METABOLISM OF CDRI-85/92, A NEW POTENT ANTI-ULCER AGENT, INVOLVING *CIS-TRANS* CONVERSION

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SUMMARY

CDRI 85/92, an anti-ulcer drug, is a new proton pump inhibitor, currently in an advanced stage of drug development. To know more about the drug it was our objective to delineate/identify the metabolic pathway as well as the enzymes responsible for the formation of metabolites. Metabolism of CDRI-85/92 (*cis*-5-styryl-2-oxazolidinone-4-carboxylic acid) was investigated in rat liver cellular fractions (S9, microsomes and cytosol) using reverse-phase HPLC and mass spectrometry techniques. Two major metabolites were produced by rat liver S9 fractions and reducing factor generating system from either untreated rats or phenobarbitone (PB)-pretreated rats. Incubation of CDRI-85/92 with postmitochondrial fraction (S9) for 24 h resulted in a *cis* to *trans* conversion (metabolite M2). Further *cis-trans* metabolizing capacity was measured separately in the cytosolic and microsomal fractions. Incubation with the cytosolic fraction resulted in an increased rate of *cis-trans* conversion, while the microsomal

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fraction showed no *cis* to *trans* conversion, thereby restricting the *cis* to *trans* conversion to Phase II enzymes, which are mainly located in the cytosol. Studies with PB-pretreated rat liver S9 fractions resulted in an increased rate of *cis* to *trans* conversion. Another metabolite was also present (M1) which was identified as an oxygenated metabolite by mass spectrometry. The major urinary metabolite from CDRI-85/92-treated Sprague-Dawley rats (20 mg/kg p.o.) was identified as M2. Studies using sulfobromophthalein and *N*-ethylmaleimide, as specific inhibitors of GST, showed a complete absence of metabolism, thus indicating the involvement of GST in the metabolism of CDRI-85/92. This study will be helpful in providing clues about factors influencing the bioavailability of CDRI-85/92 as well as drug-drug interactions.

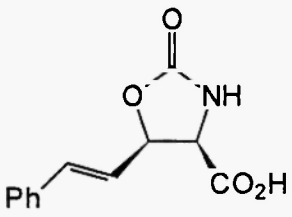
KEY WORDS

CDRI-85/92, drug metabolism, *cis-trans* conversion, glutathione-S-transferase, cytochrome P450, HPLC, mass spectroscopy, ESI-MS, MS/MS, rat

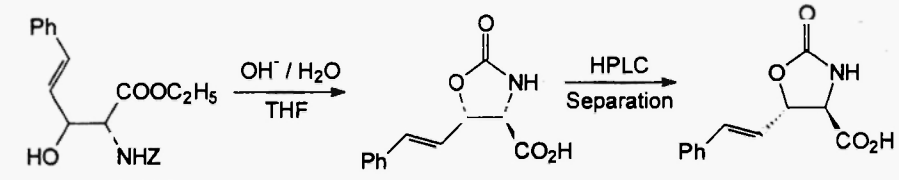
INTRODUCTION

The route of metabolism is one of the important pieces of information required in the development of a new chemical entity as a drug. The important Phase I and Phase II enzymes include cytochrome P450 and glutathione-S-transferase, respectively. CDRI-85/92 (*cis*-5-styryl-2-oxazolidinone-4-carboxylic acid) (Fig. 1A) is a new chemical entity recently evaluated for the treatment of peptic ulcer /1/. Preclinical studies have shown that it is a potent competitive proton pump inhibitor and has almost no adverse effects. The anti-ulcer activity is comparable with that of omeprazole. As developmental trials with CDRI-85/92 have advanced, it has become imperative to characterize the major route(s) of metabolism to understand the factors that could influence its bioavailability, suggest potential drug-drug interactions, and to clarify the mechanism of the formation of various metabolites.

The present study focused on understanding the metabolic fate of the drug candidate (CDRI-85/92) through various experiments including induction of Phase I (mainly CYP3A2) and Phase II (mainly



(A)



(B)

Fig. 1: **(A)** Molecular structure of CDRI-85/92. **(B)** Synthesis of its *trans* isomer, M2. THF = tetrahydrofuran.

glutathione-S-transferase [GST]) by phenobarbitone (PB) and also through *in vitro* inhibition studies using *N*-ethylmaleimide and sulfobromophthalein as specific inhibitors of GST. The metabolites were characterized by HPLC, UV spectrometry, ESI-MS and MS/MS in *in vitro* and *in vivo* urine samples.

MATERIALS AND METHODS

Chemicals

CDRI-85/92 was synthesized in-house and was >99% pure. NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, reduced glutathione, chloro-2, 4-dinitrobenzene, and PB were purchased from

Sigma Chemical Co. (St. Louis, MO). All reagents and solvents procured commercially were guaranteed reagent grade or HPLC grade.

