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Original Article

Potential herb-drug interaction of shexiang baoxin pill in vitro based on drug metabolism/transporter

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Abstract: Many researches have proved functions of anti-oxidation, endothelial protection and pro-angiogenesis efficiency of Shexiang Baoxin Pill (SBP). This study aims to investigate potential for metabolism-based interaction on CYP450s and transporter based interaction on OATP1B1, BRCP and MDR1. Human primary hepatocytes were used in this study. Probe substrates of cytochrome P450 enzymes were incubated in human liver microsomes (HLMs) with or without SBP and IC_{50} values were estimated. Inhibitive potential of SBP on activities of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4 was evaluated. Inducible potential of SBP on activities of CYP1A2, 2B6 and 3A4 was accessed. Inhibitive potential of SBP on human OATP1B1 was evaluated using cell-based assay. Inhibitive potential of SBP on human MDR1 and BCRP was also evaluated using vesicles assay. MDR1 and BCRP vesicle kit were used to determine ATP dependent uptake activity when incubated with SBP. SBP was a competitive inhibitor of CYP2B6, 2C19, while neither inhibitory nor inductive potentials toward other CYP450s were detected. No significant MDR1 inhibitory potential was estimated, while only high concentration of SBP (500 μg/ml) could inhibit activity of BCRP. Probe substrates Estradiol-17 β-glucuronide was incubated in HEK293-OATP1B1 and HEK293-MOCK cell system with different concentration of SBP and estimated IC_{50} was 179 μg/mL, which demonstrated a moderate inhibition potential against OATP1B1. In conclusion, outcome of this study suggests that SBP plays an important role in inhibition of CYP450 isozymes (including CYP2B6 and 2C9) and transporter OATP1B1. Therefore, precautions should be taken when using SBP for CYP and OATP-related herb-drug interactions.

Keywords: CYP450, shexiang baoxin pill, transporters, herb-drug interaction

Introduction

The Shexiang Baoxin Pill (SBP), indexed in the 2010 *Chinese Pharmacopoeia*, which consists of seven kinds of medicinal materials (including Moschus, Calculus Bovis, Radix Ginseng, Cortex cinnamomi, Styrax, Venenum Bufonis and Borneolum Syntheticum), and is widely used as an emergency and daily traditional Chinese medicine (TCM) in the treatment of coronary artery disease such as angina pectoris and myocardial infarction [1]. Many researches have proved its anti-oxidation, endothelial protection and pro-angiogenesis efficiency [2-4]. However, scientist proved that the SBP could accelerate the basic metabolism rate of nifedipine, leading to an unsatisfactory control of blood pressure [5]. Moreover, Liu et al. proved that patients were likely to have a bleeding tendency while undertaking the combination therapy of SBP and warfarin [6]. Although the combination of herb and drug is very popular and often proved to be superior to merely using only one of them; results from these studies have triggered adverse reactions to such an approach. Since few herb-drug interaction studies were carried out during treatments combining herbs and drugs, such lack of research directly affects the efficacy and safety leading to an unpredictable clinical outcome.

Despite of the complicated ingredients, SBP plays its pharmacological role in the form of a chemical compound in vivo. Therefore, in the process of metabolism, SBP may exert its effect on drugs and change their pharmacological characteristics and causing herb-drug interaction. An estimated incidence rate of adverse reactions varies from 1.8% to 9.1% in clinical combination of herbs and drugs [7-9]. As a consequence, it is important that possible interactions should be identified in order to avoid serious and negative effects.

Cytochrome P450s (CYP450s) are the most important phase 1 enzyme system in the metabolism of xenobiotics, including western drugs, endogenous compounds and herbal components as effective substrates, accounting for about 75% of the total metabolism [10, 11]. Many drugs may increase or decrease the activity of various CYP450 isozymes either by inducing the biosynthesis of an isozyme (enzyme induction) or by directly inhibiting the activity of the CYP450 (enzyme inhibition). This is a major source of adverse drug interactions, since changes in CYP450 enzyme activity may affect the metabolism and clearance of various drugs.

