**In Vitro Metabolism of L-corydalmine, a Potent Analgesic Drug, in Human,
Cynomolgus Monkey, Beagle Dog, Rat and Mouse Liver Microsomes**

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Abbreviations: CyLM, cynomolgus monkey liver microsomes; CYP450, cytochrome P450; DLM, dog liver microsomes; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; *l*-CDL, L-corydalmine; *l*-THP, L-tetrahydropalmatine; MLM, mouse liver microsomes; MS, mass spectrometry; PBS, phosphate-buffered saline; RAF, relative activity factor; RLM, rat liver microsomes.

Abstract

L-corydalmine (*l*-CDL) was under development as an oral analgesic agent, exhibiting potent analgesic activity in preclinical models. The objective of this study was to compare metabolic profiles of *l*-CDL in liver microsomes from mouse, rat, monkey, dog and human. Six metabolites (M1-M6) were identified using LC-Q/TOF in liver microsomes from the five species. The metabolism of *l*-CDL included *O*-demethylation (M1-3) and hydroxylation (M4-6). The desmethyl metabolites were the major ones among the five species, which accounted for more than 84%. Data from chemical inhibition in human liver microsomes (HLM) and human recombinant CYP450s demonstrated that CYP2D6 exhibited strong catalytic activity towards M1 and M2 formations, while CYP2C9 and CYP2C19 also catalyzed M2 formation. Formations of M3 and hydroxyl metabolites (M4 and M5) were mainly catalyzed by CYP3A4. Further studies showed that M1 and M2 were main metabolites in HLM. The kinetics of M1 and M2 formations in HLM and recombinant CYP450s were also investigated. The results showed that M1 and M2 formations in HLM and recombinant CYP2D6 characterized biphasic kinetics, whereas sigmoid $V_{\max}$ model was better used to fit M2 formation by recombinant CYP2C9 and CYP2C19. The contributions of CYP2D6 to M1 and M2 formations in HLM were estimated to be 75.3% and 50.7%, respectively. However, the contributions of CYP2C9 and CYP2C19 to M2 formation were only 5.0% and 4.1%, respectively. All these data indicated that M1 and M2 were main metabolites in HLM, and CYP2D6 was the primary enzyme responsible for their formations.

Key words: L-corydalmine; Metabolism; Hepatic microsomes; Cytochrome P450s; Interspecies difference

1. Introduction

L-tetrahydropalmatine (*l*-THP), an active constituent of *Stephania* and *Corydalis*, has long been recognized to have therapeutic value for treating a number of CNS-related diseases under the drug named Rotundine [1], which has been approved by the China Food and Drug Administration as a non-opioid analgesic and sedative/anxiolytic agent. The therapeutic effects of *l*-THP were considered to be partly attributing to dopamine receptor antagonism inferring its potential utility in dopaminergic dysfunctions such as cocaine addiction [2,3]. L-corydalmine (*l*-CDL), 10-desmethyl metabolite of *l*-THP, was biosynthesized through regio-selective demethylation of *l*-THP by *Streptomyces griseus* ATCC 13273 [4]. It was reported that *l*-CDL showed high affinities to dopamine receptors [5] and had better analgesic property and less significant sedative and hypnotic effects than *l*-THP [4]. Now, *l*-CDL is being developed as a potential analgesic agent in China.

The U.S. Food and Drug Administration (FDA) recommends that the metabolite profiles in humans of all new chemical entities should be characterized before initiation of largescale clinical trials ([http:// www. fda.gov/downloads/drugs/guidance compliance regulatory information/guidances/ucm292362](http://www.fda.gov/downloads/drugs/guidance/compliance/regulatory_information/guidances/ucm292362)). One of the primary aims in early preclinical investigations is to compare the metabolic fate of the new chemical entity among different species using *in vitro* methods which may provide guidance to select the right animals for safety assessment studies and to ensure that the selected experimental animals form the metabolites at adequate exposure levels as in humans [6].

The aim of the present study was to characterize the metabolites of *l*-CDL in mouse, rat, monkey, dog and human liver microsomes. The principal human cytochrome P450 (CYP) isoforms responsible for the metabolism of *l*-CDL were identified to determine the potential drug-drug interactions.

