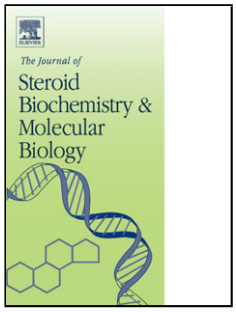

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Evaluation of the impact of 16-dehydropregnenolone on the activity and expression of rat hepatic cytochrome P450 enzymes

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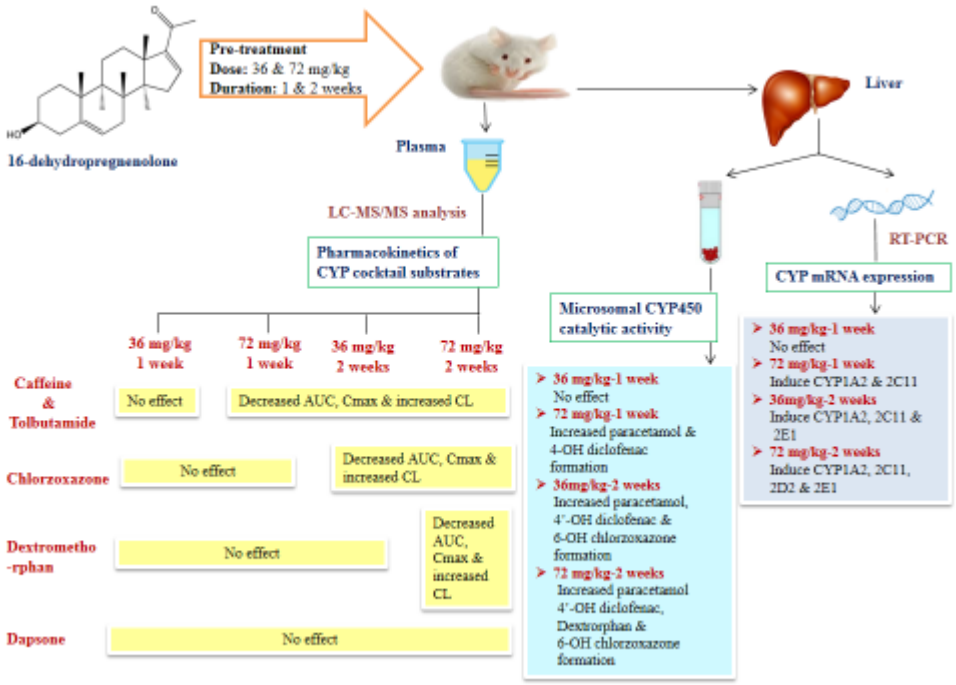
Graphical Abstract



Highlights:

- DHP decreases AUC, Cmax and increases CL of CYP1A2, 2C11, 2D2 and 2E1 substrates.
- DHP has no significant effect on pharmacokinetic parameters of CYP3A1 substrate.
- DHP increases enzyme activity of CYP1A2, 2C11, 2D2 and 2E1.
- DHP induces CYP1A2, 2C11, 2D2 and 2E1 mRNA levels with no influence on CYP3A1.
- Care should be taken when DHP is co-administered with CYP substrate drugs.

Abstract

16-dehydropregnenolone (DHP) is a promising novel antihyperlipidemic agent developed and patented by Central Drug Research Institute (CDRI), India. The purpose of the present study was to investigate whether DHP influences the activities and mRNA expression of hepatic drug-metabolizing cytochrome P450 (CYP) enzymes (CYP1A2, CYP2C11, CYP2D2, CYP2E1 and CYP3A1) in *Sprague-Dawley* (SD) rats. A cocktail suspension of CYP probe substrates which contained caffeine (CYP1A2), tolbutamide (CYP2C11), dextromethorphan (CYP2D2), chlorzoxazone (CYP2E1) and dapsone (CYP3A1) was administered orally on eighth- or fifteenth-day to rats pre-treated with DHP intragastrically at a dose of 36 and 72 mg/kg for one week and two weeks. The concentrations of probe drugs in plasma were estimated by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Alongside, the effect of DHP on CYPs activity and mRNA expression levels were assayed in isolated rat liver microsomes and by real-time reverse transcription-polymerase chain

reaction (RT-PCR), respectively. DHP had significant inducing effects on CYP1A2, 2C11, 2D2 and 2E1 with no effect on CYP3A1 in dose- and time-dependent manner, as revealed from the pharmacokinetic profiles of the probe drugs in rats. *In-vitro* microsomal activities and mRNA expression results were in good agreement with the *in-vivo* pharmacokinetic results. Collectively, the results unveiled that DHP is an inducer of rat hepatic CYP enzymes. Hence, intense attention should be paid when DHP is co-administered with drugs metabolized by CYP1A2, 2C11, 2D2 and 2E1, which might result in drug-drug interactions and therapeutic failure.

Keywords: 16-dehydropregnenolone, CYP450, cocktail, pharmacokinetics, microsomes, real time RT-PCR.

1. Introduction

16-dehydropregnenolone (DHP) (Figure 1) is a promising antihyperlipidemic agent developed and patented by CSIR-Central Drug Research Institute (CDRI), India, in its drug discovery program [1, 2]. DHP has also been identified in the circulation of pre-term neonates but not in adult human or rat [3]. It acts as a farnesoid X receptor (FXR) antagonist and has shown significant lipid lowering activity in pre-clinical studies [4]. DHP also possess indispensable pharmacological actions, notably anticancer, hypoglycemic, antiplatelet and antioxidant activities [5-7]. Chronic toxicity studies indicated that it possess a good therapeutic window without any untoward effects [2].

Cytochrome P450 (CYP) is a hemoprotein superfamily, among which CYP1, CYP2 and CYP3 play a major role in the metabolism of vast array of xenobiotics and endobiotics [8, 9]. Although the available reports hitherto on DHP shed a light on pharmacological activities, bioanalytical methods [10, 11] and pharmacokinetic studies in rats and rabbits [12, 13], there

is paucity of information regarding the impact of DHP on CYP enzymes. In the drug development process, it is highly plausible to anticipate undesirable drug-drug interactions (DDIs), the foremost contributing cause to drug failure in polypharmacy. DDIs arise when one of the concomitant drugs modulates the metabolism of co-administered drug by induction or inhibition of CYP enzymes involved in its metabolism, which may bring about detrimental consequences such as alteration of the concentration of drug in the blood, pharmacological activity and/or adverse drug reactions. [14-16] .