Besides CYP450s, membrane transporters also play an important role in the processes of drug absorption, metabolism and distribution. Transport proteins are integral transmembrane proteins, which mainly exist permanently within and span the membrane across which they transport substances. The proteins may assist in the movement of substances by facilitated diffusion or active transport.

The liver is the major clearance organ for drugs in the body where the inactivation mechanisms are composed of metabolic enzymes and drug transporters. These inactivation mechanisms are associated with the hepatic first-pass effect after oral administration and with elimination from the systemic circulation [12]. The purpose of this study was to investigate the potential for the metabolism-based interaction on CYP450s (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4) and transporter based interaction on OATP1B1, BRCP and MDR1 in vitro. Since that the results of animal studies can't be directly extrapolated to humans, while in vitro systems have been developed to replace animal studies and provide reliable predictions.

Materials and methods

Chemicals and reagents

SBP (Shanghai Hutchison Pharmaceuticals), human liver microsome (HLM, Lot No: SUBK), primary human liver cell (Lot YJM), Phenacetin (Sigma77440, STBB2177M9), bupropion (SigmaB102, 013K4612), diclofenac (SigmaUC-291, 093), dextromethorphan (SigmaD25-31, 120K1657), amodiaquine (SigmaA2799, 038F0993V), S-Mephenytoin (SigmaUC175, BCBB6110), testosterone (Sohonchemtech, 201102001), α-Naphthoflavone (SigmaN5757, 087K0067), thiophosphoramide (Sohonchemtech, 20101110), Sulfaphenazole (SigmaS07-58, 096K1462), quinidine (SigmaQ3625, 066-K2509), quercetin (SigmaQ4951, 020M1566), ticlopidine (SigmaT6654, 020M1566), Phenobarbital (Sigma P-1636), rifampicin (Sigma R-5777), ketoconazole (SigmaK1003, 121H05-24), acetaminophen (SigmaA-3035, 109H88-07), hydroxybupropion (GMH0818, 7332), 4-hydroxydiclofenac (SigmaH-3661, 058K1310), dextrorphan (SigmaUC205, 1385499), 6β-hydroxytestosterone (BD451012, 65773), Estradiol-17β-glucuronide (E17βG, MERCK, 3301-1GM), paclitaxel (Sohonchemtech, 20101019), verapamil (SigmaV4629, 048K1343), theophylline (NIFDCKYDE-W1C2), Methanol (Merck), N-Methyl quinidine (NMQ, Sigma, SBNMQ 42982876), Lucifer yellow (LFY, SigmaL0259 MKBH2142V), Osalmid (Shanghai Jinyi Chemical), β-NADPH (Sigma N-1630), Ultrapure water (Millipore), Human BCRP Vesicle Kit (GenoMembrane, Catalog#, GM0008, Lot# DMU9-G03), Human MDR1 Vesicle Kit (GenoMembrane, Catalog#, GM0015, Lot# DKA4G26), HEK293-OATP1B1&HEK293-MOCK cell strain (GenoMembrane), fluorescein (Sigma L0259-MKBH2142V), novobiocin (Sigma N6160SLB-B3208V), DMSO (Sigma D1435SHBB6757V).

SBP concentrations

The concentrations of SBP used in vitro metabolic system are expected to cover the range of concentrations occurring in vivo. Total recommended daily intake of SBP was taken by 2 pills (22.5 mg/pill), three times per day. Considering that an extreme concentration range anticipated to appear in the small intestine, 100% bioavailability minor protein binding and minor accumulation. Final dissolved concentrations were 0.1, 0.5, 1, 5, 10, 50, 100, 500 μg/ml.

Hepatocyte isolation and induction

Human primary hepatocytes were prepared from donation following the Shanghai Donation Regulation (China) and informed consent. The donors were Mongolian men from 21-35 years (Research Institute for Liver Diseases donor number: SUBK, YJM). The cause of death was trauma and all the serology analysis including HIV, RPR, hepatitis B virus and hepatitis C virus were negative.

