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In vitro Metabolism of Strychnine by Human Cytochrome P450 and Its Interaction with Glycyrrhetic Acid

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Abstract: **Objective** To investigate the metabolism of strychnine (STN) and the metabolic interaction between STN and glycyrrhetic acid (GA) *in vitro*. **Methods** Human liver microsomes (HLM) and human recombinant cytochrome P450 (CYP) isoforms were employed to study the metabolism of STN and the metabolic interaction of STN with GA *in vitro*. **Results** In HLM, the K_m, V_{max}, and clearance of STN were 88.50 μmol/L, 0.88 nmol/(mg·min), and 9.93 mL/(mg·min), respectively. STN was metabolized mainly by CYP3A4. However, STN noncompetitively inhibited CYP3A4-catalyzed testosterone 6β-hydroxylation with IC_{50} value of 5.9 μmol/L and K_i value of 5.5 μmol/L. Moreover, GA competitively inhibited STN metabolism with IC_{50} value of 10.6 μmol/L and K_i value of 17.7 μmol/L. **Conclusion** Although STN is mainly metabolized by CYP3A4 *in vitro*, STN has noncompetitive inhibition on CYP3A4-catalyzed testosterone 6β-hydroxylation. Moreover, GA could competitively inhibit STN metabolism. The present work is helpful to elucidate the metabolic interaction between STN and GA.

Key words: cytochrome P450; glycyrrhetic acid; human liver microsomes; metabolism; strychnine
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Introduction

The seeds of *Strychnos nux-vomica* L., as one of highly toxic herbal drugs, has been frequently used as an important ingredient in many traditional Chinese medicine prescriptions, such as Biqi Capsule handed down from Han Dynasty by great physician HUA Tuo, to treat joint pain, arthritic, and rheumatic diseases. In clinical application, the seed of *S. nux-vomica* was customarily used along with licorice roots to decrease its toxicity, but the detoxication mechanism was unclear yet today. Strychnine (STN), as a highly potent antagonist of glycine receptors of the vertebrate central nervous system (McCool and Chappell, 2007) and a strong blocker of various types of muscle and neuronal nicotinic acetylcholine receptors (Alcocer, Torres, and Miledi, 2005), is the major pharmacodynamic component from the seeds of *S. nux-vomica* with high toxicity. The previous studies indicated that STN was rapidly absorbed via the gastrointestinal tract, rapidly distributed to tissues with very little plasma protein binding, and eliminated mainly by hepatic metabolism to form several metabolites, 2-hydroxy, 16-hydroxy, 22-hydroxy, the 21-22 epoxide, and the *N*-oxide, which indicated that these metabolites of STN were generated differentially by different cytochrome P450 (CYP) isoforms (Boyd *et al*, 1983; Tanimoto *et al*, 1991). Glycyrrhetic acid (GA) is the major metabolite of glycyrrhizin and the major active component of licorice roots (Tian *et al*, 2006; Duan and Ji, 2007). So far, few published reports were involved in the *in vitro* metabolism of STN and the interaction with GA.

The identification of the enzymes involved in the metabolism of drug candidate is useful for better understanding of genetic polymorphism in drug clearance and for prediction of potential metabolic-based drug interactions. Currently, the risk for metabolic-based drug interactions could be predicted early during the drug discovery process using *in vitro*

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approaches (US Food and Drug Administration, 1997). The present *in vitro* study was designed to: (1) identify human CYP isoforms responsible for metabolism of STN; (2) evaluate the effect of STN on the activities of main CYP isoforms; (3) examine potential metabolic interaction between STN and GA.

Material and methods

Chemicals

Strychnine, glycyrrhetic acid, testosterone, and chlorzoxazone were purchased from National Institutes for Food and Drug Control (China). Ketoconazole (KET), diclofenac, and omeprazole were obtained from L-KT Laboratories (China). α-Naphthoflavone (ANF), 4-methylpromine (4-MET), phenacetin, and tranyl-cypromine (TRA) were from Alfa Aesar Co. (Germany). Quinidine (QUI) and sulfaphenazole (SUL) were from Sigma-Aldrich Chemical Co. (USA). NADPH was from Roche Co. (Switzerland). HPLC-grade methanol and acetonitrile were from Tedia Co. (USA). All other chemicals and solvents were of analytic grade and were obtained from common commercial sources. Stock solution of STN was prepared by dissolving a precisely weighed amount of STN in methanol and metered volume in a volumetric flask to yield a concentration of 1.9×10^4 μmol/L.

