Journal of Drug Metabolism and Toxicology

Srivastava et al., J Drug Metab Toxicol 2013, 4:5
DOI: 10.4172/2157-7609.1000157

Research Article Open Access

Pharmacokinetics, Metabolism and Excretion of Hypolipidemic DRUG: 16 -Dehydropregnenolone

Pratima Srivastava[1]*, Neha Mathur[1] and Ashim Ghatak[2]

[1]*Division of Pharmacokinetics and Drug Metabolism and CSIR-Central Drug Research Institute Sector 10, Jankipuram Extension, Sitapur Road, Lucknow-226031, UP, India*
[2]*Clinical and Experimental Medicine Division, CSIR-Central Drug Research Institute Sector 10, Jankipuram Extension, Sitapur Road, Lucknow-226031, UP, India*

Abstract

Purpose: 16-dehydropregnenolone/DHP/CDRI-80/574, a new orally effective hypolipidemic agent presently in advanced clinical trials. This paper aims to study the absorption, distribution, metabolism and excretion profiles in order to delineate the possible cause(s) behind low bioavailability of CDRI-80/574 and also strategy to enhance the same.

Methods: Metabolic, Excretion and Pharmacokinetics studies were conducted in the Sprague-Dawley rats.

Results: CDRI-80/574 exhibited log D: 4.5, absorption-half-life of 18 min and -constant 0.04/min by closed loop intestinal model in rats, was stable in gastric juice and 30% of RBCs transport/uptake was noted. CDRI-80/574 undergoes rapid metabolism in rat hepatic microsomal fractions; however extra hepatic tissues (intestinal-wall and -microvilli), showed less metabolism. IC_{50} of CDRI-80/574 was 2.22 nM, K_m=15.8 nM, $V_{max=}$0.46 nmoles/mg protein/min, $t_{1/2}$ was 3 min in microsomes. CDRI-80/574 was comparatively more stable in human hepatocytes ($t_{1/2=}$8.7 min) than rats ($t_{1/2=}$4.6 min). Aminobenzotriazole, ketoconazole, diltiazem and grapefruit juice inhibited CDRI-80/574 metabolism, whereas, its metabolism was induced by phenobarbitone. Epoxy metabolite of CDRI-80/574 was synthesized and characterized. About 15% of CDRI-80/574 and 4 metabolites (hydrolysis/hydroxylation; m/z 317/319/321/330) were detected in feces. CDRI–80/574 was 7% bioavailable and exposure increased (2 fold) by grapefruit juice. Human CL_{invivo} was predicted to be 2.7 L/h/kg (45.83 ml/min/kg) by allometric scaling.

Conclusion: CDRI-80/574 is the first hypolipidemic, synthetic compound of pregnane series, hence it is important to elucidate the major reason for its low bioavailability. The compound was fast metabolized by CYP3A4. Further the inhibitors of CYP3A4 were able to increase the bioavailability of CDRI-80/574.

Keywords: CDRI-80/574; Hypolipidemic agent; CYP3A4; Pharmacokinetics studies

Introduction

CDRI–80/574 (DHP-Figure 1) is an effective and orally active antilipidemic compound; currently it is in phase III clinical trial [1]. CDRI–80/574 decreases the LDL level in plasma resulting in hypolipidemic effect. It has good therapeutic window with no side effect [2-3]. Reports exist that Guggulsterone and CDRI-80-574 (pregnane compounds), lower lipid profile through antagonism of Bile Acid Receptor [4-5]. CDRI-80/574 due to its unique mode of action is less prone to cause drug-drug interactions.

It is evident that new drug development involves a series of developmental and evaluative steps to be carried out before getting the approval, involving pre-clinical research and development and four phases of clinical trials [6]. The purpose of pre-clinical pharmacokinetic and metabolism studies is to obtain information, which is useful for efficacy, safety and toxicity evaluation in experimental animals and in man [7-9]. Detailed pharmacokinetics aspects of CDRI-80-574 have not been in public domain. This study reports the PADMET (physiochemical characterization, absorption, metabolism, excretion and transporter) characterization of CDRI-80-574 in one of the preclinical pharmacological-toxicological relevant species, rat and its scaling to human.

Preliminary data showed that CDRI-80/574 has low bioavailability in systemic circulation (in the present study also); therefore, it was important to understand the possible reason behind the same. Studies were conducted to elucidate the important physicochemical properties, metabolic profile of CDRI-80/574 in the hepatic and extra hepatic (intestinal-wall, -microvilli) tissues of *Sprague-Dawley* rats as well as microbes, rat and human hepatocytes. Allometric scaling was conducted to predict the clearance in human. To know the involvement of specific cytochrome P450 isoforms in the metabolism of CDRI-80/574, experiments were conducted using specific inhibitors (*in vitro/in vivo*)/inducers. Excretion studies were conducted in feces,

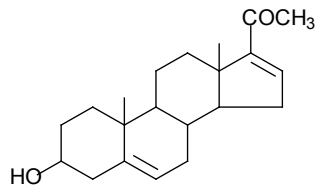


Figure 1: CDRI-80/574- 3 β-hydroxy-5, 16 pregnadiene-20-ones (DHP).

***Corresponding author:** Pratima Srivastava, Division of Pharmacokinetics and Drug Metabolism and CSIR-Central Drug Research Institute Sector 10, Jankipuram Extension, Sitapur Road, Lucknow-226031, UP, India, Tel: 919000232335; E-mail: pratimacdri@rediffmail.com

Received August 29, 2013; **Accepted** October 26, 2013; **Published** October 28, 2013

Citation: Srivastava P, Mathur N, Ghatak A (2013) Pharmacokinetics, Metabolism and Excretion of Hypolipidemic DRUG: 16 -Dehydropregnenolone. J Drug Metab Toxicol 4: 157. doi:10.4172/2157-7609.1000157

urine and bile samples. Pharmacokinetics studies were conducted with and without grapefruit juice administration. Further, major metabolite of CDRI- 80/574 i.e., its epoxy derivative was synthesized and matched with the *in vitro* and *in vivo* samples.

Material and Methods

Chemicals and reagents

CDRI-80/574 was procured from Pharmaceutical Division of Institute, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, phenobarbitone, $MgCl_2$, aminobenzotriazole, ketoconazole, diltiazem, floxetine, quinidine, sulfaphenazole, chlorozoxazone, coumarin, isoniazid, cimitidine from Sigma -Aldrich, Co. Ltd, USA. Cryopreserved human hepatocytes were procured from *in vitro* Technologies Inc. (Baltimore, MD). All the other reagents used were of the highest purity HPLC grade.

***In vivo* Experiments:** For all the *in vivo* study the protocol were reviewed and approved by the Institutional Animal Ethics Committee (IAEC).

Adult healthy male *Sprague-Dawley* rats, body wt 250 ± 25 g, were obtained from the animal house of Central Drug Research Institute (Lucknow, India). They were kept in plastic cages with clean bedding and allowed free access to food (standard pellet food from Goldmohar laboratory animal feed, Lipton India Ltd, Chandigarh) and water. Animals were maintained and monitored for good health in accordance with Test Facility SOPs and at the discretion of the laboratory animal veterinarian. Rats were acclimatized for three days prior to the study where they were maintained on an environmental controls for the animal room will be set to maintain a temperature of 22 to 25°C, humidity of 40-70% RH, and a 12-hour light/12-hour dark cycle.

Treatment Groups: Number of rats per study was 3.