Evaluating inhibitive potential of SBP on the activities of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4 in vitro

Different concentration samples of SBP (0.1, 0.5, 1, 5, 10, 50, 100, 500 μg/mL; mixture in phosphate buffer (PBS), pH 7.4 (0.1 mol/l)) were pre-incubated with human liver microsomal (HLM) at 37°C for 15 min before initiating the reaction. Thereafter, relative probe substrate solution: CYP1A2 (phenacetin, 30 μM), CYP2B6 (bupropion, 200 μM), CYP2C8 (amodiaquine, 2.5 μM), CYP2C9 (diclofenac, 25 μM), CYP2C19 (S-Mephenytoin, 100 μM), CYP2D6 (dextromethorphan, 8 μM), CYP3A4 (testosterone, 50 μM), were added respectively into all samples. Then reduced coenzyme II (β-NADPH, coenzyme of CYP450, 1 mM) was added and all the samples were incubated at 37°C, 30 min for sufficient reaction. Probes of CYPs is 30 min at 37°C, and the reaction concentration of the coenzyme is 1 mM. In order to terminate the reaction, pre-cooled methanol with internal standard was added separately. All the samples were centrifuged at 12,000 r/min for 5 min at the room temperature. The supernatants were then transferred to a separate tube and injected into LC-MS/MS metabolites analyzing.

Positive control (PC): Pre-incubation HLMs with different selective inhibitors of CYP450 isoforms: CYP1A2 (α-Naphthoflavone, 30 μM), CYP2B6 (thiophosphoramide, 200 μM), CYP2C8 (quercetin, 20 μM), CYP2C9 (Sulfaphenazole, 10 μM), CYP2C19 (ticlopidine, 5 μM), CYP2D6 (quinidine, 10 μM), CYP3A4 (ketoconazole, 5 μM) at 37°C for 15 min, and then repeat the incubation with relative probes as mentioned before.

Negative control (NC): Pre-incubation HLMs with PBS at 37°C for 15 min, the repeat the incubation procedure.

Evaluation the inhibition effect of SBP on CYP450s

The inhibition of SBP on CYP 1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4 were evaluated according to the guidance for industry drug interaction studies from FDA [13], and the inhibition rate was evaluated by analyzing the reduction of relative probe substrates.

The relative activity of CYP450s is displayed as a percentage relative to the negative control, the formula of calculating data is shown as the following:

Relative activity (%) = products concentration of test group or PC/products concentration of NC×100%.

Evaluating inducible potential of SBP on the activities of CYP1A2, 2B6 and 3A4

Human hepatocytes were prepared by a collagenase perfusion technique and were accepted for experimental use if viability was more than 70% [14]. Cryo-preserved primary human hepatocytes were plated on Falcon 24-well culture plates (0.5 ml, 0.35×10^6 cells/well) coated with collagen at 37°C, 5% CO_2. Dosing hepatocytes with different concentration samples of SBP (5, 50, 500 μg/mL), then incubate all the samples with the probe substrate of CYP1A2 (Phenacetin, 100 μM), CYP2B6 (Bupropion, 100 μM) and CYP3A4 (testosterone, 100 μM) at 37°C, 5% CO_2 for 60 min. Incubations were stopped by adding 0.5 ml ice-cold methanol. Samples were centrifuged at 1400×g for 5 min. After that, 500 μl of the supernatants were transferred and injected into LC-MS/MS metabolites analyzing.

Positive control (PC): Pre-incubation human hepatocytes with different selective inducers of CYP450 isoforms: CYP1A2 (Omeprazole, 50 μM), CYP2B6 (Phenobarbital, 1000 μM) and CYP3A4 (Rifampicin, 25 μM) at 37°C, 5% CO_2. And then repeat the incubation as described above.

Negative control (NC): Pre-incubation human hepatocytes with PBS at 37°C, 5% CO_2 and then repeat the incubation as described above.

Evaluation the induction effect of SBP on CYP450s

The induction of SBP on CYP1A2, 2B6 and 3A4 were firmly guided by USFDA Guidance [13]: ① Qualification for the test system (Hepatocytes): Activity of Positive controls (PC)>200% of Negative Controls (NC); ② Judgment of inductive potentials by test article: Potential induction >40% of PC.

Relative activity of NC (%NC) = activity of SBP treated samples or PC sample/activity of NC×100%.