Human liver microsomes (HLM) and CYP isoforms

Pooled HLM and human CYP isoforms (including CYP3A4, 1A2, 2C9, 2C19, and 2E1) were purchased from BD Biosciences (Woburn, MA, USA). All of CYP isoforms in Supersomes™ are co-expressed with human cytochrome b5 (b5) and human cytochrome C reductase by using baculovirus (*Autographa californica*) infected insect cells (BTI-TN-5B1-4). The activities of CYP3A4, 1A2, 2C9, 2C19, and 2E1 [pmol product/mg protein per min, represented by testosterone 6β-hydroxylase, phenacetin *O*-deethylase, diclofenac 4′-hydroxylase, Omeprazole 4-hydroxylase, and chlorzoxazone 6-hydroxylase catalytic activities, respectively] in HLM are 4200, 900, 2700, 39, and 2400, respectively (data were provided by GENTEST).

In vitro assay of STN metabolism

To determine the enzyme kinetic parameters of STN, kinetic assays were performed by incubation of a series of concentration of STN with HLM. All incubation was performed in triplicate. The incubation mixture consisted of STN (final concentration of 5, 10, 15, 25, 45, 65, 125, and 250 μmol/L, respectively), HLM (0.5 mg/mL microsomal protein), $MgCl_2$ (5 mmol/L), and NADPH (1 mmol/L) in a total volume of 0.2 mL PBS buffer (0.1 mmol/L, pH 7.4). The incubation time and microsomal protein concentration were chosen based on our preliminary studies to ensure the linear metabolic clearance rate (MCR) of STN. MCR was the ratio of the metabolic clearance amount of tested compound in positive sample (containing specific inhibitor) *vs* that of negative control (without specific inhibitor) after incubation reaction. The percentage of methanol in the incubation mixture was less than 1% to sustain P450 activity. The pre-incubation was performed at 37 ℃ for 3 min before adding NADPH to the incubation mixture. Reactions were initiated by the addition of NADPH to microsomal incubation system at 37 ℃ for 45 min, and terminated by the addition of 0.4 mL ice-cold methanol followed by vortexing for 3 min. After centrifugation (10 min at 10 000 × *g*), supernatant (0.5 mL) was evaporated by Biotron Speedvac Concentrators. The residue was reconstituted in 100 μL HPLC mobile phase and centrifuged at 10 000 × *g* for 10 min. The obtained supernatant was used for HPLC analysis of substrate at an injection volume of 20 μL.

Identification of major metabolic enzyme of STN

To screen the major metabolic enzyme of STN, the specific inhibitors, including ANF (CYP1A2), KET (CYP3A4), QUI (CYP2D6), sulfaphenazole (SUL) (CYP2C9), TRA (CYP2C19), and 4-MET (CYP2E1) (Bjornsson *et al*, 2003; Weaver *et al*, 2003; Kim *et al*, 2008), were co-incubated individually with STN and HLM. The concentration of the specific inhibitors used for incubation was 2, 10, 30, 10, 10, and 3 μmol/L for KET, QUI, TRA, 4-MET, SUL, and ANF, respectively, which was chosen based on the published IC_{50} or K_i values for CYP isoforms and could ensure adequate inhibitory selectivity and maximal inhibitory potency (Kim *et al*, 2003; 2006; 2008; Tang *et al*, 2004). The selected STN concentration (50 μmol/L) was less than its K_m value (Bjornsson *et al*, 2003). Other incubation conditions were the same as described above. The inhibitory effects of the specific inhibitors on the MCR of STN were evaluated respectively to screen the CYP

isoforms responsible for STN metabolism. The relative activity of CYP isoforms was calculated by dividing the peak area of STN in positive sample (incubation containing specific inhibitor) with that of negative control (incubation without specific inhibitor). After the screening assay, the human CYP isoforms with significant impact on STN metabolism were selected for further characterization. In the present study, STN (25 μmol/L) was incubated with each of 100 pmol/mL CYP isoforms and its corresponding specific inhibitor to verify the main metabolic enzyme responsible for STN metabolism. Other incubation conditions were the same as described above.

Evaluation of the inhibition of STN on human CYP isoforms

The effects of STN on CYP1A2-catalyzed phenacetin *O*-deethylation, CYP2C9-catalyzed diclofenac 4-hydroxylation, CYP2C19-catalyzed omeprazole 4-hydroxylation, CYP2E1-catalyzed chlorzoxazone 6-hydroxylation, and CYP3A4-catalyzed testosterone 6β-hydroxylation were performed individually by using CYP isoforms incubation in the absence and presence of STN (final concentration of 5－200 μmol/L). All experiments were performed in triplicate. The concentration of CYP1A2, 2C9, 2C19, 2E1, and 3A4 was 1.2, 1.6, 2.5, 6.3, and 6.1 pmol/mL, respectively (Walsky and Obach, 2004). Because the probe substrates concentration used for IC_{50} determination should be either at or near the apparent K_m values of each CYP isoforms, the probe substrate concentration of CYP1A2, 2C9, 2C19, 2E1, and 3A4 in the present work was 25, 4, 50, 75, and 50 μmol/L, respectively (Yao *et al*, 2006; Li *et al*, 2007). The incubation time was 20 min for 1A2, 2C19, and 2E1, 10 min for 3A4 and 2C9 (Walsky and Obach, 2004). Other incubation conditions were the same as described above. The inhibitory potency of STN on each CYP isoform activity was expressed as the ratio of probe substrate peak area in positive sample (containing STN) *vs* that of negative control (without STN) after incubation, and compared with that of the specific inhibitor mentioned above.

