_{max} and velocity in S9 and microsomal fraction depict the that majorily one isoform of cytochrome P-450 is involved in the metabolism of CDRI-97/78. Ketoconazole (CYP3A2 inhibitor) and Aminobenzotriazole completely inhibited the rate of reaction depicting the involvement of Phase 1 reactions particularly CYTOCHROME P450 3A2 isoform; whereas, verapamil (CYP2E1); cimetidine (CYP1A2); omeprazole (CYP2C19); quinidine (CYP2D6); sulphaphenazole and isoniazid (CYP2C9) did not affected the rate of reaction.

ABBREVIATIONS

CYP450: Cytochrome P-450; CO: Carbon monoxide; Inhibitors; Antimalarial; Trioxane derivatives.

INTRODUCTION

The roll-back /multidrug resistance in malaria has necessitated discovering novel compounds with pronounced antimalarial action. The recent armour is Artemisinin isolated from *Artemisia annua* [1]. Due to fear of endanger and scarcity of its natural habitat by increasing use, malariologist are committed to make the synthetic analogues/derivatives of its active pharmacophore having antimalarial property [2].

While trying to identify a good active candidate drug for development, it is important that it has a suitable bio-availability, metabolic stability and duration of action. This is being done as a part of preclinical absorption-distribution-metabolism-excretion (ADME) studies or Pharmacokinetic studies [3].

A good preclinical study shall provide tremendous information about the drug molecule and also provide areas where researchers

need to invest time and efforts for developing a formulation that has maximum benefit with minimum risk to human beings. Central Drug Research Institute, now-after mentioned as CDRI has synthesized a trioxane compound 97/78 [4], as a substitute to the natural occurring artemisinin (Figure 1). The compound exhibited significant antimalarial activity in pre-clinical studies and exhibit safe profile in regulatory pharmacology and systemic toxicity in rodent model. CDRI-97/78, a fully synthetic 1, 2, 4 trioxane derivative has been identified for development as a viable alternative to artemisinine derivatives for use against drug resistant *P. falciparum* and cerebral malaria cases. The compound has successfully completed preclinical efficacy and regulatory tests and Phase I clinical trials are in progress.

The present manuscript describes the detailed metabolism of an important synthetic trioxane molecule, CDRI-97/78 and its metabolite CDRI-97/63 in hepatic microsomal S9 fraction from *Sprague Dawley* rats. The molecule has shown promising properties to be a potential, highly potent anti-malarial drug molecule: viz good solubility and metabolic stability.

MATERIALS AND METHODS

Materials

H_2SO4 was from E. Merck Ltd., Mumbai, India; Formic acid from Analytical Rasayan, S.D. Fine Chemicals Ltd. and KOH from Ranbaxy Laboratories Ltd. India.

Preparation of hepatic subcellular fraction

Liver tissues were isolated from healthy male *Sprague Dawley* rats and washed thoroughly in ice cold K_2HPO_4-KCl buffer to remove the blood and homogenized. The homogenate was centrifuged at 9000 g for 20 min at 4°C to obtain the supernatant as S9 fraction or post-mitochondrial fraction. S9 preparation was centrifuged at 105000 g for 1 hr to procure microsomal pellet [5]. The pellet was suspended in the K_2HPO_4-KCl buffer and termed as the microsomal preparation. The supernatant thus obtained comprised of the cytosolic preparation. Protein content of all of these fractions was measured by Hartee-Lowry method [6]. Subcellular purity assessment was conducted as mentioned before [5].

Compounds were incubated at 250, 500 and 1000 ng at 37^{0}C with 2.5 or 5.0 mg/ml protein of the S9 or microsomal or cytosolic fraction with or without inhibitors in a final volume of 1.0 ml. The reaction was initiated by the addition of the NADPH generating system. At 0 min and after 10, 15, 30, 45, 60 and 120 min, 0.1 ml of the assay mixture was taken out and mixed in 0.3 ml ice-cold acetonitrile, vortex mixed and kept at 4^{0}C for 30 min for complete protein precipitation in eppendorf tubes. At the time of HPLC analyses, the samples were centrifuged at 10000 g for 10 min and the clear supernatant was injected to HPLC for analyses.