Animal models are routinely used in pre-clinical development of new compounds to predict their metabolic behaviour in humans, irrespective of the fact that humans differ from animals with regard to isoform composition, expression and catalytic activities of drug-metabolizing enzymes (DMEs) [17]. Moreover, quantification of CYP modulation in pre-clinical species can be tested as part of toxicological/safety evaluation before the candidate drug is selected for clinical development [18]. It has been established that human CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4/5 are homologues of rat CYP1A2, CYP2C11, CYP2D2, CYP2E1 and CYP3A1/2, respectively [19-22], which are most often implicated in DDI studies [23]. It is well-known fact that the multi-pill combination strategy is usually implemented in clinical practice. Exclusively in the treatment of hyperlipidemia, two or more drugs from different pharmacological categories are frequently co-administered to obtain desired therapeutic effects [24]. Furthermore, many antihyperlipidemic drugs are substrates of CYPs [25].

Under the scope of aforementioned evidences, it becomes imperative to elucidate the effect of DHP on regulating the hepatic CYP enzymes for better understanding its differential effect on regulating pharmacokinetic profiles of CYP substrates following oral administration in *Sprague-Dawley* (SD) rats. The changes in CYP enzyme catalytic activities were evaluated by comparing the plasma pharmacokinetics of probe substrates (caffeine for CYP1A2,

tolbutamide for CYP2C11, dextromethorphan for CYP2D6, chlorzoxazone for CYP2E1 and dapsone for CYP3A1/2) between control and DHP pre-treated groups *in-vivo*. Alongside this, the rate of formation of metabolite from specific CYP probe substrate was determined in isolated liver microsomes *in-vitro*. However, the major mechanism of CYP induction or inhibition is *via* altered rates of transcription, this biological phenomenon is addressed by studying the changes in messenger RNA (mRNA) levels using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) [26]. The outcomes of the present study would be beneficial for better forecast of drug-drug metabolic interactions mediated by DHP, which may provide valuable insights about safe and effective use of DHP with prescription drugs.

2. Experimental

2.1. Chemicals and reagents

DHP (5, 16-pregnadien-3β-ol-20-one) (purity > 99%) is a synthetic steroid molecule, which was synthesized at the Medicinal Process Chemistry Division of CDRI. Phenacetin (purity > 98%), paracetamol (purity > 98%), caffeine (purity > 98%), tolbutamide (purity > 98%), diclofenac (purity > 98%), 4′-hydroxy diclofenac (purity > 98%), dextromethorphan (purity > 98%), dextrorphan (purity > 98%), chlorzoxazone (purity > 98%), 6-hydroxy chlorzoxazone (purity > 98%), dapsone (purity > 98%), midazolam (purity > 98%), 1-hydroxy midazolam (purity > 98%), rosuvastatin (internal standard 1, IS1) (purity > 98%), chlorthalidone (internal standard 2, IS2) (purity > 98%), trizma base (Tris), β -Nicotinamide adenine dinucleotide phosphate (NADPH) reduced tetra sodium salt, magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium carboxymethylcellulose (Na.CMC), HPLC-grade tert-butyl methyl ether (TBME) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Chromatographic grade acetonitrile and methanol were obtained from Merck Chemicals

(Darmstadt, Germany). Heparin was purchased from Gland Pharma Ltd (Hyderabad, India). Ultrapure water (resistance > 18.2 mΩ) was obtained in-house using a Milli-Q PLUS PF water purifying system (Millipore, Bedford, MA). All other reagents and solvents were of analytical grade and purchased from standard chemical suppliers.

2.2. Animals

Male SD rats (220 ± 20g) were obtained from National Laboratory Animal Center (NLAC) of CSIR-CDRI. All experiments were executed according to the guidelines of Institutional Animal Ethical Committee (IAEC approval no: IAEC/2014/155) at CSIR-CDRI. The animals were acclimatized for one week in properly ventilated polypropylene cages under standard laboratory conditions with temperature (23-25 ^{o}C), relative humidity (50-70%) and day /night cycle (12/12 h) before initiation of experimental procedures. Animals were fed with standard chow diet and had free access to water *ad libitum* in hygienic conditions. Drug-free plasma containing heparin as anticoagulant was collected from adult healthy rats.

2.3 Effect of DHP on systemic exposure of CYP probe substrates

2.3.1 Study design, formulation and dosage regimen

The effect of DHP on rat CYP enzymes was studied at low dose (36 mg/kg) and high dose (72 mg/kg) administered orally for one week (short period) and two weeks (long period). Rats were randomly allocated into 5 groups (total 25 rats, n=5 per each group) to ensure equivalent mean body weight across the dose groups before dosing: vehicle control group (VCG), low dose for short period group (L-1W), high dose for short period group (H-1W), low dose for long period group (L-2W), high dose for long period group (H-2W). The VCG and other four treatment groups were administered orally 0.5% w/v Na.CMC suspension

(vehicle) and DHP suspended in a vehicle, respectively, by gavage once daily on every morning for specified treatment duration.

2.3.2 Pharmacokinetic study

On the eighth or the fifteenth day, all rats were administered orally with the cocktail substrates containing caffeine (20 mg/kg) [27], tolbutamide (15 mg/kg) [8], dextromethorphan (20 mg/kg) [20], chlorzoxazone (50 mg/kg) [28] and dapsone (20 mg/kg) [27] suspended in 0.5% w/v Na. CMC at a dose of 5 mL/kg. At pre-defined time intervals (0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 24 h), blood samples were withdrawn from each rat into heparinized polypropylene tubes *via* the oculi chorioideae vein under light ether anesthesia. The total volume of blood collected within 24 h was less than 5% of the total blood volume. Plasma was harvested by centrifuging the blood samples at 4000×g for 10 min and stored at -80 °C until LC-MS/MS analysis.

2.3.3 Sample preparation

All the biosamples were processed by one-step liquid-liquid extraction (LLE) method. To an aliquot of 50 µL plasma, 10 µL of IS (10 ng/mL) and subsequently extracted with TBME by vortexing (Vibrax VXR basic, staufen, Germany) for 5 min and centrifuging (Eppendorf, Hamburg, Germany) at 4000×g for 10 min. The clear organic supernatant was transferred to another set of clean tubes and evaporated to dryness under a nitrogen stream at 40 °C and 20 psi (Turbovap®, MA, United States). The dried residues were then reconstituted with 50 µL acetonitrile and vortex mixed. The resultant mixture was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The plasma samples were also analyzed for the presence of DHP using the previously reported extraction method and LC-MS/MS method [11].