Group 1: CDRI-80/574 was triturated in 0.5% carboxy-methyl cellulose and aqueous suspension administered at the dose of 72 and 18 mg/kg b.wt for per oral and intravenous administration respectively.

Group 2: Grapefruits were washed with running water; excess water was absorbed in filter paper. They were de-skinned and macerated. The contents were passed through sieve to get the clean watery grapefruit juice. Grapefruit Juice was given *ad libitum* overnight before the treatment of the compound.

Group 3: Phenobarbitone (80 mg/kg b.wt iv) was administered for 5 consecutive. Special care was taken to follow the code of experimentation conduct as per the norms set by the Animal ethical committee of the Institute as per GLP.

Study Design: Rats were anesthetized using gaseous anesthesia. Serial blood samples were collected through a capillary, guided in retro-orbital plexus at different time intervals (as mentioned in the results). The blood samples (0.1 to 0.2 mL) were collected in pre-labeled tubes. Blood samples were centrifuged within 15 minutes to separate plasma by centrifuging at 1540 g, 4°C for 10 minutes. The separated plasma was transferred to pre-labeled micro-centrifuge tubes and promptly frozen at – 80 ± 10°C until bioanalysis.

Liver and intestine (10 cm length after duodenum) were collected at different time interval post oral treatment of CDRI-80/574. Samples were stored at -80°C till use. Plasma and tissues [(liver, intestinal- wall and -microvilli (internal side scraping from the wall of the intestine)] from healthy untreated rats and microbes (fresh fecal matter of rats) were taken out as per the requirements.

Excretion studies were performed in bile cannulated as well as noncannulated rats. Rats were housed in modified *Bollman* cages and urine and feces collected at fixed time intervals. The urine and feces samples were collected at different intervals post dose. The samples were dried at 37°C for 24 hrs prior to analysis.

Extraction Procedure for urine samples: Rat urine (500 µl) was extracted with 3 ml of 95:5:: hexane: isopropyl alcohol, vortexed for 1 min and centrifuged at 1500g for 5 minutes. The tubes were snap-freezed at the bottom and organic layer separated. The contents were evaporated to dryness and reconstituted in 250 µl acetonitrile.

Procedure for hydrolyzing the drug conjugates: Rat urine (500 µl) sample was basified with 125 µl of 1 N NaOH, vortexed for 1 min and neutralized with 100 µl 0.6 N sulphuric acid, vortexed again and extracted the same way as mentioned above.

Extraction Procedure for Feces samples: Feces (50mg) were extracted with 2ml acetonitrile. The feces were imbibed for 15 minutes after the addition of acetonitrile and then vortexed and centrifuged (1500g for 5 min). 100 µl of supernatant was injected into HPLC without further sample cleanup.

Hepatocytes Isolation: Rats were treated with Nembutol (anaesthesia) (Na Pentothal) 50mg/kg and 50 µl of heparin (5000 U/ml) intraperitoneally. Chest and abdomen was swapped with 70% ethanol to locate the inferior venacava vein. Distal suture was placed around portal vein and catheter (16-18 GO) inserted. The outflow was connected to the perfusion pump and inferior venacava, cut above suture. Prewash solution *Hepes Buffer* was pumped through liver at 20 ml/min followed by collagenase-0.05% w/v in Hepes –5 ml/min for 20 min. Livers were removed in sterile petridish filled with cold water, teased (but not minced) and mesh filtered. Suspension was centrifuged at 500 g for 3 min in ice-cold conditions. Sediment was resuspended in after wash buffer. Cells were counted using trypan blue by hemocytometer. (Added 300 µl of PBS and 500 µl of trypan blue solution to the cell suspension of 200 µl in a 15 ml centrifuge tube. Mixed and allowed to stand for 5-15 minutes with a coverslip on hemacytometer; added 20 µl of the blue suspension to a chamber and count. Cell counts per ml are the average count per square $X10^4$). Hepatocytes were stored in liquid N_2 until use. The viability of rat hepatocytes used was 84.6 ± 8.2%.

Cryopreserved human hepatocytes were obtained from Invitrogen. The viability of human hepatocytes used was 78.9 ± 9.5%.

In vitro Experiments

Preparation of S9, microsomal and cytosolic fractions: Tissues isolated from untreated/treated male *Sprague Dawley* rats were thoroughly washed and homogenized in ice-cold 0.01 M K_2HPO_4-1.15% KCl buffer, pH 7.4. The homogenate (20% w/v) was centrifuged at 9000 g for 20 min to obtain S9 fraction or post-mitochondrial fraction. S9 fraction was re-centrifuged at 1050000 g for 60 min to obtain the microsomal pellet (microsomal pellet was suspended in 0.01 M phosphate buffer containing 0.001 M EDTA, pH 7.4 to make microsomal fractions) and cytosolic supernatant [10,11]. All the steps were carried out in cold.

HPLC validations

Analytical standards were prepared in the range of 31.25 to 1000 ng/ml from 1 mg/ml stock of CDRI-80/574 by serial dilution method. The calibration and quality control (QCs) standards ranging from 31.25-1000 ng/ml were prepared in the respective tissue samples.

Recovery was determined by comparing the response of processed quality control samples extracted with hexane containing 1% IPA.

Metabolic stability

Metabolic stability was determined in S9, microsomal and cytosolic fractions. After incubation of different concentrations of CDRI-80/574 (as mentioned in results) with enzyme source and co–factor solution (1.25 mM-NADP, 2.5 mM-G-6-P, 0.75 U/ml-G-6-PDh, 6.25 mM-$MgCl_2$) for a period of 120 min, 200 µl of the sample was taken, and extracted with 2ml of hexane (containing 1% IPA). The samples were vacuum dried, reconstituted in 200 µl acetonitrile and centrifuged at 1500 g for 10 min; clear supernatant was analyzed by HPLC at 240 nm (λ_{max} of CDRI-80/574).

Hepatocytes: Samples (0.2 ml) were double extracted with 2 ml of hexane. Hexane was evaporated by sample concentrator and samples were reconstituted in 0.2 ml of acetonitrile. These samples were centrifuged at 1200 g for 10 min and transferred in vials for analysis by using HPLC. $CL_{int\ invitro}$ value was calculated using number of hepatocytes per gram liver and the liver weight per kilogram of body weight as shown in Table 1.

***In vitro* metabolism and localization studies:** Hepatic S9, microsomal, cytosolic fractions and cofactor solutions (as mentioned above) were taken and solution of CDRI-80/574 spiked so as to produce final concentrations of 1.5, 3.0 and 6.0 µM. The samples were vortexed thoroughly after spiking and 200 µl of 0 min sample withdrawn. Immediate extraction was done with 2ml of hexane (containing 1% IPA). The test tubes were then placed in shaking water bath maintained at 37°C. 200 µl of sample was withdrawn each at 15, 30, 45, 60, 75, 90 and 120min. Similar experiments were conducted with the intestinal-wall, –microvilli and microbes (fresh fecal matter was grinded in pestle and mortar in cold conditions). The samples were extracted and processed for HPLC analyses as mentioned above.

Kinetic analysis: Hepatic S9 and cofactor solutions were taken in fifteen different test tubes. These tubes were labeled properly and CDRI-80/574 was spiked in each tube with the final concentrations of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 7.5, 9.0, 15.0, 3.0, 7.5, 15.0, 30.0 µg/ml, respectively. Processing was conducted as mentioned in the above paragraph. Reaction rates were linear with respect to protein concentration and the time of incubation. Apparent K_m and V_{max} values for the metabolism of CDRI 80/574 were estimated from Lineweaver-Burk plots using linear regression analyses [12,13].