HPLC Method development and Validation

The HPLC system used was Shimadzu with Class vp Software. The mobile phase constituted of Acetonitrile (ACN) and Triple distilled water (TDW) in the ratio 85:15. The flow rate was set at 0.8 ml/min and Λ_{max}: 254 (Shimadzu, UV-detector, SPD-10Avp). Phenomenex, Ultracarb (Reversed Phase Column), 100*4.6 mm, 5 µ pore size. column was used along-with the guard column of 5µ. A sample volume of 50 µl, gave Retention Time (RT) of 4.5 min for CDRI-97/78 and 5.5 min for its possible metabolite 97/63. Method development and validation was conducted as per FDA guidelines [7,8], for their linearity, range, specificity, sensitivity, limit of quantitation (LOQ), limit of detection (LOD), accuracy, precision, extraction recovery, stability, and robustness of the method in pure ACN as well as in calibration samples. A calibration range of 4000-62.5 ng/ml was finalized for CDRI -97/78 and 97/63 with r^2= 0.9959 and 0.9986 respectively.

Hepatic metabolic studies

Enzyme cofactor solution (1.7 mg NADP monosodium salt, 7.8 mg glucose 6 phosphate monosodium salt, 6 unit glucose 6 phosphate in 0.2 M PBS) was mixed with S9 fraction at 1:5 ratio and incubated with test compound (4 µg/ml) for 60-120 min. The reaction was stopped by protein precipitation, adding Acetonitrile (ACN) (1:3) to the above solution. The reaction mixture was centrifuged at 10,000 rpm for 10 min and the clear supernatant obtained was analyzed through HPLC. % of remaining of CDRI-97/78 and CDRI-97/63 was observed. Also, the various reaction kinetics parameters like half-life ($t_{1/2}$), Michaelis Constant (K_m), Clearance rate (Cl), Vmax and order of the reaction were monitored.

Determination of CYP 450 activity in hepatic S9 of normal rat

The microsomal fraction containing, 1-5 mg/ml of protein

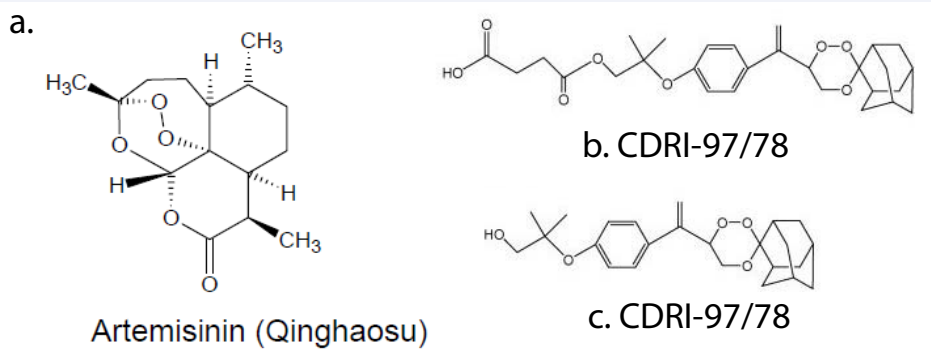

Figure 1 Structure of a. Artemisinin (plant product), b. CDRI-97/78 and c. CDRI-97/63.

was taken and to this Sodium-di-thionate, 5mg (Sigma Chemicals Co., U.S.A.) was added. This was analyzed against untreated microsomal fraction using UV-spectrophotometer (Thermo Electron Corporation, Evolution 500; EV125001). A sharp peak was observed at 412nm, denoting CYP b5. After bubbling for 30 sec with Carbon Monoxide (CO), a sharp peak was observed at 450 nm [9]. CO was generated during reaction of concentrated H_2SO_4 and formic acid and thereafter passed through saturated solution of KOH. Different concentrations of CDRI-97/78 were incubated with the S9 fraction of the liver for 10 min, and the spectra of the reduced form of the pigment obtained. % decrease in CYP 450 activity was calculated using the following formula [10].

Specific activity of CYP= OD (500-400 nm) * Dilution Factor *1000/ protein (mg/ml)* Ex. Coeff.^^91 $mM^{-1}cm^{-1}$

The activity thus obtained was plotted against different concentration of drug and the point of 50% activity inhibition was taken as the IC_{50} value.