2. Experimental

2.1. Chemicals and materials

l-CDL (purity≥99.0%) from Jiangsu Provincial Key Laboratory for TCM Evaluation and Translational Research, China Pharmaceutical University (Nanjing,

China). Lidocaine (internal standard, IS, purity $\geq 99.9\%$), diclofenac sodium, omeprazole sodium, dextromethorphan and dextrorphan from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Hepatic microsomes of Sprague-Dawley rats were prepared by differential ultracentrifugation as described previously [7]. Male ICR/CD-1 mouse (MLM, Lot. STOM), male beagle dog (DLM, Lot. LKMH), male cynomolgus monkey (CyLM, Lot. ZDD) and male Mongolia human (HLM, Lot. SUBK), as well as cDNA-expressed recombinant human CYP450 isoforms (CYP1A2, CYP3A4, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1) expressed in E.coli from Research Institute for Liver Diseases (Shanghai, China). All CYP450 isoforms were co-expressed with human CYP-reductase and supplemented with purified human cytochrome b5. D-glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6PDH), β -NADP⁺, ketoconazole, α -naphthoflavone, sulfaphenazole, quercetin, quinidine, diethyl dithiocarbamate and ticlopidine from Sigma-Aldrich (St Louis, MO, USA). 5-Hydroxyomeprazole and 4'-hydroxydiclofenac from TCI development Co. Ltd (Shanghai, China).

2.2. Metabolite profiles by HPLC/MS

Metabolite profiles of *l*-CDL in HLM, CyLM, DLM, RLM and MLM were identified by LC/MS. The incubation mixture consisted of 1 mg/ml microsomal protein, *l*-CDL (final level 20 μ M) and an NADPH-regenerating system (10 mM G-6-P, 0.5 mM β -NADP⁺, 5 mM MgCl₂, 1 U/ml G-6-PDH) in 100 mM PBS (pH 7.4) in final volume of 0.2 ml. After 5min pre-incubation at 37 °C, reactions were started by adding NADPH-regenerating system. The incubations were conducted at 37 °C for 30 min and terminated with 1ml of ethyl acetate containing lidocaine (700 ng/ml). The samples, following mixing, were centrifuged at 6200 $\times$ g for 10 min. The organic layer (0.85 ml) was transferred and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μ l water. An aliquot of 5 μ l was injected into the LC/MS system. The HPLC system consisted of a Shimadzu (Kyoto, Japan) system controller (CBM-20A), pump (LC-20ADXR), autoinjector (SIL-20ACXR), online degasser (DGU-20A3R) and column oven (CTO- 20AC). *l*-CDL and its metabolites

were separated on a reversed phase C18 column (2.0×250 mm, 5μm; Shim-pack VP-ODS; Japan) at a flow rate of 0.25 ml/min. The mobile phase A was deionized water containing 0.1 % (v/v) formic acid and 1mM ammonium formate, whereas the mobile phase B consisted of 50 % (v/v) aqueous mobile phase and acetonitrile. The gradient involved: 20 % B for 10 min; a linear increase from 20 % to 35 % B in 2 min; 35 % B for 5 min; a linear decrease from 35 % to 20 % B in 1 min; and equilibration at 20 % B for 6 min before the next injection.

Mass spectra were performed using a mass spectrometer of Shimadzu MS-2020 system (Kyoto, Japan). Detection was obtained by Select Ion Monitoring (SIM) mode using electrospray ionization in the positive ion mode for all analytes. The $[M+H]^+$ ions of *l*-CDL and these metabolites were characterized to be m/z 342 (*l*-CDL), m/z 328 (M1-3) and 358 (M4-7). The optimal conditions for analysis were set as follows: the interface voltage was kept at 4.5kV and the detector voltage was set to 1.35kV; the column oven temperature, desolvation line temperature and the heat block temperature were 40 °C, 250 °C and 200 °C; nebulizing gas and drying gas were nitrogen at a flow rate of 1.5 and 15.0 L/min, respectively; the data generated were processed using Shimadzu LabSolutions version 5.53 SP3.

Control incubations were performed without NADPH-regenerating system. Comparison of *l*-CDL metabolism in liver microsomes of five species was conducted using ratios of metabolite peak area to the internal standard peak area. All incubations were carried out in triplicate.

2.3. Metabolite identification by LC-Q/TOF

The initial screening of the *l*-CDL's metabolites and accurate mass measurements were carried out using Agilent liquid chromatography quadrupole-time-of-flight mass spectrometer (LC-Q/TOF) 6520 (Agilent Technologies, Palo Alto, CA, USA). A generic positive electrospray ionization method was used for all substrates and metabolites. The capillary voltage was 3500 V and cone voltage was 30 V. Desolvation and source temperatures was 260 °C and 150 °C, respectively. Nitrogen was used as the desolvation and cone gas with flow rates of 900 and 300 L/h. All data acquired were processed using Mass Hunter

Qualitative Analysis Software (B.04.00, Agilent).