2.3.4 Bioanalysis using cocktail LC-MS/MS method

LC was performed on Waters HPLC unit (Waters Corp., Manchester, UK) equipped to API 3200 triple quadrupole mass spectrometer with TurboIon spray interface (AB Sciex, Canada). The chromatographic separation of cocktail substrates was achieved on a Waters Symmetry-shield C_{18} reversed phase column (150 × 4.6 mm i.d., 5 μm) preceded with a guard column of same stationary phase (Thermo Scientific, USA), maintained at a temperature of 40 ^{o}C. The mobile phase comprising of solvent A (0.1% formic acid in triple distilled water) and solvent B (acetonitrile) was pumped isocratically in the ratio of 30:70, v/v at a flow rate of 0.6 mL/min. The injection volume was 10 μL and the time taken for each analytical run was 4 min. Quantitation of analytes was performed in both positive and negative multiple reaction monitoring (MRM) mode due to its selectivity and sensitivity [29]. The instrument parameters such as curtain gas, collision gas (CAD), nebulizer gas (GS1), turboIon gas (GS2) and temperature were set at 15 psi, medium, 50 psi, 50 psi and 450 °C, respectively. The dwell time was 200 ms per MRM channel under unit mass resolution in the mass analyzers. All MS/MS parameters were optimized to ensure the reproducible response and are listed in Table 1. Analyst 1.6 software (AB Sciex, Canada) controlled the equipment, data acquisition and analysis.

2.3.5 Pharmacokinetic data analysis

Plasma concentration-time profiles were analysed by non-compartmental model and pharmacokinetic parameters including maximum plasma concentration (C_{max}), area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) and clearance (CL) were determined using Phoenix WinNonlin software (version 6.3, Pharsight Corporation, Mountain view, USA). C_{max} is the maximum plasma concentration that a drug achieves in the body after dosing. AUC represents the total integrated area under the plasma concentration-

time profile and expresses the total amount of drug that enters into the systemic circulation following its administration. Clearance is the rate at which the drug is cleared or removed from the body per unit time.

2.4 In-vitro assessment of CYP-associated activities in liver microsomes

2.4.1 Drug administration and sample collection

Animal subjects, study design, formulation and dosage regimen were similar to those mentioned in section 2.3.1. Twenty-four hours after the last treatment, all rats were euthanized with anaesthetic ether and the liver tissues were excised quickly, perfused with ice-cold saline to remove blood residue, blotted dry and stored at -80 °C. The liver tissues were divided to provide samples for microsomes preparation and RNA isolation.

2.4.2 Preparation of microsomes from rat liver

Microsomes were prepared by differential centrifugation method [30]. Briefly, liver samples were homogenized in Tris-HCl buffer (50 mM, pH 7.4) containing KCl (0.15 M), EDTA (1 mM) and sucrose (0.25 M) using a Potter-Elvjhem type homogenizer. The homogenate was centrifuged (Eppendorf, Hamburg, USA) at 10,000×g for 30 minutes at 4°C. The supernatant was further centrifuged (Optima Max-XP, Beckman Coulter, USA) at 1, 05,000×g for 60 minutes at 4 °C. The microsomal pellet, thus obtained from each experimental group was washed and resuspended in microsomal storage buffer containing Tris-HCl (50 mM, pH 7.4), KCl (0.15 M), EDTA (1 mM), glycerol (20%, v/v) and aliquots were stored at -80 °C until use. Protein content in the microsomal fraction was estimated by Lowry method [31] using bovine serum albumin (BSA) as a calibration standard. Absorbance was measured at 660 nM on multimode microplate reader (Tecan, Switzerland) using Magellan ^{TM} software.

2.4.3 Measurement of microsomal CYP enzyme activities

Probe substrates are used to determine the activity of each specific CYP enzyme. Phenacetin (5 µM), diclofenac (4 µM), dextromethorphan (5 µM), chlorzoxazone (50 µM) and midazolam (5 µM) are used as probe substrates for index reactions such as phenacetin O-deethylation (CYP1A2), diclofenac 4-hydroxylation (CYP2C11), dextromethorphan O-demethylation (CYP2D2), chlorzoxazone 6-hydroxylation (CYP2E1) and midazolam 1-hydroxylation (CYP3A1/2), respectively [32] . The incubation mixture (n=5) consisted of Tris-HCl buffer (50 mM, pH 7.4), $MgCl_2$ (20 mM), microsomes (0.5 mg/ml) and NADPH (2 mM) in a final volume of 0.2 mL. Following pre-incubation in a heated water bath at 37 °C for 5 min, the enzymatic reactions were initiated by spiking substrates (0.5%, v/v organic content) and incubated for 15 min. Subsequently, the reactions were terminated by quenching with 400 µL ice-cold acetonitrile containing IS (10 ng/mL) to terminate the reaction. All samples were vortex mixed, centrifuged at 12,000×g for 20 min and the supernatant was collected for determination of metabolites (paracetamol, 4-hydroxy diclofenac, dextrorphan, 6-hydroxy chlorzoxazone and 1-hydroxy midazolam) by LC-MS/MS analysis. All metabolites were separated on Thermo Accucore aQ C18 (150×4.6 mm i.d., 2.1 µm) column with a mobile phase consisting of solvent A (0.1% formic acid in triple distilled water) and solvent B (acetonitrile with 0.1% formic acid) in the ratio 80:20, v/v at a flow rate of 0.7 mL/min. The injection volume and total run time was set to 10 µL and 7 min, respectively. The instrument parameters such as curtain gas, collision gas (CAD), nebulizer gas (GS1), turboIon gas (GS2) and temperature were set at 15 psi, medium, 45 psi, 45 psi and 450 °C, respectively. The optimized MRM transitions for all the metabolites are listed in Table 1. The enzyme catalytic activity was expressed as pmol of metabolite formation/min/mg microsomal protein.

2.5 Effect of DHP on mRNA expression of CYP enzymes

2.5.1 RNA isolation

Total RNA was extracted from 0.1-0.2g liver tissue by TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA concentration was determined, and the quality of isolated RNA was evaluated using the 260/280 nm absorbance ratio (1.8-2.0 indicates a highly pure sample) using NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). RNA integrity was confirmed by running samples on gel electrophoresis using 1% agarose gel.

2.5.2 cDNA synthesis and RT-PCR analysis

RNA samples (2 µg) were reverse transcribed to cDNA by Verso cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions using SureCycler 8800 (Agilent Technologies, USA). Further, RT-PCR analysis was performed in 96-well plates (LightCycler 480) for a 20 µL reaction mixture consisting of 2× master mix, forward and reverse primer, cDNA, and nuclease free water using DyNAmo Flash SYBR Green qPCR Kit recommendations (Thermo Scientific, USA) on Light Cycler 480 II (Roche Diagnostics). Each PCR thermo cycle consisted of initial denaturation at 95 ˚C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 30 sec and final extension at 65°C for 5 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The fold change in the expression level of target gene following DHP treatment relative to untreated control was calculated using $2^{-\Delta\Delta Ct}$ method, where ΔCt represents the differences in cycle threshold (Ct) numbers between the target gene and GAPDH, and ΔΔCt represents the relative change in the differences between treatment and control groups. The data are represented as the fold change

in gene expression relative to the control. The sequences of the forward and reverse oligonucleotide primers are summarized in the Table 2.