Animals

Male Sprague-Dawley rats weighing 250 ± 25 g were obtained from the animal house of the Institute and were fed a standard pelleted food (Goldmohar, Laboratory Animal Feed, Lipton India Ltd., Chandigarh, India) and had free access to drinking water. The rats were kept at room temperature (28°C). The Institute's animal ethics committee gave approval for this study.

Effect of CYP and GST inducer on CDRI-85/92 metabolism

Male Sprague-Dawley rats (n = 5) were treated with the Phase I and II enzyme (CYP and GST) inducer PB (80 mg/kg) intravenously once daily for 5 days in 0.2 ml of triple distilled water. On the 6^{th} day, the rat livers were isolated and processed individually, for S9 fraction preparation as detailed below (summarized in Fig. 7A). The control group of rats (n = 5) received no treatment.

Tissue preparation

Untreated and PB-pretreated rats were given ether anesthesia and sacrificed. Whole liver was perfused in ice-cold saline and homogenate was prepared using phosphate buffer (0.01 M phosphate buffer with 1.15% KCl, pH 7.4) in a Potter-Elvejzem glass-teflon homogenizer. The hepatic subcellular fraction was prepared as described /2/ from pooled rat liver. Microsomal and cytosolic proteins were quantified using a colorimetric method /3/. CYP content of the untreated and PB-pretreated rat livers was determined according to the method of Omura and Sato /4/ by the CO-binding spectra absorbance at 450 nm using a double beam spectrophotometer (Hitachi model 557), and GST activity was monitored as described by Pobst *et al.* /5/ by monitoring the disappearance of 1-chloro-2,4-dinitrobenzene.

Optimization of S9 and substrate concentration for CDRI-85/92 metabolism

To obtain an appropriate concentration of microsomes for metabolic studies, incubations were performed using 10, 20, and 40% (w/v)

S9 fraction with different concentrations of cofactor solutions and CDRI-85/92 (0.5, 1, 2, and 5 μg/ml). Enzymatic activity was compared with respect to control using a spectrophotometer. Controls were devoid of the drug but supplemented with the same volume of vehicle i.e., triple distilled water.

Metabolism of CDRI-85/92 in hepatic postmitochondrial fraction (S9)

Drug-spiked S9 fraction containing 40% w/v of post-mitochondrial fraction in phosphate buffer (0.01 M phosphate buffer with 1.15% KCl, pH 7.4) and cofactor solution (NADP, 1.2 mM; glucose-6-phosphate, 2.5 mM; glucose-6-phosphate dehydrogenase, 0.75 U/ml; $MgCl_2$, 6.25 mM) was incubated at 37°C in a shaking water-bath (Vam 908D, Vam Instruments (Pvt) Ltd., Bangalore, India). Samples were collected at 0, 4, 6, 10, 21 and 24 h in prechilled tubes and were immediately chilled to -20°C to terminate the reaction.

Incubation with GST inhibitors

GST inhibitors, viz., *N*-ethylmaleimide and sulfobromophthalein, were included in the incubations to give some insight into identifying the specific enzymes involved in CDRI-85/92 metabolism. Mixtures (containing S9/cytosol, NADPH generating factors and particular concentrations of the inhibitor) were preincubated for 10 min at 37°C before the addition of the drug. The inhibitors were separately dissolved in DMSO and used at the concentrations of 5, 10, 15, 20, 30 and 50 μg/ml /6/.

Analysis of CDRI-85/92 and its metabolites in urine

Male Sprague-Dawley rats were acclimatized to laboratory conditions prior to commencement of the study. The rats were administered a single 20 mg/kg oral dose of CDRI-85/92 and urine samples were collected. The drug and its metabolite(s) were analyzed by HPLC and ESI-MS.

Sample preparation

Before analysis, the samples were thawed at room temperature for 20 min. Protein precipitation was facilitated by the addition of

acetonitrile (3 times, v/v) and samples were kept at 4°C for 30 min for complete precipitation. Finally, the mixture was centrifuged at 4,000 *g* for 10 min at 0°C. The supernatant was analyzed by the HPLC system and the eluting peaks were monitored at 250 nm.

For ESI-MS/MS analysis, protein was precipitated by adding acetonitrile (3 times, v/v). After centrifugation the supernatant was transferred into an SPE column containing the ion exchange resin (Supelco, Supelco Park, Bellefonte, USA) fitted to a vacuum manifold (pressure maintained at -10 mm Hg), adsorbed at the solid phase by passing the sample slowly through the column, and then washed with 1 ml methanol, followed by 1 ml water and then 1 ml 1% ammonia in methanol. The columns were preactivated with 1 ml of methanol followed by 1 ml of water. Samples were obtained by eluting drug and metabolite with 2 ml 5% ammonia in methanol. The eluted samples were vacuum dried and reconstituted into a mobile phase (used for HPLC) and the drug and its metabolites were fractionated using HPLC. Each fraction was subjected to mass spectrometric analysis.