2.4. Effects of chemical inhibitors on l-CDL demethylation in HLM

The chemical inhibition study was performed by adding each of selective inhibitor of P450 enzymes into the incubation system consisted of *l*-CDL (20 μ M), 1 mg/ml HLM and NADPH-regenerating system in 200 μ l PBS. The chemical inhibitors and concentrations were selected on the basis of previous reports[8] as follows: α -naphthoflavone (5 μ M) for CYP1A2, quercetin (5 μ M) for CYP2C8, sulfaphenazole (10 μ M) for CYP2C9, ticlopidine (5 μ M) for CYP2C19, diethyl-dithiocarbamate (20 μ M) for CYP2E1, ketoconazole (5 μ M) for CYP3A4 and quinidine (0.3125~10 μ M) for CYP2D6. After incubation for 30 min, formations of *l*-CDL metabolites were measured by LC/MS as described above. All incubations were carried out in triplicate. Incubations without chemical inhibitors served as control.

2.5. Identification of human recombinant CYP450 isoforms responsible for l-CDL metabolism

Eight human recombinant CYP450s (CYP1A2, CYP3A4, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1) were used to screen human CYP450 isoforms responsible for *l*-CDL metabolism. Final concentrations of *l*-CDL and recombinant CYP450 were set to be 20 μ M and 30 pmol/ml, respectively. After incubation for 30 min, formations of metabolites were measured using LC/MS and indexed as ratios of metabolite peak area to the IS. All incubations were carried out in triplicate.

2.6. Kinetics of M1 and M2 in HLM and human recombinant CYP450s

The protein concentration and incubation time were preferentially optimized to ensure linear formations of M1 and M2. A various concentrations (0.3125~80 μ M) of *l*-CDL were incubated with 1 mg/ml of HLM or 30 pmol/ml of recombinant CYP450 and NADPH-regenerating system in 200 μ l PBS for 30 min at 37°C. The metabolites were detected using LC/MS and the amounts of the metabolites in the incubation were semi-quantified using standard curves of *l*-CDL obtained from samples without incubation. All incubations were carried out in duplicate.

2.7. Contributions of CYP2D6, CYP2C9 and CYP2C19 to the formations of M1 and M2 in HLM

Activities of CYP2D6, CYP2C9 and CYP2C19 were assessed using formations of dextrophan, 4'-hydroxydiclofenac and 5-hydroxylomeprazole from corresponding substrates, respectively. For CYP2D6, dextromethorphan (4 μ M) was incubated in the reaction mixture consisted of 0.3 mg/ml HLM or 1 pmol/ml recombinant CYP2D6 and NADPH regenerating system in 100 μ l PBS for 10 min. For CYP2C9, diclofenac(4 μ M) was incubated in the reaction mixture consisted of 0.1 mg/ml HLM or 1 pmol/ml recombinant CYP2C9 and NADPH regenerating system in 400 μ l PBS for 20 min. For CYP2C19, omeprazole(15 μ M) was incubated in the reaction mixture consisted of 0.1 mg/ml HLM or 5 pmol/ml recombinant CYP2C19 and NADPH regenerating system in 100 μ l PBS for 20 min. The amounts of dextrophan, 4'-hydroxydiclofenac and 5-hydroxylomeprazole were all measured using HPLC method [9-11]. The activities of CYP450s were indexed as ng/min/mg protein or ng/min/pmol recombinant CYP450. All incubations were carried out in triplicate.

The contributions of CYP450s to *O*-demethylation of *l*-CDL in HLM were estimated according to relative activity factor (RAF) method described previously [12]. The RAF values of CYP2D6, CYP2C9 and CYP2C19 were determined as dextrophan, 4'-hydroxydiclofenac and 5-hydroxylomeprazole formation from corresponding substrates in HLM to that in recombinant CYP system, respectively. Contributions of corresponding CYP450 isoforms to the *l*-CDL *O*-demethylation in HLM were estimated as follows:

$$\text{Contribution of CYP450i (\%)} = (\text{CL}_{\text{rec-CYP450i}} \times \text{RAF}_{\text{CYP450i}} / \text{CL}_{\text{HLM}}) \times 100 \quad (1)$$

where $\text{CL}_{\text{rec-CYP450i}}$ and CL_{HLM} was the estimated clearance of *l*-CDL *O*-demethylation in the recombinant CYP450 isoforms and the total clearance in HLM respectively. And the subscript i denoted corresponding CYP450 isoforms.