Inhibitor studies

Inhibitors stocks (1mg/ml) were prepared in acetonitrile and volume corresponding to the following concentrations taken; ketoconazole, quinidine, isoniazid, cimitidine (2.5, 5.0 and 10 µM), sulphaphenazole, floxetine (0.5, 1.0 and 2.0 µM), coumarin, chlorozoxazone and diltiazem (0.25 and 0.5 µM) and furafylline (20 µM) were separately taken in test tubes and dried in vacuum drier. Aminobenzotriazole (1mM) was used as a suicidal inhibitor of the CYP mediated metabolism.

Assay of cytochrome P- 450 activity

Cytochrome P-450 activity was assayed by its reduction with sodium dithionite followed by carbon monoxide gas binding spectra at 450 nm (14). IC_{50} value (i.e. 50% inhibition of the activity of cytochrome P_{450} observed in the absence of the inhibitor) for the inhibition of the specific activity of cytochrome P_{450} was determined.

RBC uptake studies

Spiked different concentrations of CDRI-80/574 in 5 ml of heparinised fresh whole blood (since acetonitrile can cause haemolysis, 20 µl of 1 mg/ml stock was spiked into 1 ml normal rat plasma and used for subsequent spiking) . Samples were incubated at 37°C for different time intervals (0, 5, 15, 30, 45 and 60 minutes; both Plasma and whole blood were analyzed separately and RBC uptake determined [14,15] from the following equation:

$$Crbc=\{Cb - [Cp\ (1 - Ht)]\}/Ht$$

where Cb is Drug concentration in whole blood

and Cp is Drug concentration in plasma.

Synthesis of epoxy metabolite

Dissolved 3 gm of 5, 16 pregnadiene-3β-ol-20-one acetate in 200 ml of methanol. After chilling to 15°C with 6ml of 4 N NaOH solutions, it was treated immediately with 12ml of 30 % Hydrogen peroxide and stored in refrigerator at 5°C for 23 h. Added 800 ml of water, and the resulting white solid was separated, it was washed well with water and dried. The purity of the synthesized epoxy metabolite was ensured by ESIMS and NMR spectra. Samples of the analytical and calibration standards and the test samples were analyzed with the LC-MS/MS, since lower levels of the epoxy metabolite are hard to be detected by the reversed phase HPLC (the UV sensitivity of the epoxy metabolite is less) [16,17].

HPLC conditions

HPLC (Shimadzu) system consisted of an HPLC pump, system controller, injector, U.V. detector and integrator. Mobile phase was 10 mM Ammonium acetate buffer pH 6.0: ACN:: 35:65 with a flow rate of 1.0 ml/min on C–18 reversed phase column (5 µM, 220X4.6 mm, id) with a guard column (5 µM, 30X4.6 mm, id.) [18-20].

LC-MS/MS conditions

A Perkin Elmer Series 200 HPLC system (Perkin-Elmer, USA) consisted of flow control valve, vacuum degasser, auto sampler and pump. Mobile phase consisted of ACN: Ammonium acetate buffer (10 mM pH 6.0):: 85:15 with a flow rate of 0.65 ml/min using C18, Phenomenex (reversed phase column) (5 µ 100X4.6 mm), guard column:-30X4.6 mm. API-4000 LC-MS-MS (Applied Biosystems/MDS SCIEX, Toronto, Canada) mass spectrometer was operated with standard ESI source. The software used was Analyst 1.4.2 (Applied Biosystems/MDS SCIEX, Toronto, Canada).

MS parameters: nebulizing gas was zero air (40GS 2, 10 curtain gas), declustering potential (DP) 90V (ion spray voltage, nebulizing and curtain gas conditions were used in default mode), dwell time 250 sec, mass widths: ±10amu; performed in positive ion mode (based on structures) collision gas: nitrogen, collision energies (CE): 41eV (CE optimization for the precursor to product ions transition was obtained by CE ramping by direct infusion). Sample injection volume: 20 µl,

Parameters	Rat	Human
Number of hepatocytes/g liver (cells/g liver)	120×10^6	120×10^6
Liver weight/kg body weight (g liver/kg)	40	25.7
Liver blood flow (ml/min/kg)	55.2	20.7

Data from Bayliss et al. (1999) (11) and Davies and Morris (1993) (12).

Table 1: Physiological parameters for calculation of intrinsic clearance in rat and human.

mass fragmentation m/z: 314/137. Retention times of the metabolites vis a vis m/z were compared with those of the synthetic standards to confirm identity. Unknown concentrations of the metabolites were estimated by interpolation from the standard curve (18-20).

Results

Linear least square regression analysis of the calibration graph demonstrated linearity in the range of 31.25-2000 ng/ml for CDRI-80/574 in respective biomatrices of male SD rats. There was no interference observed at the retention time (6 ± 1 min) of CDRI-80/574 after injecting processed blank and CS samples, indicating the specificity of the method as indicated in Table 2.

In situ absorption study was performed using a closed loop circulation technique in rats [21]. The absorption-rate (Ka) and –half life were 2.4 ± 0.01/hr and 18.1 ± 4.7 minutes respectively. Simulated gastric fluid study indicated that CDRI–80/574 was stable in gastric juice over a period of 4 hr at 37°C. By conventional shake flask method the Log D of the compound was estimated to be 4.52 ± 0.05, depicting high lipophilicity of CDRI-80/574.

In vitro studies on whole blood were carried out to investigate the possibility of the uptake of CDRI–80/574 by RBC. About 35% of the drug was taken up by RBCs (Table 3); the rate of uptake being instantaneous with rapid equilibrium.

CDRI-80/574 undergoes enzymatic change in hepatic subcellular fractions. (No degradation of the CDRI-80/574 was noticed in the boiled samples). The degradation constant was higher in S9 followed by microsomes and cytosol whereas; half–life depicted the reverse trend (Table 4). After 2 hr maximum amount of CDRI-80/574 was degraded in S9 fraction of liver (about 91%) followed by microsomal fraction (78%) and cytosolic fraction (16%).

Concentration dependent decrease was noticed in hepatic cytochrome P-450 activity by CDRI-80/574 (IC50 2.22 nM against CYP3A4); the Km was 15.8 nM, Vmax=0.46 nmoles/mg protein/min and intrinsic clearance=29 µl/min/mg protein.

	Low	Medium	High
Plasma			
Theoretical (ng/ml)	31.25	125	1000
% Bias-inter day	-3.12	-2.15	3.51
% Bias-intra day	-12.52	-0.12	8.61
% CV-inter day	10.91	3.31	1.21
% CV-intra day	1.20	13.51	10.24
S9			
Theoretical (ng/ml)	62.5	500	2000
% Bias-inter day	-5.12	-8.91	1.12
% Bias-intra day	-7.42	-3.56	11.25
% CV-inter day	-2.34	-8.57	10.14
% CV-intra day	-5.16	-3.46	9.21
Urine			
Theoretical (ng/ml)	80	200	500
% Bias-inter day	0.25	-0.4	2.5
% Bias-intra day	1.59	-2.1	1
% CV-inter day	3.4	3.4	1.8
% CV-intra day	9.9	7.7	6.7
Feces			
Theoretical (ng/ml)	100	200	500
% Bias-inter day	-7.41	-6.8	-3.2
% Bias-intra day	-8.29	-7.2	8.61
% CV-inter day	13.9	5.8	3.6
% CV-intra day	8.5	5.7	9.9

Table 2: Validation of CDRI-80/574 in S9, plasma, urine and feces of normal *Sprague-Dawley* rats.