HPLC analysis

HPLC analysis was carried out using a method described by Srivastava and Gupta /7,8/. The HPLC system was equipped with a pump (dual LC-10AD, Shimazdu), a manual injector (7125, Cotati, CA, USA) fitted with a fixed 20 µl loop, a photo diode array UV detector (Waters model 996) and a reverse phase C18 column (E. Merck, Darmstadt, Germany, No. S19429). The mobile phase was pumped at a flow rate of 1 ml/min. Analysis of CDRI-85/92 and its metabolites was performed with an isocratic elution. The mobile phase consisted of 30% methanol in 10 mM ammonium acetate buffer (pH 4) with 1% acetonitrile as modifier. Spectra were obtained using the photodiode array UV detector from 200 to 400 nm. The accuracy and reproducibility of the assay system was >95%. The metabolites were monitored at 250 nm. Data were processed using Waters 996 software.

Mass spectrometry

Mass spectrometry was carried out using a Micromass Quatro II Triple Quadrupole Mass Spectrometer in ESI positive mode, using Mass Lynx (version 3.1) software. Samples were introduced into the

ESI source through a syringe pump at the rate of 5 µl/min. The ESI capillary was set at 3.5 kV and cone voltage at 40 V. Nitrogen was used as both the nebulizing gas (10 l/h) and drying gas (250 l/h). The collision energy was 20 eV.

Synthesis of metabolite M2

Precursor (1) for the synthesis of metabolite M2 was prepared using a reported method /9/ and is shown in Figure 1B. The precursor (1) containing both threo- and erythro-isomers, was hydrolyzed with NaOH (3 eq) in tetrahydrofuran to give a mixture of the *cis*- and *trans*-acid from which pure *trans*-isomer was separated by preparative HPLC as a white solid, mp 152°C, IR ν_{max} (KBr) 1736, and 1595 cm^{-1}; ^1H NMR (DMSO-d6, 200 MHz) δ_H 4.45 (d, 1H J=8.5 Hz), 5.39 (t, 1H, J=8.5 Hz), 6.15 (dd, 1H, J=15.7 and 8.5 Hz), 6.76 (d, 1H, J=15.7 Hz), and 7.74-7.4 (m,5H); ^{13}C-NMR (DMSO-d6, 200 MHz) δ_C 162.0, 139.5, 136.3, 132.4, 132.0, 130.5 and 130.3; and FAB MS m/z 256 $[M+Na]^+$.

RESULTS

CDRI-85/92 inhibited CYP and GST enzymes in a concentration dependent manner (Fig. 2). At 0.5 µg/ml, 29% and 24% inhibition of

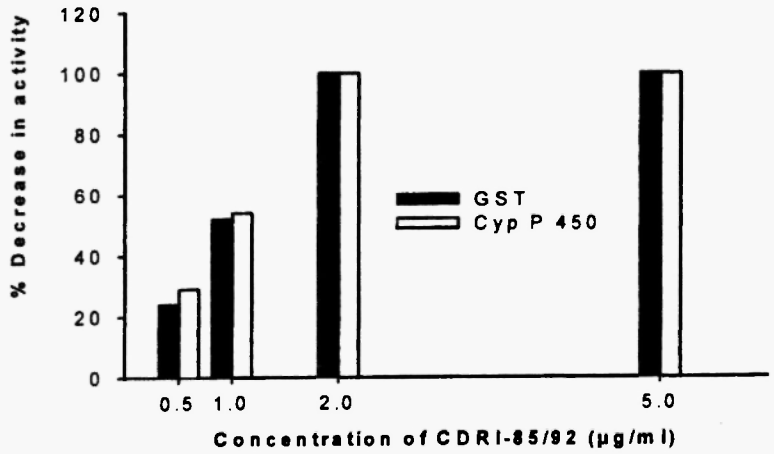


Fig. 2: Effect of CDRI-85/92 concentration on GST and CYP activity.

CYP and GST activity was observed, respectively. However, no further inhibitory effect of the drug was observed at lower concentrations. Approximately 50% inhibition of CYP and GST activities were observed with 1 μg/ml CDRI-85/92 concentration. Therefore, the *in vitro* metabolic studies were carried out at 1 μg/ml concentration of the drug, which is also its IC_{50} value. Out of various different concentrations of S9 fractions (10%, 20%, 40%, w/v), studies were carried out with 40% (w/v) S9 concentration, although this concentration metabolizes CDRI-85/92 but at an extremely slow rate. Moreover, higher concentration of cofactor solution had no effect on the drug metabolism. The time of incubation was increased till 24 h (Fig. 3). Individual compounds were well separated under the condi-