2.8. Data analysis

Kinetic parameters of metabolites were estimated by nonlinear least-squares regression on WinNonlin 6.3. Eadie-Hofstee plots were used to check for kinetic characteristics. The Michaelis-Menten equation (eq. 2) was used to calculate apparent

K_m and V_{max} values.

$$V = V_{max} \times S / (K_m + S) \quad (2)$$

The extended Michaelis-Menten equation (eq. 3) was used to estimate kinetic parameters for multi-enzyme systems.

$$V = V_{max1} \times S / (K_{m1} + S) + V_{max2} \times S / (K_{m2} + S) \quad (3)$$

where the subscript i denoted the i th component of the reaction.

A sigmoid V_{max} model equivalent to the Hill equation was used to describe Sigmoidal kinetics according to equation 4 [13].

$$V = V_{max} \times S^N / (S_{50}^N + S^N) \quad (4)$$

where V , S and V_{max} were the velocity of the reaction, the l -CDL concentration and the maximum velocity, respectively. S_{50} was the l -CDL concentration when V was half- V_{max} and N was the Hill coefficient. For Michaelis-Menten kinetics, intrinsic clearance (Cl_{int}) was calculated as V_{max}/K_m . For the extended Michaelis-Menten, Cl_{int} was defined as $V_{max,1}/K_{m,1} + V_{max,2}/K_{m,2}$. For sigmoidal kinetics, maximum clearance (Cl_{max}) was calculated as equation 5.

$$Cl_{max} = V_{max} \times (N-1) / (S_{50} \times N \times (N-1)^{1/N}) \quad (5)$$

In the present study, if the Hill coefficient was more than 1.2, the kinetic data would be fit to the Hill equation [14]. All of the data were expressed as an individual data or mean $\pm$ standard deviation (SD). One way analysis of variance (ANOVA) followed by Tukey's posthoc test was applied for the statistical data analysis and the level of significant was set at p -value of 0.05.

3. Results

3.1. Metabolite profiles of l -CDL in liver microsomes of five species

Metabolites of l -CDL in HLM, CyLM, DLM, RLM and MLM were identified. Fig. 1 showed the representative extracted ion chromatograms of the parent drug and its metabolites following 30 min incubation of l -CDL (20 μ M) with liver microsomes of human and mouse. The experiment results showed that six metabolites were identified in liver microsomes of five species, which were termed as M1, M2, M3, M4, M5 and M6. In MLM, a new minor metabolite was also detected and was termed as M7. As shown in Table1, M1, M2 and M3 were the major metabolites in liver

microsomes of the five species, which accounted for more than 84% of total metabolites. Significant species differences were observed. Formations of metabolite M1, M2 and M3 were the highest in CyLM, followed by RLM and MLM. In HLM, M1 and M2 were main metabolites, accounting for 74% and 20% of total metabolites, but M3 formation was less than 3%. The amounts of metabolites M1, M2 and M3 in DLM were similar to those in HLM, but less than those in the other three species.

3.2. Metabolite Identification

A comprehensive understanding of the fragmentation behavior of *l*-CDL can be helpful in metabolite identification using LC-Q/TOF. In the MS² spectrum, *l*-CDL displayed two diagnostic fragment ions at *m/z* 192 and 165, which were the most abundant ions (Fig. 2). The elimination of the methylradical from the two predominations formed fragments at *m/z* 177 and *m/z* 150, but with lower abundance. Tetrahydropalmatine was considered to be characterized by the retro-Diels-Alder (RDA) reaction [15]. *l*-CDL possessed the same parent nucleus as tetrahydropalmatine did, indicating that *l*-CDL also underwent RDA reaction in ion source. The fragment at *m/z* 192 resulted from RDA C-ring opening to form tetrahydroisoquinoline fragments. Fragment at *m/z* 165 probably came from RDA B-ring opening which was involved in a complex rearrangement of the B ring in one or several steps [15,16]. Several reports showed that RDA C-ring often occurred in kinds of these alkaloids [17,18].