Evaluation of the potential interaction of STN with GA

Varying concentration of GA (5－200 μmol/L) was added to one of two different concentration of STN (10 and 200 μmol/L) to assess the effect of GA on STN metabolism. Incubation was initiated by the addition of NADPH into HLM mixture containing both GA and STN. The microsomal protein concentration (0.5 mg/mL) and incubation time (45 min) used were linear with the MCR of STN. The metabolic reaction rate (V) of STN was measured in the presence and absence of GA. The reciprocal of V of STN (Y) was plotted against the concentration (X) of GA to analyze the metabolic interaction of them (Segel, 1975).

Analysis of STN and probe substrates by HPLC

The method validation was carried out according to the currently accepted *Bioanalytical Method Validation Guidance* by US Food and Drug Administration. The interference of endogenous compounds was investigated by analysis of STN or probe substrate-free incubation mixture. STN and probe substrates were separated by an Inertsil ODS-3 C_{18} column connected to a Security Guard Cartridges Phenomenex C_{18} column (4 mm × 3.0 mm, 5 μm,) with isocratic mobile phase. The flow rate was 1 mL/min and the column temperature was 25 ℃. The injection volume was 20 μL. Other chromatographic conditions used to analyze different analytes were displayed in Table 1.

Enzyme kinetic calculation and data analyses

The results are expressed as $\bar{x} \pm s$ of values obtained from three incubation preparations. The apparent kinetic parameters of STN (K_m and V_{max}) were determined by fitting the Michaelis-Menten equation or Lineweaver-Burk model by non-linear least-squares

Table 1 Chromatographic conditions

Compounds	Mobile phases	λ / nm	t_R / min
STN	methanol-water (2.5% TEA, pH 2.6 adjust with H_3PO_4) (25:75)	254	8.70
testosterone	methanol-water (70:30)	240	7.40
phenacetin	methanol-water (45:55)	243	8.22
diclofenac	methanol-water (2% acetic acid) (75:25)	276	8.80
omeprazole	methanol-water (60:40)	302	6.40
chlorzoxazone	acetonitrile-water (3% acetic acid) (42:58)	280	10.02

regression using the program SPSS Enzyme Kinetics 1.10 Demo (SPSS Inc., Chicago, IL, USA).

$V = V_{max}[S]/(K_m+[S])$ or $1/V = K_m/(V_{max}[S])+1/V_{max}$

The intrinsic clearance (CL) for *in vitro* incubation was calculated as:

$CL = V_{max}/K_m$

Where V_{max} is the maximum velocity of enzymes, K_m is the substrate concentration at which the reaction velocity is 50% of V_{max}.

For CYP isoforms inhibition studies, the activity of each CYP isoform was calculated by the MCR of its corresponding probe substrate. The MCR of the probe substrate was considered to be 100% when no STN or specific inhibitor was added in incubation assay. The inhibitory curves and IC_{50} values were evaluated using a Graghpad Prism 5 software (GrapPad Co., Ltd., USA) by non-linear regression. Type of inhibition and K_i values were evaluated with SPSS Enzyme Kinetics 1.10 Demo (SPSS Inc., USA).

Results

Method validation

The chromatograms of STN with or without HLM were shown in Fig. 1. The peak of STN was very symmetric with retention time at 8.79 min. No endogenous impurity was found to interfere the determination of STN.