Time (min)	CDRI-80/574 Concentration (µg)			Ke/p	Kb/p	% Uptake
	Blood	Plasma	RBC			
1	1.37 ± 0.11	0.71 ± 0.11	0.51 ± 0.13	0.75 ± 0.31	0.89 ± 0.13	37.03 ± 8.92
2	1.24 ± 0.02	0.74 ± 0.16	0.34 ± 0.04	0.47 ± 0.07	0.76 ± 0.03	27.60 ± 2.97
3	1.24 ± 0.09	0.79 ± 0.06	0.27 ± 0.14	0.35 ± 0.21	0.71 ± 0.10	21.33 ± 11.12
10	1.36 ± 0.15	0.81 ± 0.11	0.37 ± 0.22	0.48 ± 0.33	0.77 ± 0.15	26.57 ± 13.59
12.5	1.24 ± 0.16	0.82 ± 0.06	0.25 ± 0.07	0.32 ± 0.12	0.69 ± 0.06	20.05 ± 6.29
15	1.42 ± 0.32	0.82 ± 0.09	0.41 ± 0.20	0.49 ± 0.20	0.78 ± 0.09	28.30 ± 8.34
20	1.26 ± 0.94	0.74 ± 0.04	0.34 ± 0.06	0.47 ± 0.11	0.77 ± 0.05	27.60 ± 4.81
30	1.29 ± 0.09	0.72 ± 0.03	0.41 ± 0.05	0.56 ± 0.05	0.80 ± 0.02	31.23 ± 1.79

Values are mean ± SD of three separate sets of experiment

Table 3: RBC partitioning of CDRI-80/574.

Fractions	Degradation Constant (K)	$T_{1/2}$ (min)
S9	0.42	1.66
Microsomal	0.23	3.00
Cytosolic	0.027	25.72

Values are mean of three separate sets of experiment

Table 4: Metabolic Stability of CDRI-80/574 in S9, microsomal and cytosolic fractions.

CDRI-80/574 was metabolically more stable in human hepatocytes than rats (Table 5). It also indicates that there are very rare chances of any toxic or other metabolite(s) which can be different or more in human than in rats. The clearance (CL_{int} *in vitro*) was 32 and 11 µl/min/million hepatocytes in rat and human respectively.

CDRI-80/574 was unstable (complete loss was noticed) in the 20 and 40% w/v S9 fraction after 20 minutes. However, when the protein concentration was reduced to 10% w/v S9, appreciable amount of the parent drug could be noticed even after 30 minutes of incubation. A characteristics spectra (Figure 2) containing 0 and 30 minutes incubated 10% S9 samples showed the appearance of the 2 metabolites at 3.4 (termed M1) and 4.8 (termed M2) minutes and the decrease in the parent drug concentration. The metabolites were polar in nature as well as possess the double bond (as apparent by the absorbance at wavelength 248 nm). The relative peak heights of CDRI- 80/574, M1 and M2 are presented in Figure 3.

No extra metabolite peak was noticed when CDRI–80/574 *in–vitro* metabolism with extra hepatic tissues viz. intestinal-wall and -microvilli. Maximum metabolism as expected was in liver, followed by microbes, intestinal-wall and –microvillli (Table 6).

The comparative account of CDRI-80/574 metabolism after addition of different cytochrome P-450 isoforms specific inhibitors showed that aminobenzotriazole (ABT, suicidal inhibitor of CYPs), ketoconazole and diltiazem (specific inhibitors of CYP3A4) completely inhibited the metabolism of the CDRI-80/574, whereas, in case of other inhibitors the metabolism of CDRI-80/574 proceeded as usual with the formation of two metabolites when detected on HPLC. The concentrations of the inhibitors were chosen in the range depicts the inhibitors of the IC_{50} value.

CDRI-80/574 metabolism enhanced by 40% at 1 hr ((data not presented) with induced S9 fraction (by phenobarbitone treatment). CDRI-80//574 metabolism increased after the induction of CYP3A4; however, only 2 metabolites mentioned above could be detected on HPLC. This helped us to precisely know that majorily CYP3A4 was

Time (min)	CDRI 80-574 (% remaining)	
	Rat Hepatocytes	Human Hepatocytes
0	100	100
5	75	79
10	40	55
15	20	42
20	11	29
30	0	10

Values are mean of three independent sets of experiments

Table 5: Metabolic Stability of CDRI-80/574 in Rat and Human Hepatocytes.

responsible for the rapid metabolism of the CDRI-80/574.

One of the metabolite of CDRI-80/574 had been identified as epoxide (Figure 4). Epoxide of CDRI-80/574 was synthesized as mentioned in the Materials and Method section. The yield of the epoxy metabolite was 2.63 g (95%) with the melting point of 180-186°C. ESIMS and NMR spectra confirmed the purity of the synthesized epoxy metabolite or 5-pregnene-3β-ol-16, 17-epoxy-20-one (mol. wt 330).

The recovery of CDRI-80/574 and its epoxy metabolite in hepatic S9 fraction of male SD rats ranged from 81-91% (Tables 7 and 8). CDRI-80/574 undergoes metabolism into epoxide till 45 min; after which epoxide is being further metabolized. In microsomes, CDRI-80/574 and epoxide decreased with time (Table 9). Epoxy derivative was also continuously metabolized.

An assay method for analyzing the feces samples was developed and validated. The region of interest was found to be clean when unfortified blank feces were analyzed by this method showing good selectivity. The accuracy and precision were found to be well within the accepted limits (less than 15% at lower concentrations and less than 10% at higher). The recoveries of QC samples were consistently above 90% (Table 2). It was found that approximately 15% of the CDRI–80/574 as parent was excreted in feces, less the 0.5% in urine and about 0.07% of the dose is excreted in bile (Table 10).

Feces and microbial metabolism samples showed the presence of the 3 predicted metabolites with m/z of 317, 319, 321 and 330. To resolve the suspected metabolites a gradient system was developed and analysis of both feces and urine samples with this showed three peaks complying with the results of Mass analysis.