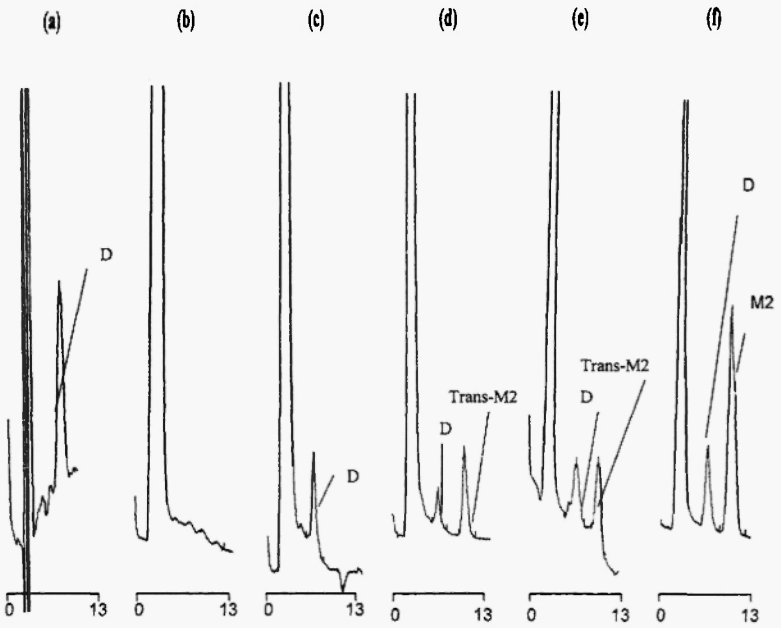


Fig. 3: High performance liquid chromatograms of **(a)** an analytical standard containing 500 ng/ml of CDRI-85/92, **(b)** an extract of drug-free S9 incubated for 24 h, **(c)** S9 containing 1,000 ng/ml of CDRI-85/92 incubated for 10 h, **(d)** S9 containing 1,000 ng/ml of CDRI-85/92 incubated for 24 h, **(e)** an analytical standard containing 250 ng/ml of *cis*-CDRI-85/92 and synthetic *trans*-CDRI-85/92, and **(f)** S9 containing 250 ng/ml of *cis*-CDRI-85/92 and synthetic *trans*-CDRI-85/92 incubated for 24 h.

tions used. Moreover, the extraction procedure and the chromatographic conditions yielded a clean chromatogram for the compound and its metabolites and endogenous impurities did not interfere with the elution of the compound and its metabolites.

Localization of the site of metabolism in the subcellular fractions was ascertained by analyzing the drug-incubated samples obtained from the microsomal and cytosolic samples separately. In the microsomal fraction, there was no change in the concentration of the drug up to 24 h (Table 1). Analysis of samples incubated with the cytosolic fraction for 21 h and 24 h showed 16.6% (83.4% drug concentration) and 86% (14% drug concentration) inhibition of the drug concentration, respectively, with the appearance of metabolite M2 corresponding to the *trans*-isomer. Moreover, metabolite M2 was observed as the major metabolite excreted in the urine after 12 h of a single 20 mg/kg oral dose of CDRI-85/92 (130 ng/ml of urine, of the 18 ml of urine in the time period 0-12 h).

On incubation with the PB-pretreated rat liver S9 fraction, there was an ~4-fold increase in CYP activity, and GST activity was also increased significantly (Fig. 4A). Analysis of the drug after 10, 21, and 24 h of incubation with PB-pretreated rat liver S9 fractions showed the appearance of another peak, designated as M1, towards the polar end (t_R, 6.3 ± 0.2 min), as well as M2 (t_R, 10.5 ± 0.2 min), and the drug peak (D; t_R, 7 ± 0.2 min). Concentration of M1, M2 and the parent drug at different time intervals is given in Table 2.

TABLE 1

Conversion of CDRI-85/92 to its *trans* isomer on incubation with microsomal and cytosolic fractions

Time (h)	% concentration of CDRI-85/92 on incubation with	
	Microsomal fraction	**Cytosolic fraction**
10	100	100
21	100	83.4
24	100	14

Values are means of three separate observations.