CYP isoforms inhibition

Final concentrations of 50 or 25 µM were used in case of different inhibitors. However, for Ketoconazole, a 100 and 50 µM concentration were chosen, based on its high solubility and toxicity. The weighed compounds were dissolved in DMSO and vacuum dried for 30 min. S9 (10%) containing the co-factor solution (1:5 ratio) was used. Samples were incubated in shaking water bath at 37°C and solution was withdrawn every 15 min till 1 hr. The reaction was stopped using ACN and analyzed by HPLC-PDA under the optimized HPLC conditions. The metabolites' peaks obtained in the process were also analyzed. Inhibition was calculated on the basis of K, $t_{1/2,}$ dc/dt at t= 30 min. CDRI-97/78 was found to follow first-order kinetics; therefore, log C vs t was plotted. The slope of the plot was used to determine the K (K=-slope/2.303) and $t_{1/2}$= 0.693/K. The dc/dt (at t= 30 min) = rate of reaction at t= 30 min = $-KC_{30}$. Here C_{30}= concentration remaining at t= 30min.

Finally, % inhibition on the basis of K =

$$\frac{\left(K_{\text{without inhibitor}} - K_{\text{with inhibitor}}\right)}{K_{\text{without inhibitor}}} \times 100$$

Similarly, % inhibition on the basis of $t_{1/2}$ and dc/dt for t= 30 min were calculated.

Metabolite identification

A Perkin Elmer Series 200 HPLC system (Perkin-Elmer, USA) consisting of flow control valve, vacuum degasser, pump and autosampler was used to deliver mobile phase. The mobile phase conditions were similar to HPLC conditions; API-4000 LC-MS-MS (Applied Biosystems /MDS SCIEX, Toronto, Canada) mass spectrophotometer was operated with standard ESI source coupled with a LC separation system. The software used was Analyst 1.4.2 (Applied Biosystems/MDS SCIEX, Toronto, Canada). For optimization of MS parameters, approximately equimolar solutions of each analyte were prepared in ACN. The Zero air (GS1, 25 psi) was used as nebulizing gas. The declustering potential was 50V (ion spray voltage, nebulizing and curtain gas conditions were used in default mode) for a dwell time of 0.2 s and mass width of ±10 amu, performed in positive ion mode (based on structures). Nitrogen was used as the collision gas at 30 eV of collision energies (CE). The CE optimization for the precursor to product ions transition was obtained by CE ramping by direct infusion. Sample injection volume was 20 µl.

Pure analytical standards of CDRI- 97/78 and 97/63, S9 samples (blank, CDRI- 97/78 and 97/63 were incubated with S9 fractions and samples taken at 0 and 60 min by giving 10 consecutive injections in HPLC and collecting the various fractions for the peak of RT at 4-5 min and 5-6 min in separate tubes labelled CDRI-97/78 and CDRI-97/63 respectively. These samples were vacuum dried in HETO vacuum drier, reconstituted in ACN, filtered through Millipore filter and thereafter injected in LC-MS/MS for analysis. The results were studied for mol. wt. and fragmentation pattern of any sample with respect to that of analytical standards.

RESULTS AND DISCUSSION

Irrespective of the malaria having a history of 4000 years, it is still a major challenge for human health [11]. There is always a growing demand of new antimalarials [12]. Antorotane (OZ277) is a leading entity of trioxane class of antimalarial being recently on Phase II clinical trials [13]. CDRI-97/78 and 97/63 are new trioxane class of antimalarial compounds; therefore, studies were conducted to know its metabolic stability profile in the hepatic tissue and involvement of specific CYP450 isoforms in the metabolism through the aid of specific inhibitors/inducers of CYP450.

The % of parent compound disappearance in different liver fractions (S9, microsomal and cytosolic) was compared to determine the subcellular fraction and the Phase(s) of metabolism in which CDRI-97/78 and CDRI-97/63 have appreciable degradation and maximum stability (Table 1).

The reaction was first-order in all the three subcellular fractions; the $t_{1/2}$ of CDRI- 97/63 was 150, 100 and 94 min and that of CDRI-97/78 was 10, 376 and 10 min in microsomal, cytosolic and S9 fraction respectively. In other words metabolism of CDRI-97/63 was almost alike and quite slow in all the three matrices whereas, CDRI-97/78 was mostly metabolized by Phase 1 reactions.

Further the data was subjected to double reciprocal method (Line-weaver Burk plot [14,15] to determine the Michaelis constant (K_m) as well as maximum rate of drug degradation (V_{max}) and Clearance rate (Cl) for the entire time course of the reaction as per below mentioned reaction:

$$\frac{1}{v} = \frac{1}{V\max}\left(\frac{Km}{V\max}\right) * \left(\frac{1}{s}\right)$$

Table 1: Metabolic Stability of CDRI-97/78 and CDRI-97/63 in different fractions of rat liver.