The structure of M1-6 was deduced using the mass change of the molecular ion and similarity/change of fragmentation patterns, compared with those of *l*-CDL. Metabolite profiles of *l*-CDL were summarized in Fig. 2.

M1 and M2 eluted at 9.4 min and 10.1 min with [M+H]⁺ ion at *m/z* 328. The same product ions were observed at *m/z* 328, 178 and 151, corresponding to a loss of 14 Da from the product ions of *l*-CDL (*m/z* 342, 192 and 165). Fragments *m/z* 163 produced by elimination of the methylradical from ion at *m/z* 178, similar to cleavage pathway of *l*-CDL (*m/z* 192→177). All these results indicated that for M1 and M2, demethylation occurred at C-2 or C-3 position of A ring. The exact demethylation position cannot be determined by the current data.

M3 was eluted at approximately 13.1 min with [M+H]⁺ ion at *m/z* 328 which

was 14 Da less than that for *l*-CDL, suggesting the desmethly product of *l*-CDL. The productions at m/z 192, 177, 165 and 150 were consisted with tetrahydroisoquinoline fragments of *l*-CDL (m/z 192, 177, 165, 150). If the demethylation occurred at C-2 or C-3 position of A- ring, the product ion at m/z 178 would be observed. Hence M3 was deduced to be C-9 demethylation of *l*-CDL.

M4 was detected at 9.8 min and displayed an $[M+H]^+$ ion of m/z 358, corresponding to a gain of 16 Da on *l*-CDL (m/z 342). Like M3, the product ions at m/z 192 and 165 were obviously formed by the RDA reaction, suggesting that oxidation occurred on C or D ring. It was tentatively deduced that hydroxylation occurred at C-8 position, resulting in M4. Thus, when RDA reaction (C ring) occurred, an transition ion at m/z 167 formed. The transition ion translated immediately into the ion at m/z 149 by loss of a H_2O molecule (-18 Da). The subsequent loss of the CO moiety (-28 Da) from the ion at m/z 149 produced a fragment at m/z 121. All these results further indicated that M4 was 8-hydroxyl- *l*-CDL.

M5 had a retention time of 10.5 min with $[M+H]^+$ ion at m/z 358, getting extra 16 Da on *l*-CDL. Two product ions at m/z 192 and 177 resulted from RDA C-ring opening, which were consisted with the cleavage behavior of *l*-CDL and indicated that oxidation did not happened on A or B ring. No more characteristic fragments could be observed, indicating hydroxylation possibly happened on D ring.

M6 eluted at approximately 13.3 min on HPLC and produced protonated molecular ion at m/z 358, 16 Da higher than that for *l*-CDL. In addition, fragment ion at m/z 192 was also observed, suggesting that the oxidation occurred on C or D ring. The base product ion at m/z 338 (20 Da less than protonated M6 ion at m/z 358) was possibly formed by a complex rearrangement of the C ring with loss of H_2O molecule and di-dehydrogenation (-20 Da), leading to the formation of substituted protoberberine. Therefore, M6 was tentatively identified as 13-hydroxyl-*l*-CDL.

3.3. CYP450 isoforms responsible for metabolism of *l*-CDL in HLM

As mentioned above, *O*-desmethyl metabolites of *l*-CDL (M1, M2 and M3) were the main metabolites of *l*-CDL. The contribution of individual human P450 isoforms to formations of the three metabolites was assessed using selective chemical inhibitors.

The results (Fig. 3A) showed that among the tested selective chemical inhibitors, only quinidine (10 μ M) strongly inhibited M1 and M2 formations by 85% and 60%, respectively. Ketoconazole inhibited M3 formation by 46%. Other CYP450 chemical inhibitors did not show inhibitory effects on demethylation of *l*-CDL in HLM. Concentration-dependent inhibitory effects of quinidine on M1 and M2 formations were also observed (Fig. 3A). IC₅₀ values of M1 and M2 formations were estimated to be 0.65 μ M and 1.76 μ M, respectively.

Eight human recombinant P450 isoforms were used to further identify CYP450 isoforms responsible for *l*-CDL metabolism (Fig. 3B). The result showed that CYP2D6 exhibited strong catalytic activity towards M1 formation. M2 formation was mainly mediated by CYP2C9, CYP2C19 and CYP2D6, although CYP1A2 and CYP3A4 also showed weak catalytic activity. CYP3A4 also catalyzed formations of M3 and hydroxyl metabolites (M4 and M5).