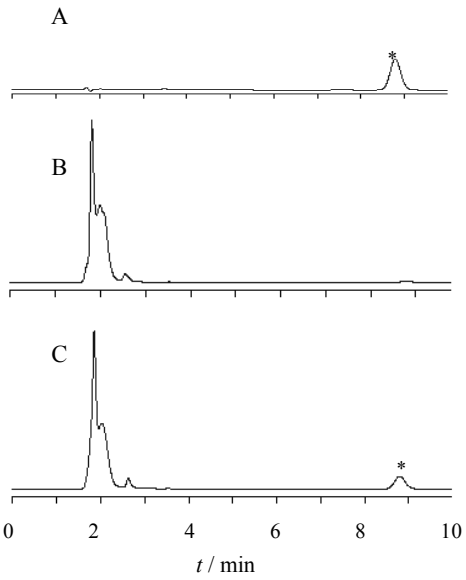

Fig. 1 Chromatograms of STN in HPLC mobile phase (A), HLM incubation solution without STN (B), and HLM incubation solution spiked with 100 μmol·L^{-1} STN (C)

*: STN

The calibration curve of STN showed good linearity in the concentration range of 2—280 μmol/L. The mean regression equation was: $Y = 26.343X - 6.044$ ($R = 0.9999$, $n = 6$), where Y stands for the peak area of STN, and X refers to STN concentration in NADPH-free HLM incubation systems. The lower limit of quantification (LLOQ) for STN was proved to be 0.66 μmol/L and the lower limit of detection (LLOD) was 0.20 μmol/L.

The extraction recoveries of the quality control (QC) samples at 20, 120, and 280 μmol/L were (93.8 ± 0.57)%, (97.6 ± 0.71)%, and (92.9 ± 0.23)%.

Enzyme kinetics of STN in HLM

The incubation, the V of STN at different reaction concentration (C) was measured. The Michaelis-Menten plot and the Lineweaver-Burk plot ($1/V$ *vs* $1/C$) were shown in Fig. 2. Based on the kinetic plots, the K_m and V_{max} values of STN in HLM were 88.5 μmol/L and 0.8785 nmol/(mg·min), respectively. The *in vitro* CL was 9.93 mL/(mg·min).

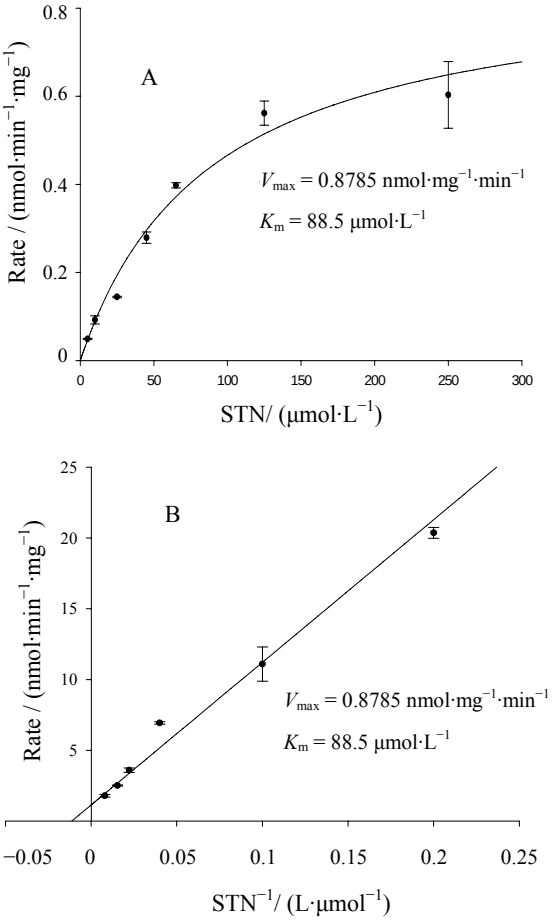

Fig. 2 Plots of Michaelis-Menten (A) and Lineweaver-Burk (B) for STN metabolism in HLM

Major metabolic CYP isoforms of STN

The inhibitory effects of CYP-specific inhibitors on the MCR of STN in HLM were shown in Fig. 3. Concentration of specific inhibitors used was 2, 10, 30, 10, 10, and 3 μmol/L for KET, QUI, TRA, 4-MET, SUL, and ANF, respectively Among the six inhibitors tested, CYP3A4 specific inhibitor KET (2 μmol/L) remarkably inhibited STN metabolism in HLM with 12%－16% MCR of that of the control. CYP2C19 inhibitor TRA and CYP2C9 inhibitor SUL also had slight inhibition on STN metabolism in HLM, respectively. The results indicated that CYP3A4 was likely to be the major enzyme responsible for STN metabolism, CYP2C19 and CYP2C9 play ancillary roles in STN metabolism.