2.6 Statistical analyses

Experimental data were represented as mean ± standard error of mean (SEM). Statistical comparisons were executed using GraphPad Prism 5.0 software (GraphPad Inc., USA). Statistical significant differences were evaluated between control and treatment groups using student's unpaired t-test and/or one-way ANOVA with post-hoc dunnett test when appropriate. *P*-values less than 0.05 are considered to be significantly different for all tests.

3. Results

3.1 Effect of DHP on the pharmacokinetics of CYP probe substrates

3.1.1 Effect of DHP on CYP1A2

The effect of different dosage regimens of DHP on pharmacokinetic parameters of caffeine in SD rats are represented in Table 3. Mean plasma concentration-time profiles of caffeine in different groups are depicted in Figure 2. After pretreatment with DHP, the $AUC_{0-\infty}$ and C_{max} of caffeine in H-1W group were significantly reduced by 31.2% and 33.8%, respectively, and the CL of caffeine was significantly accelerated by 30.3% in comparison to VCG. The $AUC_{0-\infty}$ and C_{max} of caffeine in L-2W group were decreased significantly by 85.9% and 54.6%, respectively and CL of caffeine was increased significantly by 84.5% compared to those of VCG. The $AUC_{0-\infty}$ and C_{max} of caffeine in H-2W group were decreased significantly by 151.1% and 98.0%, respectively and CL was increased by 149.1% in comparison to VCG. The pharmacokinetic parameters of caffeine showed no significant difference between L-1W group and VCG.

3.1.2 Effect of DHP on CYP2C11

Pharmacokinetic profiles of tolbutamide in control and treatment groups were used to describe the activity of CYP2C11. Conferring to the results shown in Table 3 and Figure 2, the influence of DHP on pharmacokinetic parameters of tolbutamide in L-1W group was insignificant when compared to those of VCG. After pretreatment with DHP at high dose for short period in H-1W group, the $AUC_{0-\infty}$ and C_{max} of tolbutamide were decreased significantly by 4.6% and 3.9%, respectively compared to those of VCG, whereas CL of tolbutamide was increased significantly by 6.3%. On the other hand, the $AUC_{0-\infty}$ and C_{max} of tolbutamide in L-2W group were decreased significantly by 9.4% and 7.8%, respectively and CL of tolbutamide was increased significantly by 9.4% compared to those of VCG. The $AUC_{0-\infty}$ and C_{max} of tolbutamide in H-2W group were decreased significantly by 18.4% and 13.8%, respectively and CL was significantly increased by 18.4% in comparison to VCG.

3.1.3 Effect of DHP on CYP2D2

CYP2D2 activity was assessed by comparing the pharmacokinetics of dextromethorphan in the study groups. The mean plasma concentration-time curves of dextromethorphan and the corresponding pharmacokinetic parameters are presented in Table 3 and Figure 2, respectively. In all DHP pre-treated groups except H-2W group, the $AUC_{0-\infty}$, C_{max} and CL of dextromethorphan were not modulated significantly, as compared to those observed in the VCG. Interestingly, it was found that the $AUC_{0-\infty}$ and C_{max} were lessened significantly by 87.5% and 36.3%, respectively; in contrast CL was enhanced by 77.2% for dextromethorphan in H-2W group compared to those of VCG.

3.1.4 Effect of DHP on CYP2E1

CYP2E1 activity was determined by comparing pharmacokinetic behaviours of chlorzoxazone between control and DHP treatment groups. The impact of DHP on mean

plasma concentration-time profile and pharmacokinetic parameters of chlorzoxazone in rats are given in Table 3 and Figure 2, respectively. The effect of DHP at low and high dose given for one week on $AUC_{0-\infty}$, C_{max} and CL of chlorzoxazone was nondistinctive, while a marked difference existed at same doses administered for two weeks, in comparison to VCG. It is noteworthy that the $AUC_{0-\infty}$ and C_{max} of chlorzoxazone in L-2W group were declined significantly by 44.0% and 15.5%, respectively and CL of chlorzoxazone was augmented by 43.8%. But in H-2W group, the $AUC_{0-\infty}$ and C_{max} of chlorzoxazone were significantly dropped by 102.57% and 43.8%, respectively, however CL of chlorzoxazone was enhanced by 103.6%.

3.1.5 Effect of DHP on CYP3A1

The pharmacokinetic parameters and mean plasma concentration-time profiles of dapsone in rats from different study groups are presented in Table 3 and Figure 2, respectively. There was no significant change in the systemic exposure levels ($AUC_{0-\infty}$ and $C_{max)}$ and CL of dapsone between VCG and treatment groups.

3.2 Effect of DHP on CYP activities in rat liver microsomes

The potential influence of DHP on CYP enzyme activities was investigated with the liver microsomes prepared from the rats pre-treated with DHP and the results are represented in Figure 3. Compared with the VCG, the formation of paracetamol from phenacetin by CYP1A2 was unconspicuous in L-1W group but it was significantly increased to 1.79-fold, 2.07-fold and 2.80-fold in H-1W, L-2W and H-2W groups, respectively. Similar trend was observed for CYP2C11 catalyzing the formation of 4-hydroxy diclofenac from diclofenac in which the activity was remarkably augmented to 1.28-fold, 1.36-fold and 1.40-fold in H-1W, L-2W and H-2W groups, respectively, but with no effect on the activity in L-1W group in comparison to VCG. The formation of dextrorphan from dextromethorphan was not changed

significantly in L-1W, H-1Wand L-2W groups; besides, a 1.91-fold increase in the activity was recorded in H-2W group compared with the VCG. The activity of CYP2E1 towards the metabolism of chlorzoxazone to 6-hydroxy chlorzoxazone was insignificantly affected by DHP in L-1W and H-1W groups, but markedly increased to 1.45-fold and 2.0-fold in L-2W and H-2W groups, respectively, in comparison to VCG. DHP had no significant effect on the activity of CYP3A1 at low and high dose administered for short and long period compared to that of VCG.