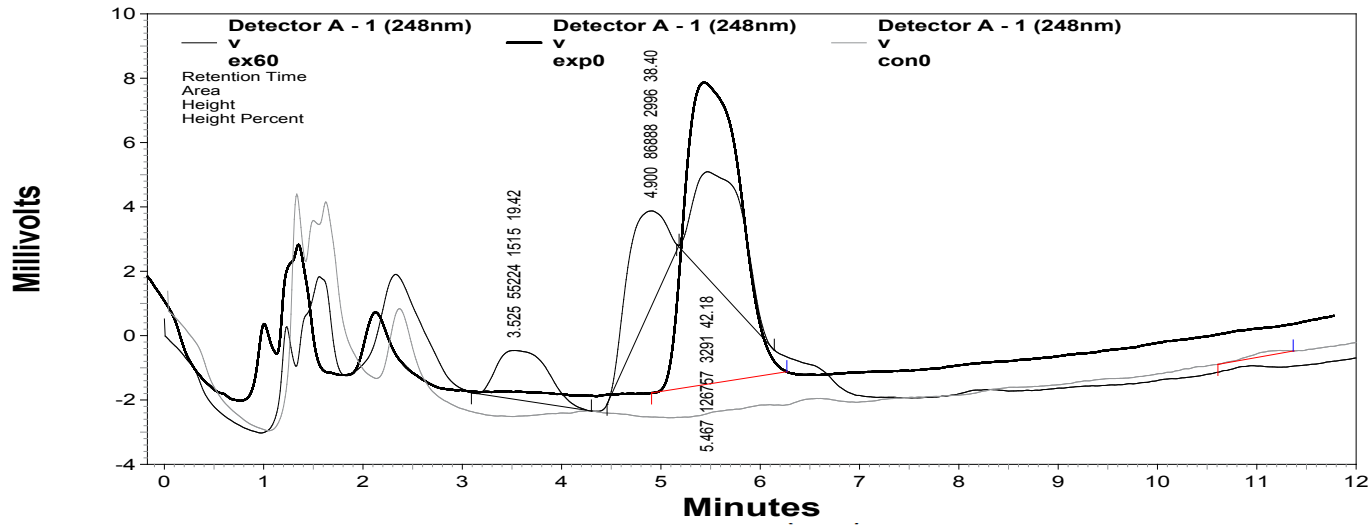


Figure 2: *In vitro* metabolism of CDRI-80/574: Appearance of two metabolites after 60 minutes. M1 is formed at 3.4 and M2 at 4.8 min (solid black lines).

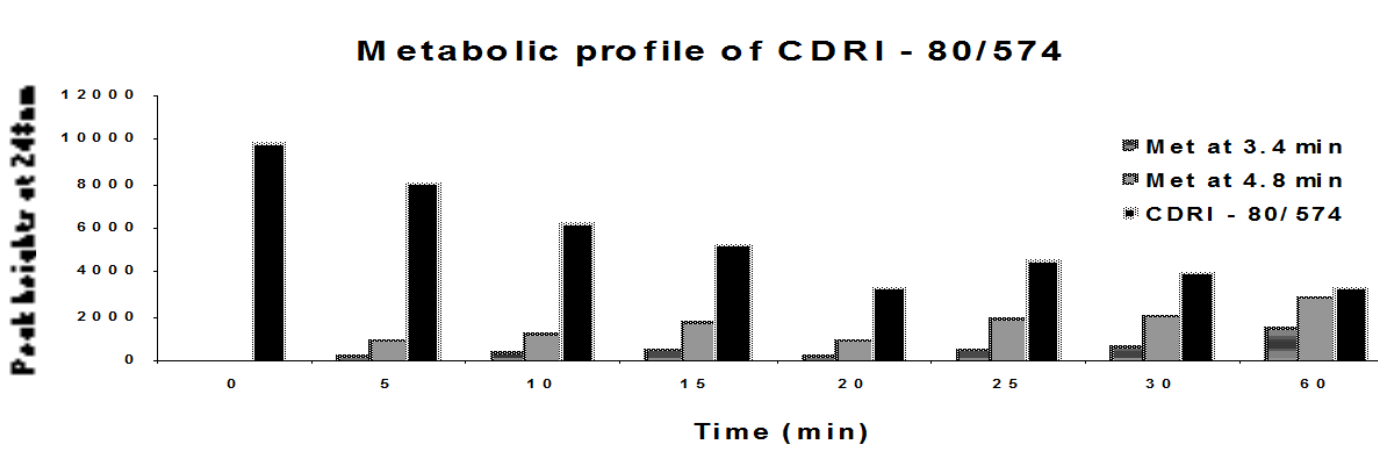


Figure 3: Peak heights of CDRI-80/574, M1 (3.4 min) and M2 (4.8 min) in the microsomal fractions at different time of incubation.

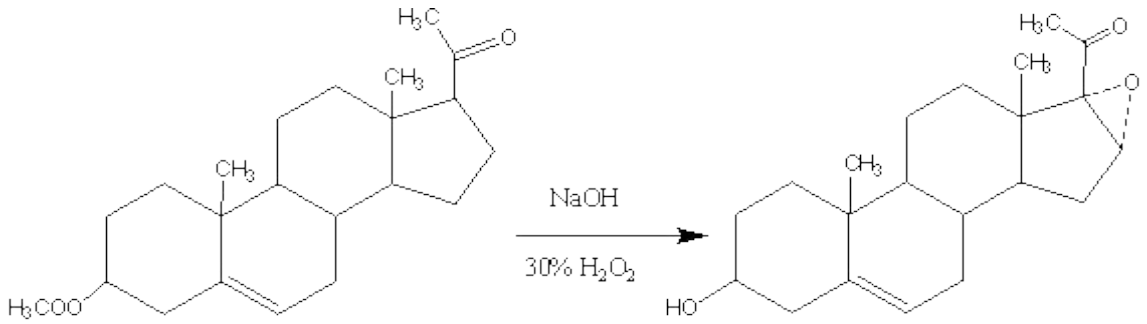
Time (min)	% decrease in CDRI-80/574			
	liver	intestinal-wall	-microvilli	microbes
0	0	0	0	0
15	43.41	4.28	3.22	2.58
30	56.90	8.52	7.21	4.85
45	60.98	13.34	11.45	21.45
60	84.55	18.98	16.71	43.35

Values are mean of three independent sets of experiments

Table 6: CDRI-80/574 metabolism in liver, intestinal-wall, -microvilli and microbes.

Please refer to the Figure 5 proposed metabolic pathway of CDRI-80/574.

Pharmacokinetics was studied after 18 mg/kg i.v. dose of CDRI–80/574 in male *Sprague Dawley* rats. The plasma concentration time profile followed a two compartment open model. CDRI–80/574 was traced up to 3 hours and the plasma concentration time profile best fitted to a two compartment open model with first order elimination (correlation >0.99) (Figure 6).



5, 16 Pregegnadiene-3β-ol-20-one acetate — 16-Dehydropregnenolone epoxide

Figure 4: Conversion reaction of CDRI-80/574 into its epoxide metabolite.

Inhibitors	Concentration (μM)	CYP(s) Isoforms	% CDRI-80/574 metabolism	Metabolite(s)
Ketoconazole	2.50	**3A4, 5, 7**	12.95	Nil
Diltiazem	0.25	**3A4, 5, 7**	10.49	Nil
Quinidine	2.50	2D6	95.10	Two
Sulfaphenazole	0.50	2C9	22.66	Two
Chlorozoxazone	0.25	2E1	53.79	Two
Isoniazid	2.50	2C9	82.07	Two
Cimitidine	2.50	1A2	54.83	Two
Floxetine	0.25	2C19	52.26	Two
Coumarin	0.25	2A6	52.96	Two
Aminobenzotriazole	1.0	all	Nil	Nil

Values are average of two different sets of experiments

Table 7: Effect of different CYP450 isoform inhibitors.

Spiked concentration of CDRI-80/574 (ng/ml)	% of	
	CDRI-80/574	Epoxy metabolite
12.5	87.41 ± 3.15	91.23 ± 3.12
25	86 ± 4.14	81.43 ± 4.24
50	89.4 ± 4.05	83.23 ± 5.82

Values are mean ± SD of three separate sets of experiments

Table 8: Formation of epoxy metabolite from CDRI-80/574 in hepatic S9 of male *Sprague Dawley* rat.

Time (min)	S9 (0.9μg/ml)		S9 (1.8μg/ml)		Microsomes (0.45μg/ml)	
	CDRI-80/574	Epoxy	CDRI-80/574	Epoxy	CDRI-80/574	Epoxy
0	100	0	100	0	100	0
15	64	30	47	50	35	68
30	51	45	30	65	20	79
45	10	80	25	77	12	84
60	0	100	3	100	0	100
75	0	71	0	84	0	82
120	0	62	0	70	0	68
150	0	62	0	70	0	0

Values are mean of three separate sets of experiments

Table 9: Formation of epoxy metabolite from CDRI-80/574 in hepatic S9 (2 protein concentrations) and microsomal fraction of male *Sprague Dawley* rat.