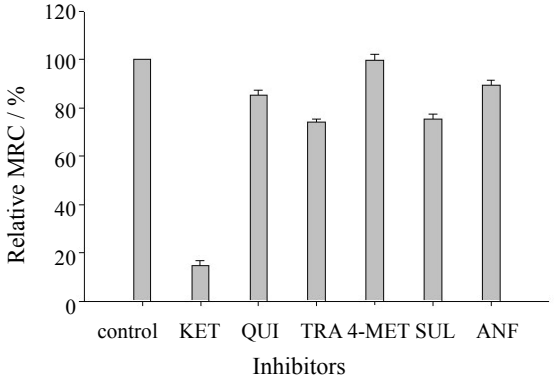

Fig. 3 Inhibition of CYP-specific inhibitors on MCR of STN (50 μmol·L^{-1}) in HLM

To verify the results stated above, heterologously expressed recombinant human CYP3A4, 2C19, and 2C9 (100 pmol/mL) were selected to be incubated individually with STN (25 μmol/L) in the presence and absence of the corresponding specific inhibitor for 45 min at 37 ℃ in triple experiments. CYP3A4 specific inhibitor KET inhibited the MCR of STN by approximately (95.7 ± 2.9)%. Both CYP2C9 specific inhibitor (SUL) and CYP2C19 specific inhibitor (TRA) have no significant effects on the MCR of STN. The results substantiated that CYP3A4 was the major metabolic enzyme of STN in HLM.

Effect of STN on activities of CYP isoforms

The inhibitory effects of STN and the IC_{50} values of the specific inhibitors on the activities of CYP isoforms were shown in Fig. 4, Fig. 5, and Table 2. At the concentration up to 50 μmol/L, STN inhibited CYP3A4-catalyzed testosterone 6β-hydroxylation, CYP1A2-catalyzed phenacetin *O*-deethylation, and CYP2E1-catalyzed chlorzoxazone 6-hydroxylation with the inhibitory potencies up to 85%. The highest inhibition was observed for CYP3A4-catalyzed testosterone 6β-hydroxylation with IC_{50} value of 5.9 μmol/L. The inhibitory curves were obtained by using Graphpad Prism 5 software.

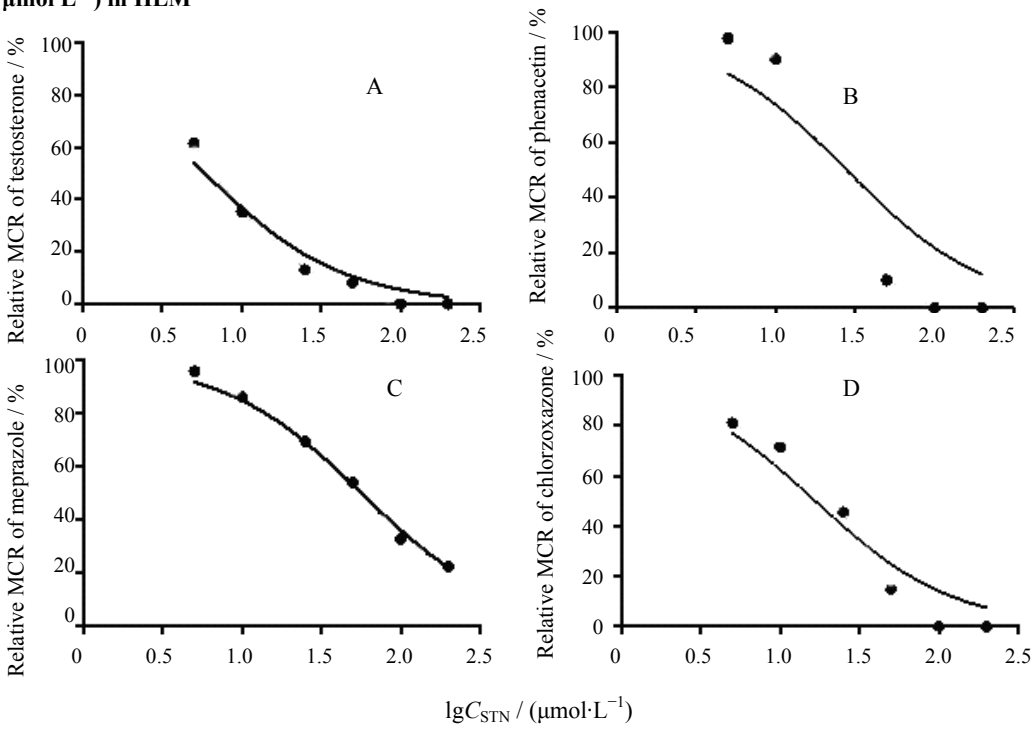

Fig. 4 Inhibition of STN on CYP3A4-catalyzed testosterone 6β-hydroxylation (A), CYP1A2-catalyzed phenacetin *O*-deethylation (B), CYP2C19-catalyzed Omeprazole 4-hydroxylation (C), and CYP2E1-catalyzed chlorzoxazone 6-hydroxylation (D) reactions