3.3 Effect of DHP on mRNA expression of CYP enzymes

As represented in Figure 4, the mRNA expression level of CYP1A2 in H-1W group, L-2W group and H-2W group was significantly increased to 1.75-fold, 1.96-fold and 2.26-fold to that observed in the VCG, respectively. However, the mRNA expression level of CYP2C11 in H-1W group, L-2W group and H-2W group was significantly up-regulated to 1.45-fold, 1.57-fold and 1.63-fold to that observed in the VCG, respectively. In addition, no significant differences in the mRNA expression level of CYP2D2 were observed between VCG and DHP treated groups except H-2W group in which the expression level was increased to 2.12-fold. Compared to VCG, DHP treatment increased the CYP2E1 mRNA expression to 1.58-fold and 2.03-fold in L-2W group and H-2W group, respectively, without any significant effect in L-1W group and H-1W group. The mRNA expression for CYP3A1 showed no significant difference among all treatment groups.

4. Discussion

A wide variety of drugs are metabolized by CYP enzymes in the liver, and more than 90% of DDIs occur at the CYP enzyme-catalyzed step [33]. During the drug development process, investigators often conduct two types of drug metabolism studies to assess the potential for CYP450-based drug interactions. One type of study characterizes the metabolic pathway of

the new drug and the potential for other drugs to modify the metabolism of the new drug. The other type of study evaluates the potential for the new drug to alter the metabolism of other drugs which is more challenging than the first type of study [34]. The present report sought to investigate the potential of DHP to alter the metabolism of CYP probe substrate drugs. The modulation (induction or inhibition) of CYPs is recognized as the major mechanism of metabolic DDIs [35]. Drug regulatory authorities of United States, Europe and China require that metabolic research based on the CYP system should be included in new drug evaluations. Thus, a full characterization of DHP for its influence on CYP enzymes is necessary in order the best to understand the likely extent of DDIs.

It has been reported that DHP had very low bioavailability (~3%), quick absorption and rapid elimination ($t_{1/2}$ = 2-3h) at an oral dose of 72 mg/kg in male SD rats. 5-pregnene-3β-ol-16, 17-epoxy-20-one (M1) is the major metabolite in rat plasma [12]. The plasma concentration-time profile of DHP following oral (36, 72, 100 and 150 mg/kg) and intravenous (1, 5 and 10 mg/kg) administration has revealed that DHP exhibited multiple peak phenomenon at ~0.25, ~1.5-2, ~3-4 and ~7-8 h post dose [13]. Preliminary studies on CYP mediated metabolism of DHP have been performed in-house (data not shown) wherein we found that CYP3A was mainly responsible for its metabolism among CYP1A2, 2C11, 2D2 and 2E1. However, the detailed metabolic pathway of DHP needs further investigation.

DHP exhibited antihyperlipidemic activity at a dose range of 36-72 mg/kg in SD rats [13]. Based on the dose proportionality studies, it was proved that both pharmacokinetics and pharmacodynamic activity of DHP exhibited saturation above 72 mg/kg dose [12, 13]. Hence, in accordance to the literature, we have chosen 36 mg/kg as low dose and 72 mg/kg as high dose for consecutive 7 days (short period) or 14 days (long period) treatment to evaluate the effect of DHP on the five pivotal rat hepatic CYP enzymes, notably, CYP1A2, 2C11, 2D2, 2E1 and 3A1 based on the fact that they are homologous to human CYP1A2, CYP2C9,

CYP2D6, CYP2E1 and CYP3A4 by 78%, 76%, 77%, 71% and 78%, respectively [36]. Currently, DHP is in pre-clinical stage for which regulatory toxicology studies are ongoing in male *Sprague-Dawley* (SD) rats. The present study was performed in male SD rats such that the findings could be correlated well with the outcomes of toxicological studies.

The study represented herein is the first attempt in demonstrating the effect of DHP on five major CYP enzymes in rats using a probe drug cocktail approach. The selection of different probe drugs for *in vitro* and *in vivo* evaluation of CYP enzyme activity was based on the recommendations given by United States Food and Drug Administration (USFDA) regulatory authority and the literature preference [22, 27, 34, 37-40]. Previous reports have demonstrated the selection criteria of probe substrates for CYPs which includes the availability of substrate and metabolite standards, selectivity, sensitivity, sufficient metabolite production at low substrate concentrations, effect of organic solvents, inter-substrate interactions, metabolic and kinetic properties, etc [41, 42]. In each case, the substrate is metabolized exclusively or primarily by its specific enzyme. The activity levels of the multiple enzymes are less than 10% of the activity of the main enzyme, which indicate that the substrates are specific for the desired enzyme [43]. The results showed that DHP at a low dose of 36 mg/kg given for one week had no effect on any of the CYPs studied, but when the treatment duration increased from one week to two weeks, the metabolic clearance of caffeine, tolbutamide and chlorzoxazone was increased and consequently, their plasma levels were dropped to several folds. The rate of formation of paracetamol, 4-hydroxy tolbutamide and 6-hydroxy chlorzoxazone from the respective CYP1A2, 2C11 and 2E1 substrates was increased in liver microsomes, indicating the rise in the CYP enzyme catalytic activity in rats treated with DHP at low dose given for two weeks. Interestingly, the gene expression study revealed that the mRNA expression levels of CYP1A2, 2C11 and 2E1 were clearly enhanced to varying degrees, which were consistent with the outcomes of *in vivo* pharmacokinetic and

in vitro microsomal studies. Furthermore, at high dose (72 mg/kg) given for seven days, the effect of DHP was observed only on CYP1A2 and 2C11. But, after fourteen days treatment with the high dose DHP had accelerated the activities and mRNA levels of CYP1A2, 2C11, 2D2 and 2E1. Hence, DHP has the potential to induce rat CYP1A2, 2C11, 2D2 and 2E1 in a dose- and time-dependent manner. On the other hand, CYP3A which represents the major isoenzyme in the liver [44, 45] and responsible for metabolism of vast array of xenobiotics, was not significantly affected by DHP as reflected from pharmacokinetic parameters of dapsone, the rate of 1-hydroxy midazolam formation and the mRNA expression level of CYP3A1 in control and DHP-treated rats. Also, it was found that DHP and its metabolites were completely eliminated from rats demonstrating the absence of accumulation when CYP substrates were administered. Hence, the possibility of competition between DHP and dapsone and/or other CYP substrates could be ruled out, which was confirmed by insignificant alteration of AUC, Cmax and CL of dapsone in DHP treated rats compared to those of control rats. Thus, it is speculated that a typical dose of DHP is unlikely to have clinically relevant effects on the pharmacokinetic disposition of drugs primarily dependent on CYP3A pathways for metabolism.