	% of CDRI-80/574 dose recovered					
	Bile duct cannulated			Noncannulated		
Time (h)	0-12	12-24	Total	0-12	12-24	Total
Urine	0.24	0.09	0.33	0.32	0.15	0.47
Feces	11.85	1.23	12.08	12.21	2.76	14.97
Bile	0.06	0.01	0.07	-	-	-

Table 10: Excretion Profile of CDRI-80/574 in rat.

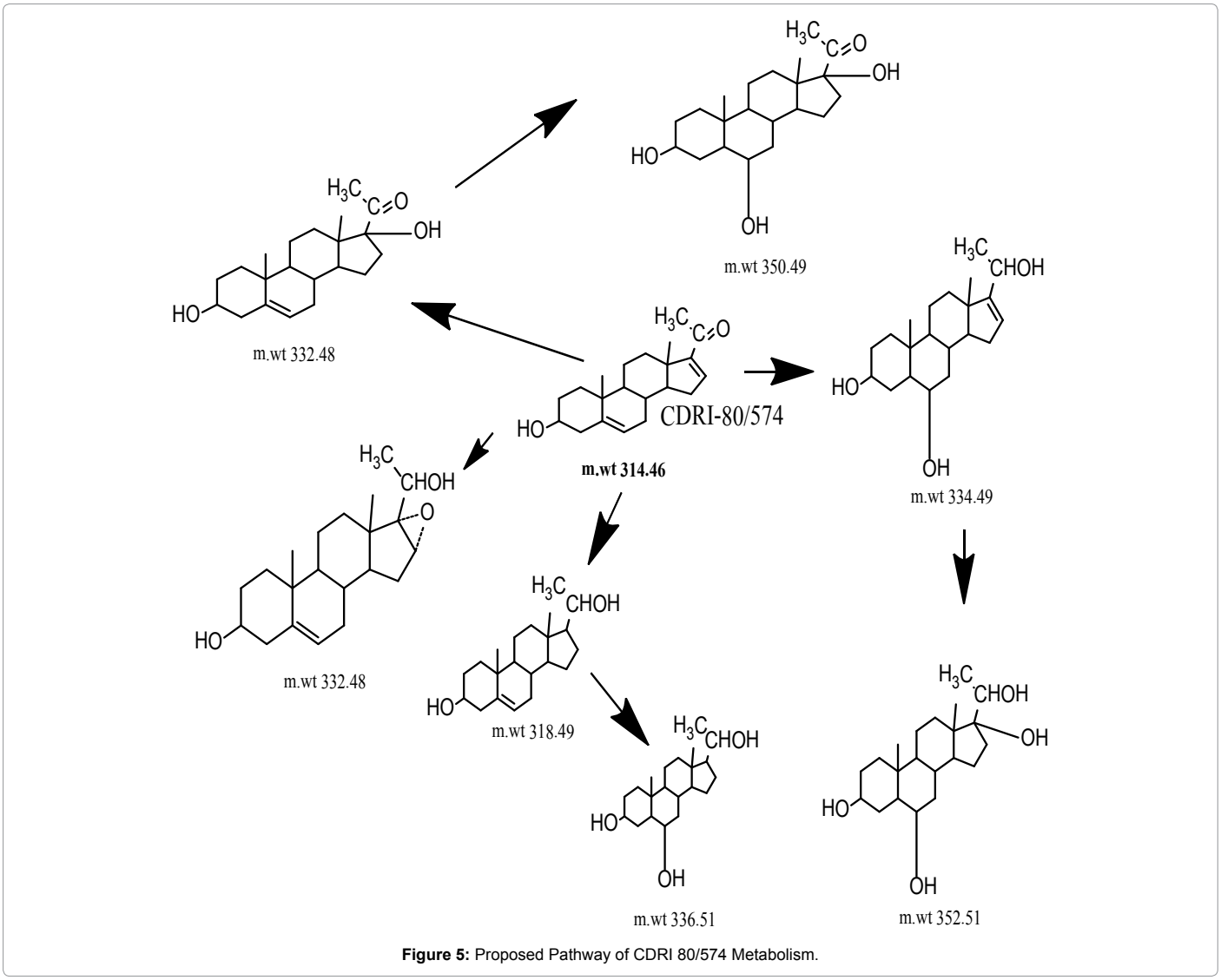


Figure 5: Proposed Pathway of CDRI 80/574 Metabolism.

The elimination half-life ($t_{1/2}\beta$) was 19 min in which β is the elimination rate constant for the terminal exponential phase, and mean residence time (MRT) was estimated to be 28.66 min. Volume of distribution (Vd) and total clearance (Cl) were 2 L and 7.5 L/h/kg, respectively (Table 11). No level of CDRI–80/574 was detected in the systemic circulation after 72 mg/kg oral dose in rats. Thus samples were collected from portal vein after oral administration to generate a plasma concentration time profile; the C_{max} was found to be 250 µg/ml at 90 min with the AUC of 240 µg. min/mL, respectively. The bioavailability was found to be about 7%. Further, grapefruit juice influence was well noted on the p.o. pharmacokinetics of CDRI-80/574, the C_{max} and AUC increased by 2 folds (Table 11).

Discussion

CDRI-80-574 or DHP, developed by CDRI, is an orally acting hypolipidemic agent, which is currently under advanced stage of clinical trials. This compound is proposed to decrease the LDL levels in plasma thus producing hypolipidemic effect [20]. It is also known to inhibit the recirculation of bile acids thereby enhancing their excretion as well as conversion of cholesterol to bile acids. This drug is known to be free from any untoward side effects and has good therapeutic window.

Development and validation of an analytical method employed for the quantitative determination of drugs and their metabolites in biological samples are the key determinants to generate reproducible

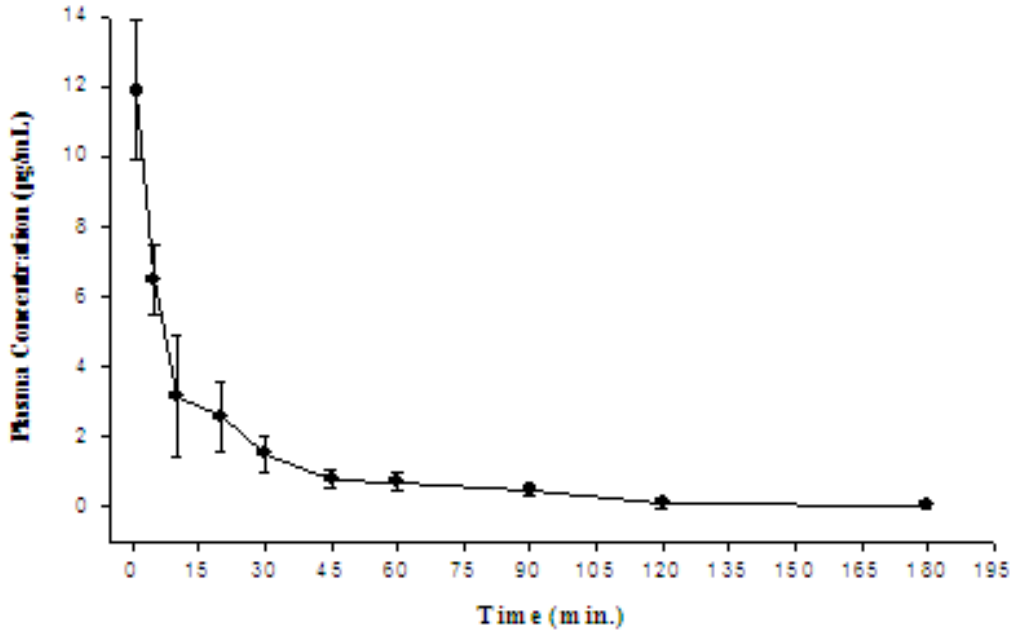


Figure 6: CDRI-80/574 concentration in plasma following an i.v. dose of 18 mg/kg in rats (n=5).