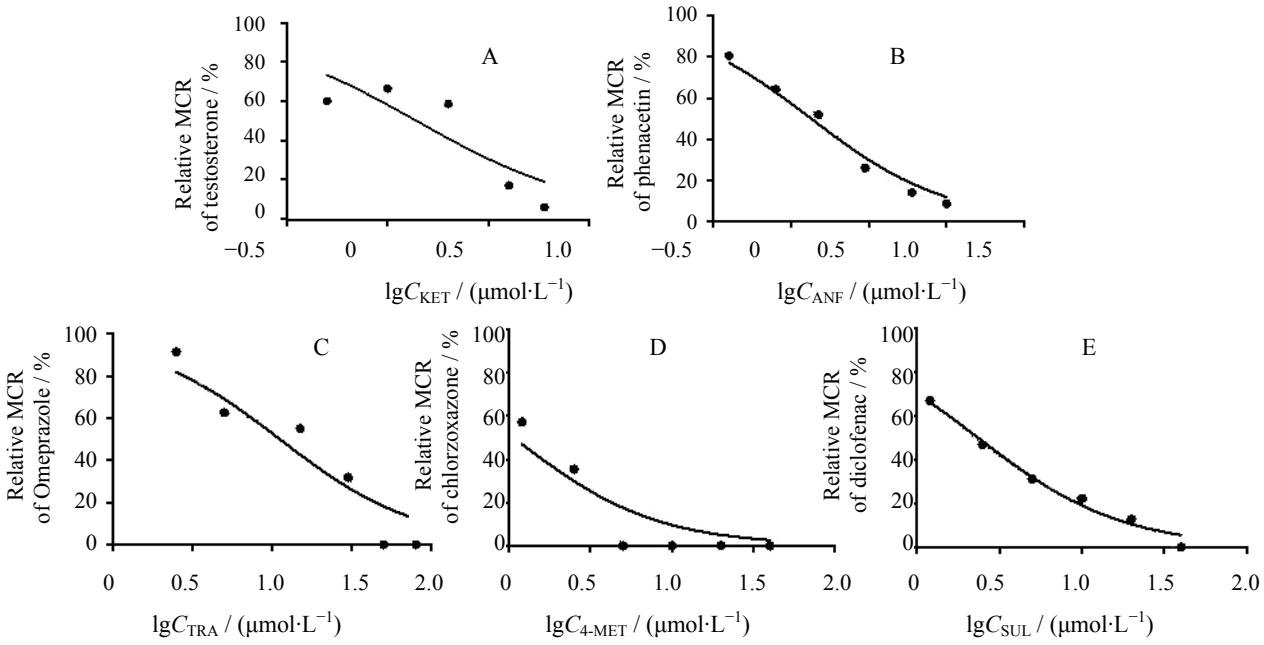

Fig. 5 Inhibition of specific inhibitors on CYP3A4-catalyzed testosterone 6β-hydroxylation (A), CYP1A2-catalyzed phenacetin *O*-deethylation (B), CYP2C19-catalyzed Omeprazole 4-hydroxylation (C), CYP2E1-catalyzed chlorzoxazone 6-hydroxylation (D), and CYP2C9-catalyzed diclofenac 4-hydroxylation (E) reactions

Table 2 IC_{50} values of STN and specific inhibitors for CYP isoforms

CYP isoforms	STN / specific inhibitors	IC_{50} / (μmol·L^{-1})	Standard error (lgIC_{50})
3A4	STN / KET	5.9 / 1.4	0.06 / 0.13
1A2	STN / ANF	28.0/ 1.4	0.21 / 0.04
2C19	STN / TRA	56.6 / 11.2	0.02 / 0.12
2E1	STN / 4-MET	16.7 / 1.1	0.10 / 0.13
2C9	STN / SUL	— / 2.4	— / 0.03

Inhibitory type of STN on testosterone metabolism

To determine the inhibitory type of STN on the metabolism of testosterone, a series of concentration of STN (0, 5.0, and 10 μmol/L) and testosterone (50, 40, 30, and 20 μmol/L) was co-incubated with CYP3A4 (final concentration 12.5 pmol/mL) at 37 ℃ for 20 min, respectively. The type of inhibition and the K_i value were evaluated with the program SPSS Enzyme Kinetics 1.10 Demo. According to the reference (Bjornsson *et al*, 2003), if the inhibitory type is noncompetitive, the IC_{50} value will be the same as its K_i value under the condition of the probe substrate concentration at its K_m value. In the present work, the concentration of testosterone was chosen at the apparent K_m value (50 μmol/L) based on known literature value (Walsky and Obach, 2004), and the IC_{50} value (5.9 μmol/L) and K_i value (5.5 μmol/L) of STN for CYP3A4 were found almost the same (Fig. 6). The result indicated that STN noncompetitively inhibited the metabolic reaction of testosterone catalyzed by CYP3A4.