Based on the findings, it is anticipated that the prolonged treatment with DHP could induce CYP1A2, 2C9, 2D6 and 2E1 in humans which leads to a reduction in concentration of concomitant drug in the blood due to increased metabolism and makes it hard to achieve the therapeutic effect at target site. Enzyme induction may also result in increased toxicity if the metabolites formed during biotransformation are chemically reactive. The clinical significance of a metabolic drug interaction will depend on the magnitude of the change in the concentration of active species (parent drug and/or metabolites) at the site of pharmacological action and the therapeutic index of the drug. The smaller the difference between toxicity and efficacy, the greater the likelihood that a drug interaction will have

serious clinical consequences [46]. Numerous studies demonstrated that the induction of drug metabolizing enzymes contributes to approximately 30% of drug interactions [35]. Thus, the modulating effects described herein might have substantial clinical consequences for the evaluation of potential risk of drug interactions with DHP. Knowledge about the effect of induction on CYPs by DHP could guide clinical co-administration of drugs reasonably well, and prevent therapeutic failure due to decrease in subtherapeutic drug concentrations or toxicological implications due to higher levels of a toxic metabolite [47, 48].

5. Conclusion

In conclusion, the current investigation provides ample scientific evidences that DHP up-regulate the activities and expression of CYP1A2, 2C11, 2D2 and 2E1 in rats. Hence, we could not disregard that the co-medication of DHP with drugs metabolized by human CYP1A2, CYP2C9, CYP2D6 and CYP2E1 may induce metabolism of these drugs and alters the plasma levels of these drugs, thereby leading to relevant drug-DHP interactions. However, the insignificant effect of DHP on CYP3A1 implies that there could be no clinically relevant drug-DHP interactions between the drugs metabolized by human CYP3A4 and DHP when both are administered concomitantly. These findings provide a safety reminder for precautious co-administration of DHP and the drugs metabolized by CYP1A2, CYP2C9, CYP2D6 and CYP2E1. Further studies in human *in vitro* systems are ongoing in our research laboratory to delineate the clinical relevance of DHP-mediated impact on CYP enzymes. Our results may serve as a valuable tool to provide a basis for the prediction of clinical drug-drug interactions observed during early/late drug development process. Besides, these findings may provide the rational basis for the selection of suitable antihyperlipidemic drugs in the development of combination therapy with DHP for the treatment of hyperlipidemia.

6. Conflict of interest

There is no potential conflict of interest.

7. Acknowledgements

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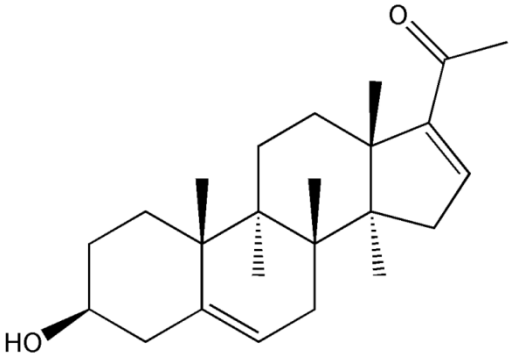

Fig. 1.: Chemical structure of 16-dehydropregnenolone

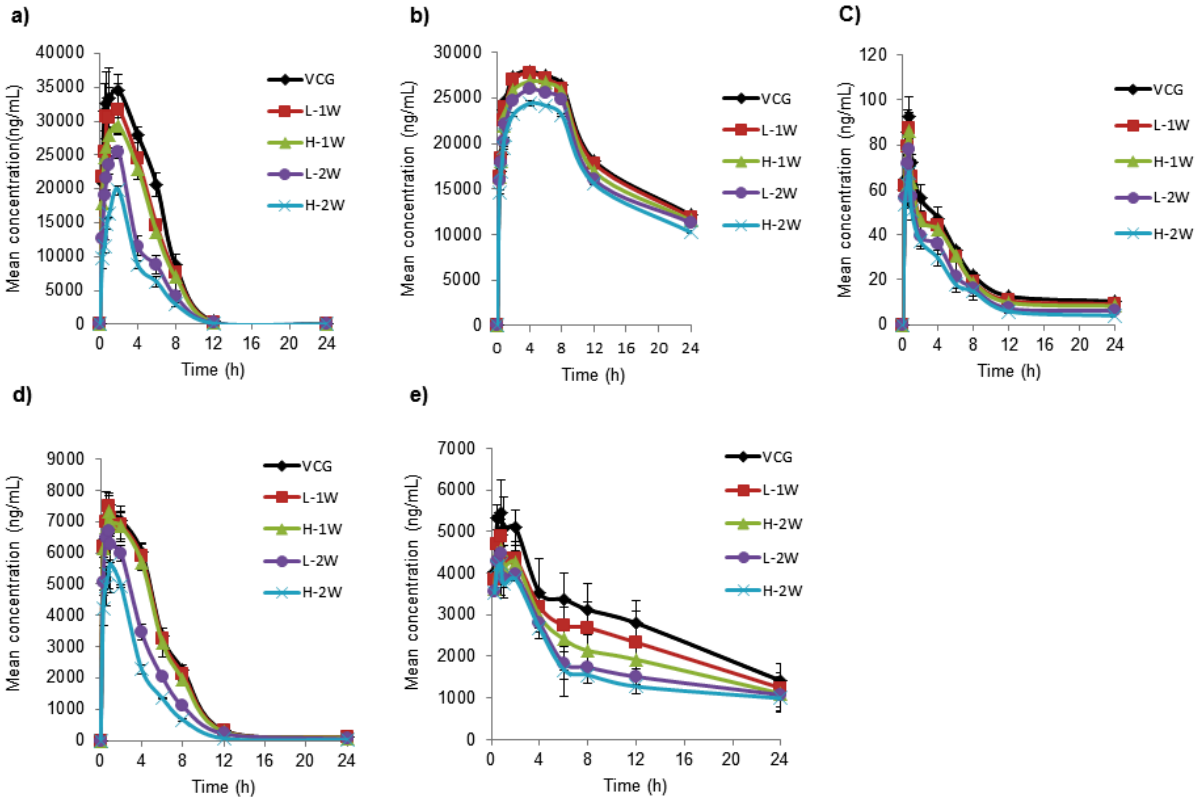

Fig. 2.: Mean plasma concentration-time profiles of caffeine (a); tolbutamide (b); dextromethorphan (c); chlorzoxazone (d); dapsone (e) in SD rats. VCG: vehicle control group; L-1W: low dose (36 mg/kg) given for 1 week; H-1W: high dose (72 mg/kg) given for 1 week; L-2W: low dose (36 mg/kg) given for 2 weeks; H-2W: high dose (72 mg/kg) given for 2 weeks. Error bars represent SEM (*n*=5).