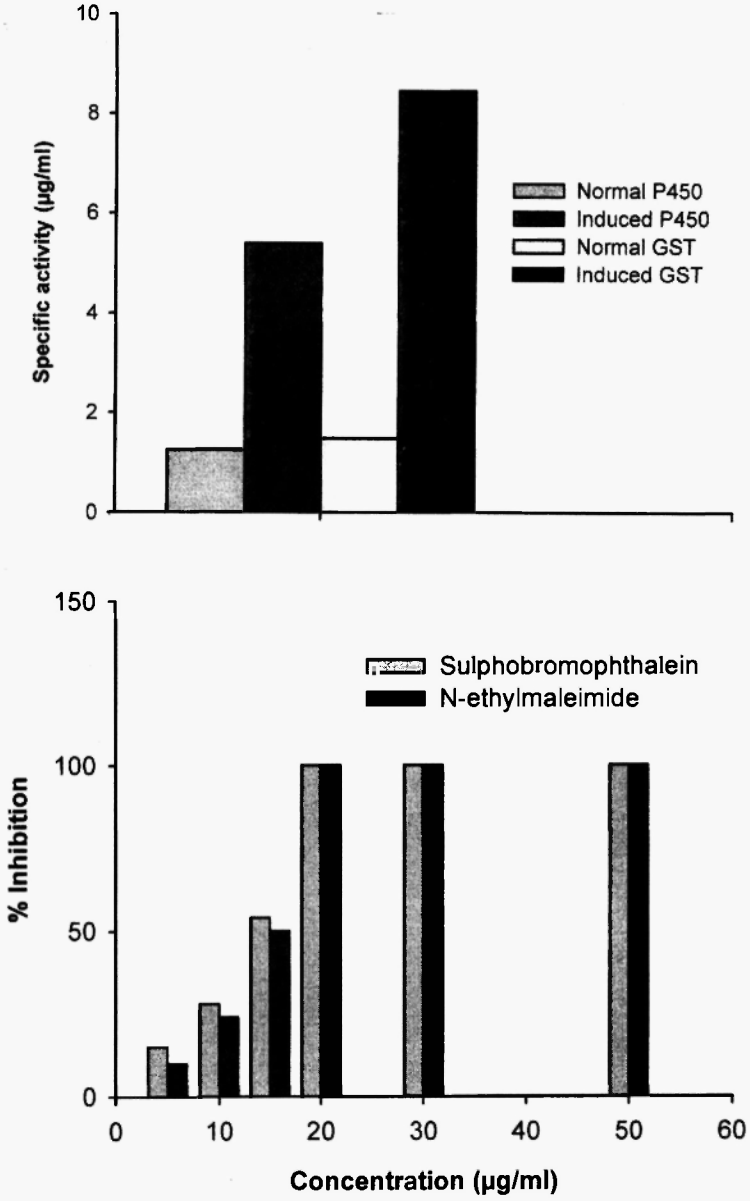


Fig. 4: (A) Effect of phenobarbitone on CYP and GST activity in the metabolism of CDRI-85/92. (B) Effect of GST inhibitors, *N*-ethylmaleimide (dark bars) and sulfobromophthalein (light bars) on GST activity in CDRI-85/92 metabolism [not done at 40 µg concentration].

TABLE 2

Levels of CDRI-85/92 and its metabolites M1 and M2 at different times after incubation with phenobarbitone-pretreated rat liver S9 fraction

Time (h)	Concentration*		
	M1	M2	CDRI-85/92
0	–	–	100
6	–	–	100
10	61	76	88
21	280	394	44
24	556	632	12

Values are means of three separate observations.
* Concentration in terms of ng/mg protein in case of M1 and M2 and percentage for CDRI 85/92.

Effect of GST inhibitors

The results of CDRI-85/92 metabolism from male Sprague-Dawley rat liver S9 fraction after incubation with various GST inhibitors are presented in Figure 4B. These studies showed that sulfobromophthalein and *N*-ethylmaleimide completely inhibited GST activity at 20 μg/ml concentration. No change in the drug concentration was found between 0 and 24 h in incubated biological samples containing these inhibitors. In a separate investigation, an insignificant change in GST activity was observed up to 24 h at 37°C.

Identification of metabolites

A product ion mass spectrum of the drug was obtained to facilitate structural characterization of the new metabolites by mass spectroscopy. The molecular ion was observed as MH^+ with m/z 234, as MNH_4^+ with m/z 251 (Fig. 5A).