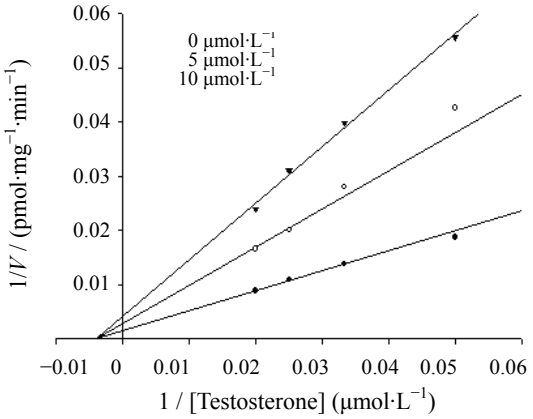

Fig. 6 Dixon plot of co-incubation of variant concentrations of testosterone with STN (0, 5.0, and 10 μmol·L^{-1}) in presence of CYP3A4 (12.5 pmol·mL^{-1}), respectively

Metabolic interaction of STN and GA

The effect of GA on STN metabolism was illustrated in Fig. 7. At the lower concentration of STN (10 μmol/L), GA (10.6 μmol/L) inhibited the MCR of STN in HLM by approximately 50% (Fig. 7A). In order to determine the inhibitory type of GA on STN

metabolism, $1/V$ (Y) of STN at 10 and 200 μmol/L was plotted against a serial of GA concentrations (X), respectively (Fig. 7B). The results indicated that GA competitively inhibited the metabolism of STN in HLM with IC_{50} value of 10.6 μmol/L and K_i value of 17.7 μmol/L.

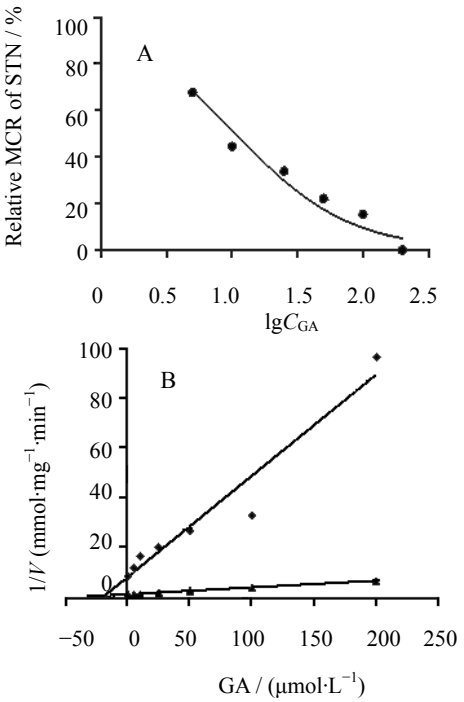

Fig. 7 Inhibition of GA on STN metabolism in HLM

A: inhibitory curve of GA on STN metabolism obtained by using Graghpad Prism 5 software

B: Dixon plot of co-incubation of variant concentration of GA with 10 (◆) and 200 μmol·L^{-1} STN (▲), respectively

Discussion

Based on the results of the present study, the K_m, V_{max}, and CL for STN metabolism in HLM were 0.88 nmol/(mg·min), 88.50 μmol/L, and 9.93 mL/(mg·min), respectively. The enzyme kinetics of STN in HLM followed Michaelis-Menten kinetics.

The CYP450-mediated drug-drug interaction could profoundly affect the safety and efficacy of drug therapies (Halpert, 1995; Guengerich, 1997), especially for the drugs with narrow therapeutic index and inherent toxicity (Scripture and Figg, 2006). The important findings of this study showed that STN was metabolized mainly by CYP3A4 which was the most abundant CYP isoforms in HLM and had broad substrate specificity. The activity of CYP3A4 could be affected by a variety of xenobiotics (Wang *et al*, 2006). Interaction between CYP3A4 substrates is complicated because of allosteric characteristics of CYP3A4 (Shou *et al*, 1999; Wang *et al*, 2000). The usual interaction between two different substrates for the same enzyme is competitive inhibition. However, the present study demonstrated that STN noncompetitively inhibited the CYP3A4-catalyzed testosterone 6β-hydroxylation with IC_{50} value of 5.9 μmol/L. In addition, we also found that GA had a competitive inhibitory action on STN metabolism with K_i value of 17.7 μmol/L in this study.

References

Alcocer GG, Torres MA, Miledi R, 2005. Strychnine induces embryotoxicity in rat neurulation. *Neurotoxicol Teratol* 27(6): 855-859.

Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, Kumar G, Mcleod J, Obach RS, Oberts S, Roe A, Shah A, Snikeris F, Sullivan JT, Tweedie D, Vega JM, Walsh J, Wrightion SA, 2003. The conduct of *in vitro* and *in vivo* drug-drug interaction studies: A pharmaceutical research and manufacturers of America (PhRMA) perspective. *Drug Metab Dispos* 31(7): 815-832.