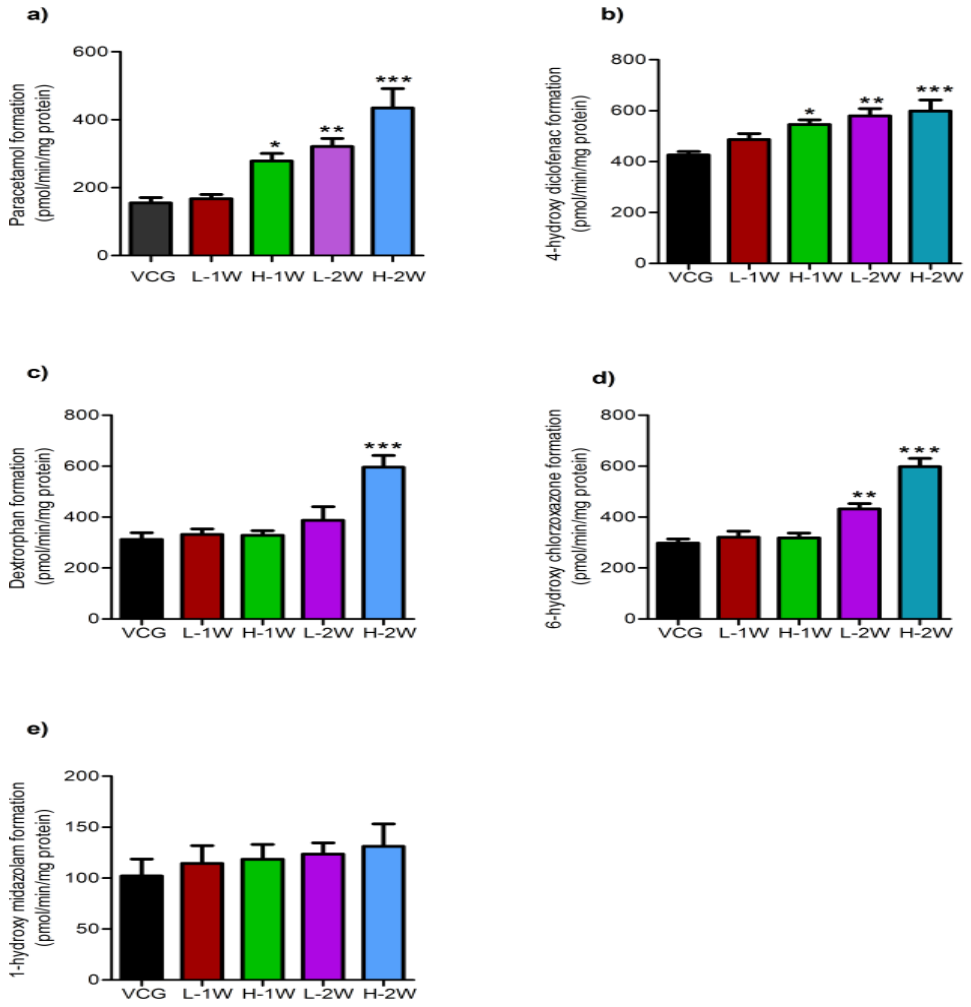

Fig. 3.: Changes in liver microsomal CYP activities in rats administered with DHP. VCG: vehicle control group; L-1W: low dose (36 mg/kg) given for 1 week; H-1W: high dose (72 mg/kg) given for 1 week; L-2W: low dose (36 mg/kg) given for 2 weeks; H-2W: high dose (72 mg/kg) given for 2 weeks. Phenacetin O-deethylation (CYP1A2) (a); diclofenac 4-hydroxylation (CYP2C11) (b); dextromethorphan O-demethylation (CYP2D2) (c); chlorzoxazone 6-hydroxylation (CYP2E1) (d); midazolam 1-hydroxylation (CYP3A1) (e). Results are expressed in mean ± SEM (n=5). *Significantly different from VCG, $P < 0.05$; ** significantly different from VCG, $P < 0.01$ and *** significantly different from VCG, $P < 0.001$.