Parameters	iv treatment	Oral treatment	Oral Treatment after grapefruit juice
C_{max} (µg/L)	-	250.0 ± 45.1	791.4 ± 19.2
T_{max} (h)	-	1.5	1.5
AUC_{last} (µg.h/L)	1386 ± 371.21	784.1 ± 48.1	1287.1 ± 84.8
$T_{1/2}$ (min)	18.7 ± 1.20		
V_z (L)	2.02 ± 0.21		
Cl (L/h/kg)	7.49 ± 1.59		
MRT (min)	28.66	140	258
%F		7	

Values are mean ± SD of 6 rats

Table 11: Pharmacokinetics Profile of CDRI-80/574 after pretreatment with grapefruit juice.

and reliable data having significant role in evaluation of various pharmacokinetic parameters [18,19].

The study also gives an idea about the partition coefficient of the drug between plasma and the erythrocytes, the transport system with a high capacity and low affinity compared to plasma proteins. Thus erythrocytes are loaded first and unloaded first. The transport function of red cells is influenced by physiological factors as the role of erythrocytes become more apparent when protein binding is saturated [21-23]. Therefore less bioavailability of CDRI-80/574 can be an artifact when conducted using matrices devoid of RBCs, also, chances for erythrocytic metabolism of CDRI-80/574 cannot be ruled out.

Metabolic stability depicts the readiness of the drug to undergo metabolic oxidative metabolism [24]. It gives a subjective estimate of drug's stability in different biometrics. % loss/degradation of CDRI-80/574 in liver subcellular fractions was compared to determine the biomatrix in which drug has shown appreciable biodegradation. The results show that the main pathway for CDRI–80/574 degradation was Phase I reactions and that it was comparatively stable in cytosol. Contrary to microsomes, intact hepatocytes contain Phase I (P450), Phase II (conjugative) enzymes, their cofactors as well as active transporters. Therefore, isolated hepatocytes were used to study the integrated metabolism and distribution of compounds. CDRI-80/574 was metabolically more stable in human hepatocytes than rats.

Human CL_{invivo} were predicted based on human $CL_{int\ invitro}$ using rat scaling factor [25-28]. For compounds cleared almost exclusively by P450-mediated pathways, scaling from human liver microsomes was as predictive as single-species scaling of clearance based on data from rat, dog, or monkey. Both the below mentioned equation were used for allometric scaling of clearance.

$$\text{Human } CL_{invivo} = \text{Rat } CL_{invvivo} * \text{Human } CL_{int\ invitro} / \text{Rat } CL_{int\ invitro}$$

or

$$\text{Human } CL_{invivo} = \text{Rat } CL_{invivo} * \text{Human } Qh_{human} / \text{Rat } Qh_{rat}$$

Human CL_{invivo} was found to be 2.75 L/h/kg (45.83 ml/min/kg) and 2.72 L/h/kg (45.33 ml/min/kg) by utilizing $CL_{int\ invitro}$ and Qh values respectively. Since we have used only a species for scaling, it was important to see the confidence in the same by *in vitro* intrinsic clearance as well as hepatic blood flow (Qh). The agreement or nearness by both the methods increases the reliability to predict high clearance of CDRI-80/574 in human, predominantly by liver.

Due to the less bioavailability of CDRI 80/574, its metabolism in

extrahepatic tissues viz.-intestinal-wall and -microvilli was monitored. The above extra hepatic tissues as well as fresh fecal matter containing microbes (representative of the protozoan class) were chosen because they are responsible for major portion of drug metabolism when drug is given orally and it also can undergoes primitive drug metabolism. No extra metabolite peak was noticed when CDRI–80/574 *in–vitro* metabolism was monitored in the extra hepatic tissues viz. intestinal-wall and -microvilli. Maximum metabolism as expected was in liver, followed by microbes, intestinal-wall and –microvillli. Further, it also confirmed that the metabolism of CDRI–80/574 was not by the exclusive CYP450 isoforms present in either hepatic or extrahepatic tissues, but the CYP450 isoform, which predominates in liver, intestine and microbes. Presence of presystemic GIT metabolism was also confirmed by microbial metabolism studies.

Depending upon the class and the chemical nature, different compounds have different affinity towards the specific cytochrome P-450 isoform [29-30]. There are a number of drugs, which act as a substrate towards the CYP3A4. Therefore, it was important to see the inhibitory profile. Aminobenzotriazole (suicidal inhibitor of CYPs), ketoconazole and diltiazem (specific inhibitors of CYP3A4) completely inhibited the metabolism of the CDRI-80/574. It clearly indicated that CDRI-80/574 was metabolized predominantly by CYP3A4, which contributed to about 70% of total CYPs at a very faster rate; thereby giving reason for its less bioavailability. In other words, CDRI 80/574 undergoes rapid fast pass metabolism [31] and therefore its exposure to the systemic circulation becomes affected in the oral treatment. Use of specific inhibitor of CYP3A4 can increase the bioavailability and efficacy of CDRI-80/574.

Many studies have been conducted on grapefruit juice's inhibitory potential against CYP 3A4 activity [32-34]. CDRI-80/574 was fairly stable in the S9 fraction of grapefruit juice treated rats. However only 13% the specific activity of CYP450 remained in grapefruit juice vs. untreated by CDRI-80/574. Phenobarbitone (mainly induces subfamily 3A4 of CYP450 system) was used as inducer for the determination of Phase I enzymatic activity. There was about 3-fold enhancement in the levels of the cytochrome P-450 after phenobarbitone treatment. Also, the activity of Glutathione-S-transferase, taken as a marker of the Phase II drug metabolism was also found to be induced.

High clearance or disposition or excretion of CDRI-80/574 may also contribute towards less exposure. It was found that approximately 15% of the CDRI–80/574 as parent was excreted in feces, less the 0.5% in urine and about 0.07% of the dose is excreted in bile. In line with other drugs of the same class, the urinary and biliary excretions of CDRI–80/574 were negligible while a significant portion of the orally administered dose (up to 15 %) was excreted unchanged in feces. The mass analysis of the samples, both feces and microbial metabolism samples, showed the presence of the 3 predicted metabolites (m/z of 317, 319, 321 and 330). Analysis of feces and urine samples showed three peaks complying with the results of Mass analysis.

There are many hypolipidemic drugs viz. lovastatin, simvastin etc, which have poor %F (Table 12). Same profile of enhancement in pharmacokinetics profile was noticed in case of simvastatin [30].The bioavailability was found to be about ~7%. Further, grapefruit juice influence was well noted on the p.o. pharmacokinetics of CDRI-80/574, the C_{max} and AUC increased by 2 folds (Table 11).

Conclusion

80/574 is a CDRI candidate novel steroidal hypolipidemic agent.