3.4. Kinetics for M1 and M2 formations in HLM and recombinant CYP450s

The present study showed that M1 and M2 were main metabolites in HLM. Therefore, kinetics for M1 and M2 formations in HLM and recombinant CYP450s were further characterized (Fig. 4). M1 and M2 formations in both HLM and recombinant CYP2D6 were characterized biphasic kinetics, suggesting that two components were involved in the M1 and M2 formations in HLM and recombinant CYP2D6. The Cl_{int} values were estimated as follows. In HLM for M1, Cl_{int1}: 0.46 μ l/min/mg protein and Cl_{int2}: 0.09 μ l/min/mg protein; for M2 Cl_{int1}: 1.48 μ l/min/mg protein and Cl_{int2}: 0.39 μ l/min/mg protein. In recombinant CYP2D6 for M1, Cl_{int1}: 25.87 μ l/min/nmol CYP450 and Cl_{int2}: 1.65 μ l/min/nmol CYP450; for M2, Cl_{int1}: 59.25 μ l/min/nmol CYP450 and Cl_{int2}: 3.42 μ l/min/nmol CYP450. Although V_{max2} for the low-affinity component was larger than V_{max1} for the high affinity component, Cl_{int2} of the low affinity component was significantly lower than Cl_{int1} of the high-affinity component, whose contributions to total clearance in HLM and recombinant CYP2D6 were less than 20% and 6%, respectively. Affinities of M1 and M2 in HLM, indexed as K_m, were close to those of recombinant CYP2D6, inferring that kinetic characteristics of M1 and M2 formations in HLM were similar to those by

CYP2D6. It was different from M2 formation by CYP2D6 that a sigmoid $V_{\max}$ function was more suitable for the data of M2 formation mediated by CYP2C9 and CYP2C19. The estimated S_{50} values of M2 formation by CYP2C9 (27.92 μM) and CYP2C19 (37.23 μM) were markedly higher than K_{m1} (1.68 μM) of M2 formation by CYP2D6, and the estimated maximum clearances ($Cl_{\max}$) of M2 formation for CYP2C9 and CYP2C19 were 2.79 and 5.44 $\mu\text{l}/\text{min}/\text{nmol}$ CYP450, respectively, less than that of CYP2D6 (62.67 $\mu\text{l}/\text{min}/\text{nmol}$).

3.5. Contributions of CYP2C9, CYP2C19 and CYP2D6 to l-CDL O-demethylation in HLM

The total clearances of M1 formation in HLM and in recombinant CYP2D6 were estimated to be 0.55 $\mu\text{l}/\text{min}/\text{mg}$ proteins and 27.52 $\mu\text{l}/\text{min}/\text{nmol}$ CYP2D6, respectively. Formations of dextrothorphan from dextromethorphan in HLM and in recombinant CYP2D6 were measured to be 16.93 ng/min/mg proteins and 1.12 ng/min/pmol CYP2D6, respectively. The RAF_{CYP2D6} was calculated to be 15.12 pmol/mg proteins and the contribution of CYP2D6 to M1 formation in HLM was estimated to be 75.3 %.

The clearances of M2 formation in HLM, recombinant CYP2D6, recombinant CYP2C9 and recombinant CYP2C19 were measured to be 1.87 $\mu\text{l}/\text{min}/\text{mg}$ protein, 62.67 $\mu\text{l}/\text{min}/\text{nmol}$ CYP2D6, 2.79 $\mu\text{l}/\text{min}/\text{nmol}$ CYP2C9 and 5.44 $\mu\text{l}/\text{min}/\text{nmol}$ CYP2C19, respectively. Formations of 4'-hydroxydiclofenac from diclofenac in HLM and recombinant CYP2C9 were measured to be 59.60 ng/min/mg proteins and 2.26 ng/min/pmol CYP2C9. The RAF_{CYP2C9} was estimated to be 26.32 pmol/mg proteins. Formations of 5-hydroxylomeprazole from omeprazole in HLM and recombinant CYP2C19 were measured to be 13.04 ng/min/mg protein and 1.16 ng/min/pmol CYP2C19, respectively. RAF_{CYP2C19} was estimated to be 11.23 pmol/mg proteins. Therefore, the contributions of CYP2D6, CYP2C9 and CYP2C19 to M2 formation in HLM were estimated to be 50.7%, 5.0% and 4.1%, respectively, suggesting that M2 formation was mainly attributed to CYP2D6.