Boyd RE, Brennan PT, Deng JF, Rochester DF, Spyker DA, 1983. Strychnine poisoning. Recovery from profound lactic acidosis, hyperthermia, and rhabdomyolysis. *Am J Med* 74(3): 507-512.

Department of Health and Human Services, 1997. US Food and Drug Administration. Drug metabolism/Drug interaction studies in the drug development process: Studies *In Vitro*, April.

Duan WQ, Ji SL, 2007. Advances in studies on anti-viral activity of glycyrrhizic acid. *Chin Tradit Herb Drugs* 38(4): a1-a3.

Guengerich FP, 1997. Role of cytochrome P450 enzymes in drug-drug interactions. *Adv Pharmacol* 43: 7-35.

Halpert JR, 1995. Structural basis of selective cytochrome P450 inhibition. *Annu Rev Pharmacol* 35: 29-53.

Kim H, Yoon YJ, Kim H, Kang S, Cheon HG, Yoo SE, Shin JG, Liu KH, 2006. Characterization of the cytochrome P450 enzymes involved in the metabolism of a new cardioprotective agent KR-33028. *Toxicol Lett* 166(2): 105-114.

Kim KA, Kim MJ, Park JY, Shon JH, Yoon YR, Lee SS, Liu KH, Chun JH, Hyun MH, Shin JG, 2003. Stereoselective metabolism of Lansoprazole by human liver cytochrome P450 enzymes. *Drug Metab Dispos* 31(10): 1227-1234.

Kim KB, Seo KA, Yoon YJ, Bae MA, Cheon HG, Shin JG, Liu KH, 2008. *In vitro* metabolism of a novel PPAR agonist, KR-62980, and its stereoisomer, KR-63198, in human liver microsomes and by recombinant cytochrome P450s. *Xenobiotica* 38(9): 1165-1176.

Li XY, Chen XY, Li Q, Wang LL, Zhong DF, 2007. Validated method for rapid inhibition screening of six cytochrome P450 enzymes by liquid chromatography—tandem mass spectrometry. *J Chromatogr B* 852(1/2): 128-137.

McCool BA, Chappell A, 2007. Strychnine and taurine modulation of amygdala-associated anxiety-like behavior is "state" dependent. *Behav Brain Res* 178(1): 70-81.

Scripture CD, Figg WD, 2006. Drug interactions in cancer therapy. *Nat Rev Cancer* 6(7): 546-558.

Segel IH, 1975. *Biochemical Calculations*. Wiley: New York.

Shou M, Mei Q, Ettore MR, Dai R, Baillie TA, Rushmore TH, 1999. Sigmoidal kinetic model for two co-operative substrate-binding sites in a cytochrome P450 3A4 active site: An example of the metabolism of diazepam and its derivatives. *Biochem J* 340(Pt3): 845-853.

Tang J, Usmani KA, Hodgson E, Rose RL, 2004. *In vitro* metabolism of fipronil by human and rat cytochrome P450 and its interactions with testosterone and diazepam. *Chem Biol Interact* 147(3): 319-329.

Tanimoto Y, Ohkuma T, Oguri K, Yoshimura H, 1991. A novel metabolite of strychnine, 22-hydroxystrychnine. *Xenobiotica* 21(3): 395-402.

Tian QL, Guan YP, Zhang B, Liu HZ, 2006. Research advances on pharmacological activities of components in licorice. *Nat Prod Res Dev* 18(2): 343-347.

Walsky RL, Obach RS, 2004. Validated assays for human cytochrome P450 activities. *Drug Metab Dispos* 32(6): 647-660.

Wang RW, Newton DJ, Liu N, Atkins WM, Lu AYH, 2000. Human cytochrome P-450 3A4: *in vitro* drug-drug interaction patterns are substrate-dependent. *Drug Metab Dispos* 28(3): 360-366.

Wang YG, Wang SQ, Liu YX , Yan LP , Dou GF, Gao Y, 2006. Characterization of metabolites and cytochrome P450 isoforms involved in the microsomal metabolism of aconitine. *J Chromatogr B* 844(2): 292-300.

Weaver R, Graham KS, Beattie IG, Riley RJ, 2003. Cytochrome P450 inhibition using recombinant proteins and mass spectrometry-multiple reaction monitoring technology in a cassette incubation. *Drug Metab Dispos* 31(7): 955-966.

Yao HT, Chang YW, Lan SJ, Chen CT, Hsu JTA, Yeh TK, 2006. The inhibitory effect of polyunsaturated fatty acids on human CYP enzymes. *Life Sci* 79(25): 2432-2440.