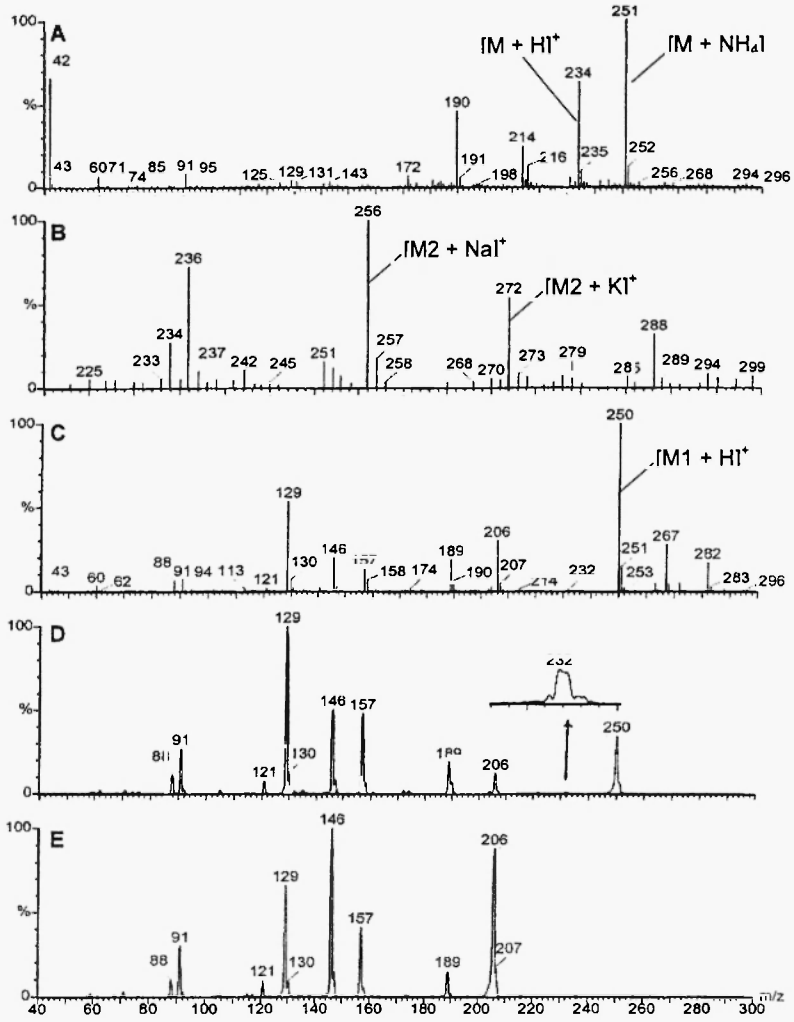


Fig. 5: Mass spectra of **(A)** product ion of CDRI-85/92, **(B)** metabolite M2 from cytosolic sample incubated for 24 h, **(C)** metabolite M1 from phenobarbitone-pretreated rat liver S9 fraction incubated for 24 h, **(D)** MS-MS of metabolite M1, and **(E)** MS-MS of daughter ion at m/z 206.

Following HPLC isolation, metabolites were characterized by ESI-MS, MS/MS and UV spectroscopy. The UV spectrum of M2 showed maximum absorption at 250 nm, similar to the parent compound. MS

analysis of the metabolite M2 from the cytosolic 24 h sample yielded efficient $[M+Na]^+$ and $[M+K]^+$ peaks at m/z 256 and 272, respectively (Fig. 5B). These ion-associated peaks demonstrated that the molecular weight of metabolite M2 was the same as that of the parent drug. These results in combination with thin layer chromatography (TLC) and spiking studies with synthetic *trans*-isomer strongly confirmed M2 as the *trans*-isomer of the drug. Metabolite M2 was, therefore, identified as *trans*-5-styryl-2-oxazolidinone-4-carboxylic acid.

The characterization of M1 was based on UV, ESI-MS and MS/MS data only, as the amount of this metabolite was not sufficient for NMR analysis. The maximum UV absorption of M1 was at 209 nm and may be due to loss of conjugation. The ESI-MS analysis of M1 showed a prominent MH^+ ion with m/z 250 (Fig. 5C). Its molecular mass suggested that it contained an additional oxygen atom. MS/MS fragmentation of the MH^+ ion yielded the various daughter ions (Fig. 5D). MS/MS analysis of the parent drug was also done in order to understand the possible fragmentation of the metabolite. A peak at m/z 232 $[250-18]^+$ indicated that the product ion is formed by the loss of a water molecule (Fig. 5E). The second daughter ion at m/z 206 $[250-44]^+$ resulted from the cleavage of the ring and subsequent loss of the CO_2. This kind of stable product ion formation, resulting in the loss of a water molecule and ring cleavage, was also seen in the collision induced dissociation spectrum of the parent drug. A similar fragmentation pattern of oxozolidinone losing CO_2 has been reported previously /10/. A peak at m/z 91 is indicative of the presence of a benzyl group in the metabolite. This also confirmed that the site of oxygenation was not in the phenyl ring of the parent drug. The MS/MS pattern of the metabolite permitted us to conclude that the site of oxygenation is on the styryl double bond carbon atom. Thus, metabolite M1 was identified as oxostyryloxaazolidinone carboxylic acid. A summary of the possible fragmentation pattern and MS/MS spectra supporting this structure of M1 are shown in Figure 6.