Matrices	CDRI-97/78		CDRI-97/63	
	% Remaining after 60 min	Half-life (min)	% Remaining after 60 min	Half-life (min)
Cytosol	88.38	376.14	92.00	100.30
Microsomes	1.48	9.80	74.01	150.46
S9	1.15	9.32	64.63	94.04

Here, v = rate of degradation and s = concentration. Figure 2 and Table 2, gives a complete picture of this equation.

The results were also analyzed for time dependent, % decrease in CDRI-97/78 and CDRI-97/63 levels (Table 3, Figure 3-5). As a metabolite of CDRI-97/78, % increase in concentration of CDRI-97/63 was monitored. CDRI-97/78 was found to be metabolized primarily in the microsomal fraction.

Titration was conducted to find suitable S9 and cofactor solution amount in which CDRI-97/78 concentration could be noticed even at 30 minutes. The results obtained showed a concomitant increase/appearance of the metabolite (at 5.5 min) in the 10% S9 fraction with time and with the decrease in the parent drug (Figure 3). An additional peak at RT similar to CDRI-97/63 was observed (Table 1, Figure 1-5).

Degradation of CDRI-97/78 by 11.6% in cytosol against 98.5% in microsomes indicate that the main pathway metabolism is through Phase I enzymes. However, on the contrary, CDRI-97/63 concentration was almost the same in 3 fractions even after 1hour, without any definitive kinetics. The reason behind this might be speculated that the concentration of CDRI-97/63 was saturable in 45 min. Also, it's metabolite was non-detectable under applied conditions. It was possible that the polar metabolite being formed, eluting in the initial of each run.

From Line-weaver Burk plot, the maximum rate at which enzyme(s) will degrade the CDRI-97/78 was 1.11 µg/ml. In other words, it suggests that 0.08 ml of blood reaching liver gets cleared of the drug per minute. This was important as this can further help to know the hepatic toxicity level of the drug.

Metabolite Identification: The LC-MS/MS spectra obtained indicates that CDRI-97/78 (m/z = 500) metabolizes to CDRI-97/63 (m/z = 400). The spectra revealed that CDRI-97/78 is degrading into CDRI-97/63. It could also be concluded from the data that CDRI-97/63 was further metabolizing but the identity of that metabolite arising from CDRI-97/63 could not be conclusively ascertained with this data.

Efforts were put as a small experiment to know what drug does to liver enzymes. Here, effect of CDRI-97/78 and CDRI-97/63 on CYP450 enzymes was studied. A linear decrease in the activity of CYP450 was observed and IC_{50} was calculated. This is an important parameter to define the dose of the drug within safety limits. The results obtained showed that, there was a concomitant decrease in the specific activity of the hepatic cytochrome P-450 with the rise in the drug concentration. Higher % reduction in the activity (with respect to control) of CYP450 was noticed when higher concentration of drug was spiked. It was observed that the compound caused a concentration

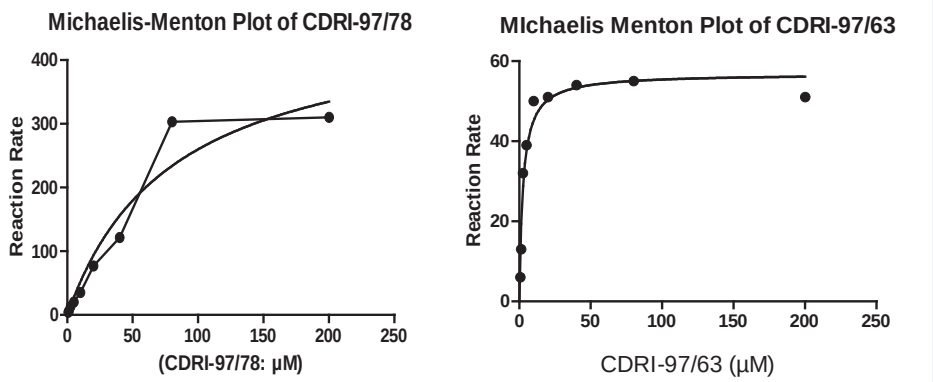

Figure 2 Kinetics of Metabolic Reaction of CDRI-97/78 and CDRI-97/63.

Table 2: Kinetics of CDRI-97/78 and CDRI-97/63 in different fractions of rat liver.

Parameters	S9 Fraction		Microsomal Fraction	
	CDRI-97/78	CDRI-97/63	CDRI-97/78	CDRI-97/63
Km (µM)	259.43	56.79	81	2.6
Vmax (µmoles/min/mg protein)	1.45	0.55	470	57
Clint (ml/min)	0.050	0.009	0.030	0.008

Table 3: Metabolic Stability of CDRI-97/78 in different subcellular fractions of rat liver and appearance of metabolite (CDRI-97/63).