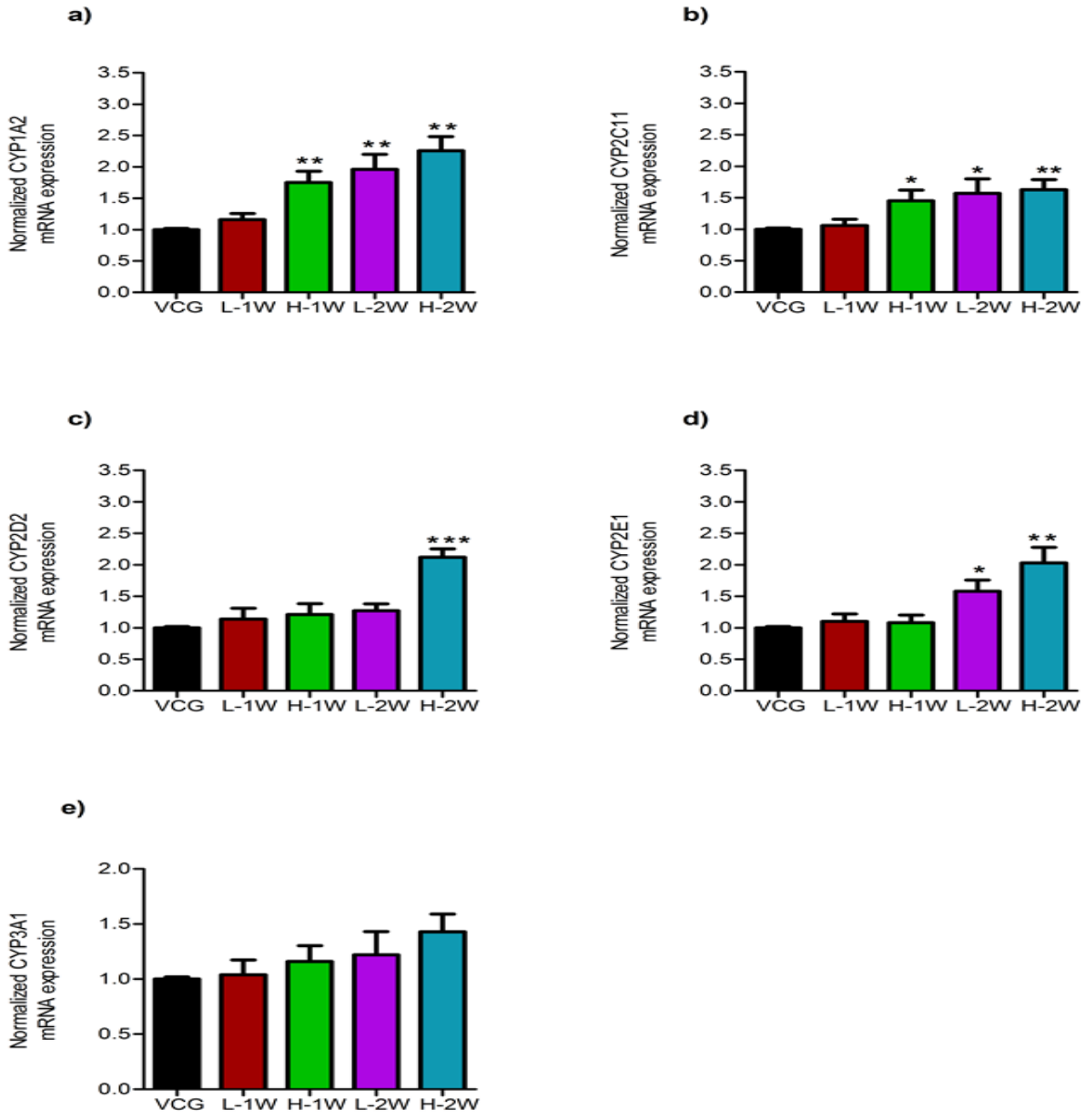

Fig.4.: Changes in mRNA expression of hepatic CYP1A2 (a); CYP2C11 (b); CYP2D2 (c); CYP2E1 (d); and CYP3A1 (e) in rats administered with DHP. VCG: vehicle control group; L-1W: low dose (36 mg/kg) given for 1 week; H-1W: high dose (72 mg/kg) given for 1 week; L-2W: low dose (36 mg/kg) given for 2 weeks; H-2W: high dose (72 mg/kg) given for 2 weeks. Results are expressed in mean ± SEM (n=5). *Significantly different from VCG, $P < 0.05$; ** significantly different from VCG, $P < 0.01$ and *** significantly different from VCG, $P < 0.001$.

Tables

Table 1: Optimized MS/MS parameters of CYP probe substrates and metabolites.

Compound	MRM Transition	Polarity	DP (eV)	CE (eV)
DHP	315.1→137.5	+	55	37
Caffeine	195.1→138.0	+	56	19
Tolbutamide	271.0→91.0	+	36	50
Dextromethorphan	272.2→171.0	-	53	54
Chlorzoxazone	167.6→132.0	+	-52	-30
Dapsone	249.0→156.0	+	53	16
Paracetamol	152.1→110.0		45	21
4'-hydroxydiclofenac	312.1→230.1	+	41	42
Dextrorphan	258.2→157.1	+	61	50
6-hydroxychlorzoxazone	183.8→120.0	-	-35	-28
1'-hydroxymidazolam	342.1→324.0	+	56	26
Rosuvastatin (IS1)	482.1→258.2	+	63	25
Chlorthalidone (IS2)	336.9→146.0	-	-58	-32

MRM: multiple reaction monitoring; DP: declustering potential;

CE: collision energy.

Table 2: Oligonucleotide primers used for real-time RT-PCR.

CYP	Gene accession no	Forward primer sequence	Reverse primer sequence
1A2	NM_012541.3	**CTACAACTCTGCCAGTCTCCAG**	**CCTCTCAACACCCAGAACACT**
2C11	U33173.1	**GGAGGAACTGAGGAAGAGCA**	**AATGGAGCATATCACATTGCAG**
2D2	NM_012730.1	**GAAGGAGAGCTTTGGAGAGGA**	**AGAATTGGGATTGCGTTCAG**
2E1	AF061442.1	**CTGACTGTCTCCTCATAGAGATGG**	**TCACAGAAACATTTTCCATTGTGT**
3A1	U09742.1	**ACCCGTCTGGATTCTAAGCA**	**TGGAATTATTATGAGCGTTCAGC**
GAPDH	M17701.1	AGCTGGTCATCAATGGGAAA	ATTTGATGTTAGCGGGATCG

Table 3: Main pharmacokinetic parameters of CYP substrates following oral administration to rats (n=5, mean ± SEM).

Parameter	VCG	L-1W	H-1W	L-2W	H-2W
Caffeine (CYP1A2)					
$AUC_{0-\infty}$ (µg.h/mL)	224.35±11.52	200.17±9.23	171.00±3.80***	120.70±1.76***	89.36±0.86***
C_{max} (µg/mL)	39.25±2.86	34.75±2.43	29.32±1.13**	25.39±0.90***	19.82±0.67***
CL (L/hr/kg)	0.225±0.012	0.265±0.020	0.293±0.006**	0.415±0.006***	0.560±0.005***
Tolbutamide (CYP2C11)					
$AUC_{0-\infty}$ (µg.h/mL)	736.46±7.15	726.48±7.54	704.08±9.43*	673.07±8.25***	622.04±7.76***
C_{max} (µg/mL)	27.98±0.16	27.87±0.23	26.91±0.24*	25.96±0.26***	24.58±0.20***
CL (L/hr/kg)	0.0204± 0.00019	0.0207± 0.00022	0.0217± 0.00029*	0.0223± 0.00027***	0.0241± 0.00030***
Dextromethorphan (CYP2D2)					
$AUC_{0-\infty}$ (µg.h/mL)	0.69±0.054	0.57±0.063	0.59±0.093	0.47±0.069	0.37±0.029*
C_{max} (µg/mL)	0.092±0.0091	0.095±0.0079	0.087±0.0033	0.077±0.0052	0.067±0.0016*
CL (L/hr/kg)	29.330±2.26	36.430±3.81	36.087±5.23	44.839±6.28	51.958±4.03*
Chlorzoxazone (CYP2E1)					
$AUC_{0-\infty}$ (µg.h/mL)	49.29±1.32	47.88±1.44	46.30±0.82	34.22±0.46***	24.33±1.22***
C_{max} (µg/mL)	7.87±0.18	8.36±0.27	7.60±0.14	6.82±0.19*	5.48±0.45***
CL (L/hr/kg)	1.017±0.026	1.047±0.033	1.081±0.020	1.462±0.019***	2.071±0.107***
Dapsone (CYP3A1)					
$AUC_{0-\infty}$ (µg.h/mL)	102.77±10.31	92.71±17.36	90.61±31.01	94.16±19.54	95.63±11.86
C_{max} (µg/mL)	6.19±0.43	5.59±0.17	5.48±0.42	4.84±0.35	4.66±0.54
CL (L/hr/kg)	0.200±0.017	0.244±0.051	0.310±0.096	0.235±0.036	0.241±0.070

$AUC_{0-\infty}$: area under the plasma concentration-time curve from zero to infinity; C_{max}: maximum plasma concentration; CL: total body clearance. VCG: vehicle control group; L-1W and L-2W: low dose (36 mg/kg) given for 1 week and 2 weeks, respectively; H-1W and H-2W: high dose (72 mg/kg) given for 1 week and 2 weeks, respectively. *Significantly different from VCG, $P < 0.05$; ** significantly different from VCG, $P < 0.01$ and *** significantly different from VCG, $P < 0.001$.