DISCUSSION

In this study, the metabolism of CDRI-85/92 was investigated by incubation of CDRI-85/92 with S9 fractions *in vitro* and *in vivo* from male Sprague-Dawley rats. The *in vitro* metabolic study was initiated

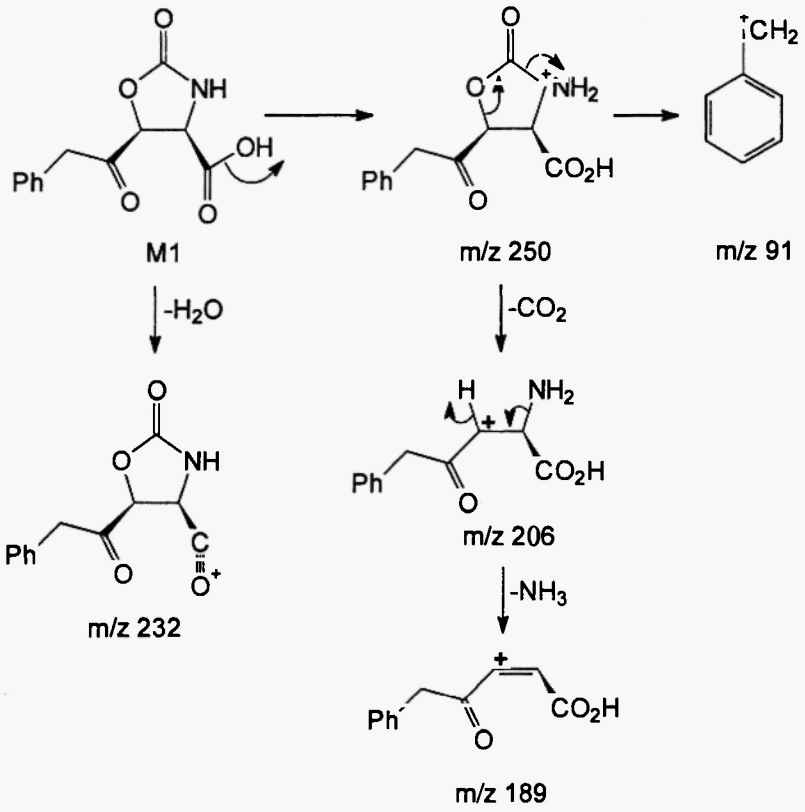


Fig. 6: Fragmentation pattern of metabolite M1 of CDRI-85/92.

with the standardization of the concentrations of S9, CDRI-85/92, cofactors and the time of incubation. Based on the observations, the 40% rat liver S9 fractions, which metabolize CDRI-85/92 but at an extremely slow rate, were used. The candidate drug inhibits CYP and GST activities in a concentration-dependent manner, being approximately 50% at 1 μg/ml (Fig. 3), which is its IC_{50} value. The Michaelis-Menten equation could not be used here because CDRI-85/92 was found to be an inhibitor of CYP and GST, instead of a substrate; therefore, we employed the concentration which inhibited GST activity by 50%, so that there was equilibrium between metabolism and the enzyme level. An increase in cofactor concentration did

not increase the metabolism of CDRI-85/92. Therefore, the *in vitro* metabolic studies were carried out with 40% rat liver S9 fractions, 1 μg/ml of drug concentration, with the cofactors mentioned in the Methods section. Following standardization of *in vitro* metabolism conditions, three approaches were used to identify the Phase I and Phase II enzymes involved in the metabolic transformations: (1) studies with separate cytosolic and microsomal fractions; (2) study with the CYP and GST inducer, PB; and (3) studies with selective chemical GST inhibitors. All experiments were carried out in triplicate. One set of experiment was done with vehicle, or without S9 fraction, only S9 fraction and S9 fractions devoid of the drug but supplemented with the same volume of vehicle i.e., triple distilled water. Additionally, the S9 fractions were found active even after 24 h of incubation, as there was no detectable loss in enzyme activity.

Untreated male Sprague-Dawley rat livers S9 fractions converted CDRI-85/92 to a major metabolite M2 (Fig. 7A). Moreover, analysis of samples incubated for 24 h showed 73% reduction of the drug concentration with the appearance of an additional peak at t_R 10.5 ± 0.2 min (Fig. 3d). TLC in methanol-water (50:50, v/v) and HPLC analysis of the synthetic *trans*-isomer indicated it to be the *trans*-isomer of CDRI-85/92 (Fig. 3f). The formation of this metabolite was higher in the cytosolic fraction as compared to the S9 fraction, whereas no conversion was observed with microsomal fractions. This increased rate of *cis-trans* conversion indicates metabolism by cytosolic enzymes. This shows the involvement of cytosolic GST in the isomerization reaction in which the *cis* form of the drug is converted to its *trans* isomer. The role of GST as isomerase has been reported in the case of Δ^5-androstene-3,17-dione metabolism /11/. *Cis* to *trans* conversion of retinoic acid has also been reported to be catalyzed by GST, as the site of the GST action is the chiral center present in the ring /12,13/. Another important perspective is the relatively long time required for the metabolism/conversion of *cis* to *trans* form by GST. The reason for this given in the literature is that rat liver possesses slow activity of GST /15-17/.