Drugs	% F	Urinary excretion %	Plasma Protein Binding %	Cl (ml/min/kg)	Vd (liters/kg)	Half-life (hr)
Gemfibrozil	98 ± 1	< 1	> 97	1.7 ± 0.4	0.14 ± 0.03	1.1 ± 0.02
Lovastatin	< 5	Negligible	95	4-18	____	1.1-1.7
Pravastatin	18 ± 8	47 ± 7	43-48	3.5 ± 2.4	0.46 ± 0.04	1.8 ± 0.8
Simvastatin	< 5	Negligible	94	7.6	____	1.9
Clofibrate	95 ± 10	57 ± 2 .1	96.5 ± 0.3	0.12 ±.01	0.11 ± 0.02	13 ± 3

Table 12: HumanPharmacokinetic profile of some current hypolipidemic drugs.

Studies were carried out to elucidate the fate of the drug after oral administration. The experiments reported in the present manuscript state that CDRI–80/574 was metabolized at a faster rate. The metabolism of the compound was localized to the phase I of drug metabolism and completely inhibited by CYP3A4 specific inhibitors. Since about 70% of the CYP450 is in the form of CYP3A4, this explains the reason behind the very rapid metabolism of CDRI–80/574 and its low bioavailability, when given orally. Metabolites identified preliminary as hydrolysis and hydroxy products *in vitro* (S9 fraction) and *in vivo* (urine and feces). Human Cytochrome P450 activity was inhibited in a concentration dependent manner by CDRI–80/574.

It is therefore suggested that the inhibitor of CYP3A4 can be supplemented as on add-on to increase the bioavailability of CDRI – 80/574.

Acknowledgements

The author would like to thank Director, and Head, Drug Metabolism and Pharmacokinetics, CDRI for their valuable suggestions and interest in the study. CDRI Communication No.: 6411.

References

1. CTRI/2011/05/001761 (2011) Comparative assessment of tolerability, efficacy and safety of combination of compound CDRI 80/574 and Atorvastatin versus Atorvastatin in subjects with Hyperlipidemia (A Randomized, Double-blind, Parallel-group, Active-comparator Controlled, Phase II Clinical Study)
2. Ghatak A, Asthana OP (1995) Recent trends in hyperlipoproteinemias and its pharmacotherapy. Ind Pharmacol, 27: 14-29.
3. Pratap R, Gupta RC, Chander R, Khanna AK, Srivastava AK, et al. (2000) Medicaments for hyperlipidemic and hyperglycemic conditions. Eur Pat EP, 1020191.
4. Wu J, Xia C, Meier J, Li S, Hu X, et al. (2002) The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. Mol Endocrinol 16: 1590-1597.
5. Verma AK, Khemaria P, Gupta J, Singh DP, Joshi BS, et al. (2010) Biotransformation of FXR antagonist CDRI 80/574. ARKIVOC, 9: 1-11.
6. Rowland M, Tozer TN (1995) Clinical Pharmacokinetics: Concepts and applications. Lippincott Williams &Wilkins.
7. Gibaldi M, Perrier D (1982) Pharmacokinetics, Marcel Dekker, Inc., Second edition, Page 3.
8. Bauer L (2008) Applied Clinical Pharmacokinetics, 2nd ed., McGraw-Hill.
9. Guraratna C (2000) Drug Metabolism and Pharmacokinetics in Drug Discovery: A Primer for Bioanalytical Chemists: Part 1 .Current Separations 19: 18-23.
10. Roe S (2001) Protein purification applications: a practical approach. Oxford University Press.
11. Bayliss MK, Bell JA, Jenner WN, Park GR, Wilson K (1999) Utility of hepatocytes to model species differences in the metabolism of loxtidine and to predict pharmacokinetic parameters in rat, dog and man. Xenobiotica 29: 253-268.
12. Davies B, Morris T (1993) Physiological parameters in laboratory animals and humans. Pharm Res 10: 1093-1095.
13. Copeland RA (2000) Enzymes: a practical introduction to structure, mechanism, and data analysis. 2nd Edition Wiley and sons.

14. OMURA T, SATO R (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 239: 2370-2378.

15. Bergqvist Y, Domeij-Nyberg B (1983) Distribution of chloroquine and its metabolite desethyl-chloroquine in human blood cells and its implication for the quantitative determination of these compounds in serum and plasma. J Chromatography 272: 137-148.

16. A publication of Reliable methods for the preparation of organic compounds. Organic Synthesis.

17. Smit WA, Bochkov AF, Caple R (1998) Organic synthesis: the science behind the art. Royal Society of Chemistry (Great Britain).

18. Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, et al. (2000) Bioanalytical method validation--a revisit with a decade of progress. Pharm Res 17: 1551-1557.

19. US Department of Health and Human Services; FDA; CDER; CVM, BP; Guidance for Industry; Bioanalytical Method Validation, May 2001.

20. Third Report of the National Cholesterol Education Program (NCEP) (2002) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Final Report. Circul, 106:3143.

21. Srivastava P, Gupta RC (2003) In situ absorption and protein binding characteristics of CDRI-85/92, an antiulcer pharmacophore. Int J Pharm 257: 97-102.

22. Marroum PJ, Curry SH (1993) Red blood cell partitioning, protein binding and lipophilicity of six phenothiazines. J Pharm Pharmacol 45: 39-42.

23. MAREN TH, ROBINSON B, PALMER RF, GRIFFITH ME (1961) The binding of aromatic sulfonamides to erythrocytes. Biochem Pharmacol 6: 21-46.

24. Thompson TN (2001) Optimization of metabolic stability as a goal of modern drug design. Med Res Rev 21: 412-449.

25. Hosea NA, Collard WT, Cole S, Maurer TS, Fang RX, et al. (2009) Prediction of human pharmacokinetics from preclinical information: comparative accuracy of quantitative prediction approaches. J Clin Pharmacol 49:513-533.

26. Wilkinson GR, Shand DG (1975) Commentary: a physiological approach to hepatic drug clearance. Clin Pharmacol Ther 18: 377-390.

27. Bickett DJ, MacKenzie PI, Veronese ME, Miners JO (1993) In vitro approaches can predict human drug metabolism. Trends Pharmacol Sci 14: 292-294.

28. Mordenti J (1986) Man versus beast: pharmacokinetic scaling in mammals. J Pharm Sci 75: 1028-1040.

29. Halpert JR, Guengerich FP, Bend JR, Correia MA (1994) Selective inhibitors of cytochromes P450. Toxicol Appl Pharmacol 125: 163-175.

30. http://pharmacology.georgetown.edu

31. Thummel KE, O'Shea D, Paine MF, Shen DD, Kunze KL, et al. (1996) Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. Clin Pharmacol Ther 59: 491-502.

32. Benton RE, Honig PK, Zamani K, Cantilena LR, Woosley RL (1996) Grapefruit juice alters terfenadine pharmacokinetics, resulting in prolongation of repolarization on the electrocardiogram. Clin Pharmacol Ther 59: 383-388.

33. Lilja JJ, Kivisto KT, Neuvonen PJ (2000) Duration of effect of grapefruit juice on the pharmacokinetics of the CYP3A4 substrate simvastatin. Clin Pharmacol Ther 68: 384-390.

34. Kimura Y, Ito H, Ohnishi R, Hatano T (2010) Inhibitory effects of polyphenols on human cytochrome P450 3A4 and 2C9 activity. Food Chem Toxicol 48: 429-435.