Time (min)	S9		Microsomes		Cytosol	
	CDRI-97/78	CDRI-97/63	CDRI-97/78	CDRI-97/63	CDRI-97/78	CDRI-97/63
0	100	7.94	100	4.092	100	4.83
10	47.52	39.95	45.15	73.54	96.13	41.33
15	32.77	52.87	9.86	73.72	94.24	60.46
30	10.74	59.16	5.15	82.68	92.37	88.84
45	3.51	100	1.88	100	90.55	100
60	1.15	64.63	1.48	74.01	88.38	92.00

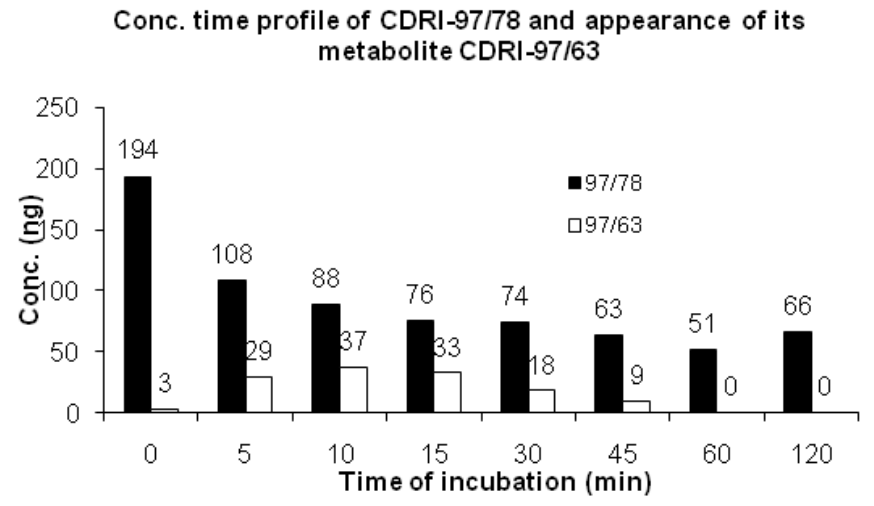

Figure 3 Concentration-time profile of CDRI-97/78 metabolism and appearance of CDRI-97/63.

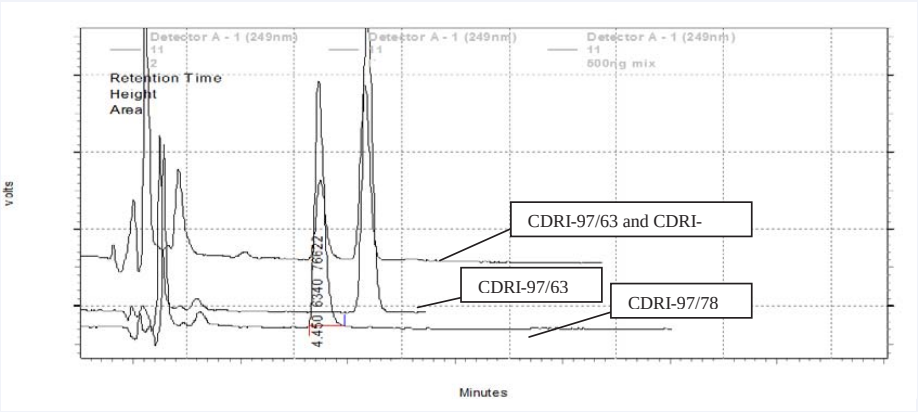

Figure 4 Simultaneous chromatography of CDRI-97/78 and 97/63 (chromatogram 3) for CDRI - 97/78 (chromatogram 1) and 97/63 (chromatogram 2).