Liver microsomes from rats pretreated with PB, an inducer of CYP1A2, CYP3A2, CYP2B1 and CYP2B2 /14,17-20/, metabolized CDRI-85/92 to another major metabolite (M1) in addition to M2 (Table 2). On the basis of multi-wavelength HPLC, ESI-MS, MS/MS

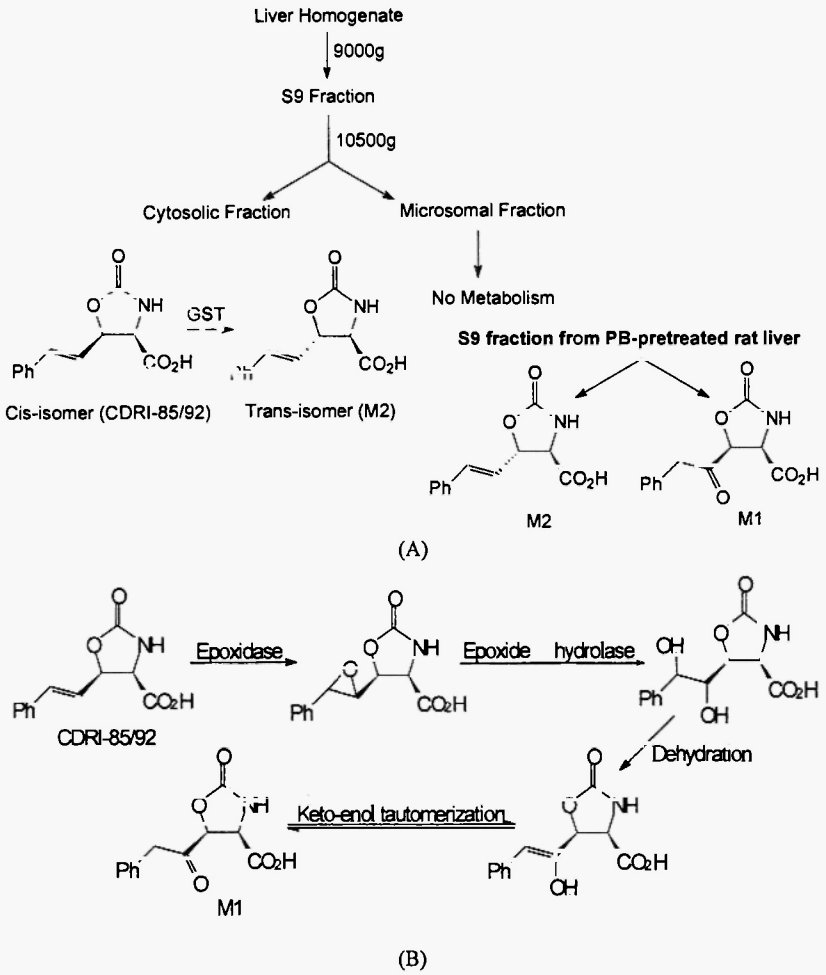


Fig. 7: Pathways of **(A)** isolation of CDRI-85/92 and its metabolites, and **(B)** proposed biotransformation of CDRI-85/92 to metabolite M1.

and UV analysis, both metabolites were identified. M2 was identified as the *trans* isomer of CDRI-85/92. The proposed biotransformation pathway of CDRI-85/92 to M1 is given in Figure 7B. Based on the above-mentioned characteristics, metabolite M1 was identified as oxostyryloxaazolidinone carboxylic acid.

The amount of CDRI-85/92 metabolites produced by rat liver microsomes was influenced by pretreatment with the CYP and GST

inducer, PB (Table 2). The formation of metabolites M1 and M2 points to the involvement of enzymes other than CYP in the metabolism of CDRI-85/92, since M2 was produced in untreated rat liver S9 fractions. GST seems to be involved in the formation of M2, as GST inhibitors completely inhibited the formation of M2. The glutathione (GSH)/GST system is a primary cellular mechanism protecting against cytotoxic and genotoxic stress. GST isozymes are Phase II detoxification enzymes, which catalyze the conjugation of cytotoxic agents to glutathione (γ-glutamyl cysteinyl glycine) producing less reactive chemical species /13/. In cellular detoxification, reduced GSH acts by conjugating with electrophilic drugs, either spontaneously or through catalysis by GST, rendering the compound more water soluble and, thus, more easily eliminated /13/. Although GST dependent conversion of CDRI-85/92 required a relatively long time for the formation of M2, it is interesting to note the formation of the *trans* isomer (M2) of the parent drug, in corroboration of the fact that the main site of GST action is the chiral center present in the ring. Therefore, the formation of this metabolite probably proceeds via initial ring opening and subsequent ring closing by the involvement of GST.

The results presented here therefore provide a metabolic pathway involving GST by which the *cis* form of CDRI 85/92 is converted into its *trans* form. This study will be helpful in providing clues about factors influencing the bioavailability of CDRI-85/92 as well as drug-drug interactions.

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