4. Discussion and conclusions

In vitro studies demonstrated that l-CDL was metabolized to at least 6

metabolites in liver microsomes of five species. *O*-desmethyl (M1-3) and hydroxyl (M4-6) metabolites were both deduced based on the data from LC-TOF-MS (Fig. 2). Synthesized reference compounds of metabolites are needed to confirm their chemical structures.

Relative amounts of six metabolites (M1-6) were compared among five species. CyLM and RLM formed all detectable metabolites in adequate levels compared with HLM, which indicated that monkey and rat may be more suitable for long-term safety assessment of *l*-CDL.

The kinetics data showed that two components were involved in M1 and M2 formations from *l*-CDL in HLM and recombinant CYP2D6, but contributions of low affinity component to M1 and M2 formation were minor. The kinetic characteristics of M1 and M2 formations in HLM were similar to those by recombinant CYP2D6, demonstrating the high contribution of CYP2D6 to M1 and M2 formations in HLM, which was evidenced by the result of chemical inhibition studies. Data from recombinant CYP450s showed that CYP2C9 and CYP2C19 also catalyzed M2 formation, which were characterized by a sigmoidal $V_{\max}$ kinetics. However, $Cl_{\max}$ values by CYP2C9 and CYP2C19 were less than 10% of CYP2D6, inferring minor contributions of CYP2C9 and CYP2C19 to M2 formation in HLM. The estimated contributions of CYP2D6 to M1(75.3%) and M2 (50.7%) formations were consistent with findings in chemical inhibitors, while contributions of CYP2C9 and CYP2C19 to M2 formation were only 5.0% and 4.1%, respectively, which may explain the finding that neither sulfaphenazole nor ticlopidine inhibited M2 formation in HLM.

Moreover, CYP2C9, 2C19 and 2D6 are most important polymorphic CYPs [19-21], indicating that these CYPs polymorphisms are more likely to influence an individual response to *l*-CDL treatment.

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Table 1

Comparison of M1-6 of *l*-CDL incubated with NADPH-regenerating system in human, dog, monkey, rat and mouse microsomes for 30 min at 37 °C

	Human	Dog	Monkey	Rat	Mouse
M1	0.46±0.01	0.44±0.01	7.86±0.11	5.11±0.32	1.04±0.01
M2	1.69±0.01	0.72±0.04	8.50±0.09	2.96±0.19	3.04±0.06
M3	0.07±0.01	0.39±0.05	1.54±0.20	1.08±0.05	1.15±0.08
M4	0.05±0.01	0.02±0.02	0.34±0.01	0.31±0.03	0.15±0.01
M5	0.02±0.00	0.04±0.00	0.22±0.01	0.31±0.02	0.08±0.01
M6	0.01±0.00	0.01±0.00	0.36±0.01	1.05±0.08	0.05±0.00

Data are documented as ratios of metabolite peak area to the internal standard peak area and presented as mean ± SD (n = 3).

Figure captions:

Fig.1. Extracted ion chromatograms for *l*-CDL and its metabolites produced following incubation with pooled human and mouse liver microsomes in the presence of a NADPH-regenerating system.

Fig.2. MS² spectrum of *l*-CDL and CID spectra of protonated M1-6.

Fig.3. Effects of chemical inhibitors on the formations of metabolites (M1, M2 and M3) after incubation of *l*-CDL (20 µM) with HLM (1 mg/ml) for 30min and varying concentrations (0.3125-10 µM) of quinidine used in the inhibition studies (A); Metabolisms(M1-M6) of *l*-CDL by human recombinant P450 enzymes after incubation of *l*-CDL (20 µM) with human recombinant CYP450_s (30 pmol/ml) for 30min(B). Data are reported as mean ±SD (n = 3). *P<0.05 and **P<0.01 indicate a significant difference from the control.

Fig.4. Formation kinetics of M1 in HLM (A) and recombinant CYP2D6 (C); Formation kinetics of M2 in HLM (B), recombinant CYP2D6, 2C9 and 2C19 (D, E, F) over the *l*-CDL concentration range of 0.3 to 80 µM. Their respective Eadie-Hofstee plots and kinetic parameters were obtained. K_m was indexed as µM and V_{max} was indexed as pmol/min/mg HLM or µl/min/nmol recombinant CYP450. The results are presented as mean of duplicate data.

Figure(s)

Human

Mouse

Figure(s)

Figure(s)

Figure(s)