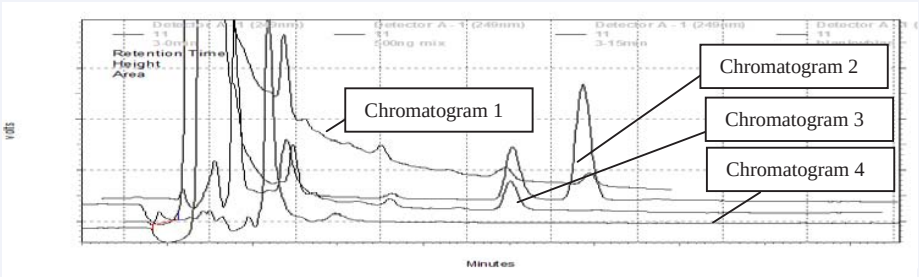

Figure 5 Representative chromatograms of CDRI-97/78 metabolism by S9 fraction of rat liver 1- after 15 minutes of incubation;2- standard of CDRI-97/78 and 97/63; 3-CDRI-97/78 at 0 min of incubation; 4-blank.

dependent inhibition in the activity of hepatic cytochrome P-450. The IC_{50} value was 17.12 µg/ml for CDRI-97/78 and 18.99 µg/ml for CDRI-97/63 (Figure 6).

The main pathway for degradation of CDRI-97/78 is likely to be through CYP3A4. This enzyme is responsible for hydroxylation of parent compound. Thus, CDRI-97/78 is likely to be converted to a metabolite CDRI-97/63. (As observed when samples containing CDRI-97/78 were allowed to degrade). The results when analyzed using LC-MS/MS technique, showed that the metabolite obtained has a structure similar to CDRI-97/63.

Secondly, this study gave us an important clue to increase the efficacy of the drug, within safety limits. This was possible, as

CDRI-97/78 can be given with any drug inhibiting CYP3A4. The latter will cause a decrease in the degradation rate of the CDRI-97/78 and hence will increase it's stability ($t_{1/2}$ will increase). Thus, same amount of drug will show it's effect for longer duration.

A decreased rate of reaction was observed with ketoconazole, (specific inhibitor of 2C19, 3A4) and erythromycin (specific inhibitor of 3A4) [16,17]. With verapamil (specific inhibitor of 1A2) and Quinidine (specific inhibitor of 2D6), not much difference in rate of reaction was observed at different concentrations. However, no concentration dependent profile was observed with Troleandomycin and chlorpromazine, hence the data had not been included in the (Table 4).

CONCLUSION

The results imply that CDRI-97/78 metabolizes at a faster rate. The metabolism of the compound is mainly by phase I of drug metabolism. The metabolism of the drug results in formation of only one metabolite observable under the conditions, we have used. Although possibility for at least one more metabolite (much more polar than both CDRI-97/78 -and CDRI-97/63 being observed) exists. This phase-I metabolism is occurring in microsomal fraction of the liver and the metabolite being formed during this metabolism is surprisingly showing more of non-polar nature. The degradation of this metabolite with time is indicating formation of one more metabolite, not observed under specified conditions. This is possibly more polar in nature, and is thus eluting in the very early phase with the protein portion of the Liver fraction (not resolved). The metabolism of CDRI-97/78 was completely inhibited by CYP3A4 specific inhibitors. Since 70% of the CYP450 is in the form of CYP3A4, this explains the reason behind the very rapid metabolism of CDRI-97/78 and its low bioavailability. This also explains, the reason for drug metabolism in microsomal fraction, as most of CYP's are located in microsomes only. It is therefore suggested that the inhibitor of CYP3A4 can be supplemented as an add-on to increase the bioavailability of CDRI-97/78.

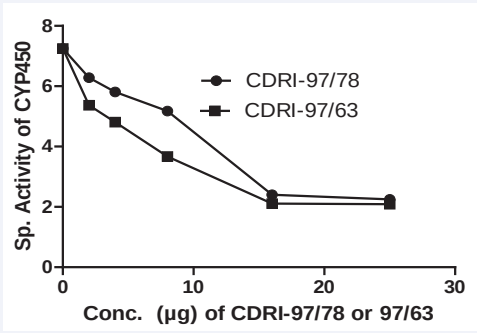

Figure 6 Inhibition of CYP450 by CDRI-97/78 and CDRI-97/63.

Table 4: Comparative data of inhibition for CDRI-97/78.

Inhibitor		% Inhibition (On basis of following parameters)		
Name	Intial Conc (µM)	K	T-1/2 (half-life)	dc/dt at t= 30min
Ketoconazole	K(100)	15.28	18.03	13.81
	K(50)	2.99	3.08	0.89
Erythromycin	E(50)	21.93	28.08	27.10
	E(25)	5.05	5.315	0.67
Verapamil	V(50)	33.22	49.75	13.14
	V(25)	12.62	14.44	15.91
Sulphaphenazole	S(50)	34.885	53.57	37.3
	S(25)	4.98	5.24	0.42
Quinidine	Q(50)	35.88	55.95	52.56
	Q(25)	11.29	12.73	46.47

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