Table 1. Regression data

Analyte	Linear regression equation	r
Phenacetin	y = 0.32x+0.000886	0.9976
Bupropion	$y = 0.0177x-1.15e^{-0.005}$	0.9997
Amodiaquine	$y = 0.000322x+5.36e^{-0.005}$	0.9973
Diclofenac	$y = 9.71e^{-0.005}x-0.000462$	0.9934
S-Mephenytoin	$y = 0.00142x-7.36e^{-0.005}$	0.9996
Dextromethorphan	$y = 0.0202x-3.34e^{-0.005}$	0.9997
Testosterone	y = 0.00156x-0.000316	0.9987

Relative activity of PC (%PC) = (Activity of SBP treated samples-Activity of negative control)/(Activity of positive control-Activity of negative control)×100%.

Evaluating inhibitive potential of SBP on human OATP1B1 by cell-based assay in vitro

HOATP1B1-expressing cell line (HEK293-OATP1B1) was used in this study. MOCK cells: HEK293 incorporated with corresponding empty vector for negative control (HEK293-MOCK).

HEK293-MOCK cells and HEK293-OATP1B3 cells were grown on 75 cm^2 cell culture flasks at 37°C, 5% CO_2, by using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 1 mg/ml of geneticin. The cells were cultured in 24-well plates for assaying and were plated 48 to 52 h before assaying. Prior to assay, the media was removed from the plate and the cells washed two times (2×0.25 ml) with pre-warmed transportation buffer (pH 7.4). Left the second wash buffer in the wells and placed the plate at 37°C for 5 min before initiating incubation for uptake assays. Removed the second buffer from the wells immediately and started the assay by adding 0.15 ml pre-warmed (37°C) mixture solution (E17βG with SBP (0, 0.5, 5, 50 and 500 μg/mL or rifampin). The plates were returned to the incubator at 37°C. After 10 min, the incubation solution was removed and the cells were washed three times (3×0.25 ml) with ice-cold transportation buffer (pH 7.4). After removing the third washing buffer, 0.1 mL NaOH (1 M) was added to each well and allows standing for 30 min at room temperature to lyse the cells. Then added 0.1 mL HCl (1 M) to the lysate. The protein content of cell lysates was determined using the Pierce® BCA Protein assay kit according to the manufacturer's instructions. Measure the amount of E17βG transported into the cell by LC-MS/MS.

Evaluation the inhibition effect of SBP on human OATP1B1

The uptake rate (U, pmol/mg/min) of E17βG is calculated using the following equation:

$$U = \frac{C_{lysate}}{P \times T}$$

C_{lysate} is the E17βG concentration in cell lysate (pmol/ml); P is the protein content (mg/ml); T is the time of incubation (min).

The net uptake rate of E17βG is calculated using the following equation:

Net uptake rate = $U_{OATP1B1}$-Mean of U_{Mock}

The inhibition of the test article on hOATP1B3 was evaluated by using relative net uptake activity (% of NC), calculated as following:

$$\%\ of\ NC = \frac{Net\ uptake\ rate\ with\ test\ artical}{Mean\ of\ Net\ uptake\ rate\ without\ test\ artical} \times 100\%$$

Evaluating inhibitive potential of SBP on human MDR1 and BCRP by vesicles assay in vitro

The potential inhibitory study was based on the method of membrane vesicle transportation with ATP/ADP equilibrium with vesicles assay [15-17]. Probe substrate of MDR1: N-Methylquinidine (NMQ); probe substrate of BCRP: Lucifer yellow (LFY).

Pre-incubation SBP (0.5, 5, 50 and 500 μg/mL) or reference controls with Human MDR1 and BCRP vesicles (Human MDR1 and BCRP Vesicle Kit with Buffer, GenoMembrane) at 37°C for 5 min, and followed by incubation with NMQ (50 μM) or LFY (10 μM) at 37°C for 2 min in the presence of ATP or AMP (4 mM). Reactions were stopped by ice-cold Buffer B2 solution together with NC and PC samples. All samples were transferred to 96-well glass-fiber filter plate and filtered by using vacuum pump. Washed the filter 5 times with 0.2 mL ice-cold Buffer B2.

The MDR1 vesicles on filters were dissolved by adding 50 μL 80% methanol and centrifuged at 2000 r/min, 5 min for twice. The dis-

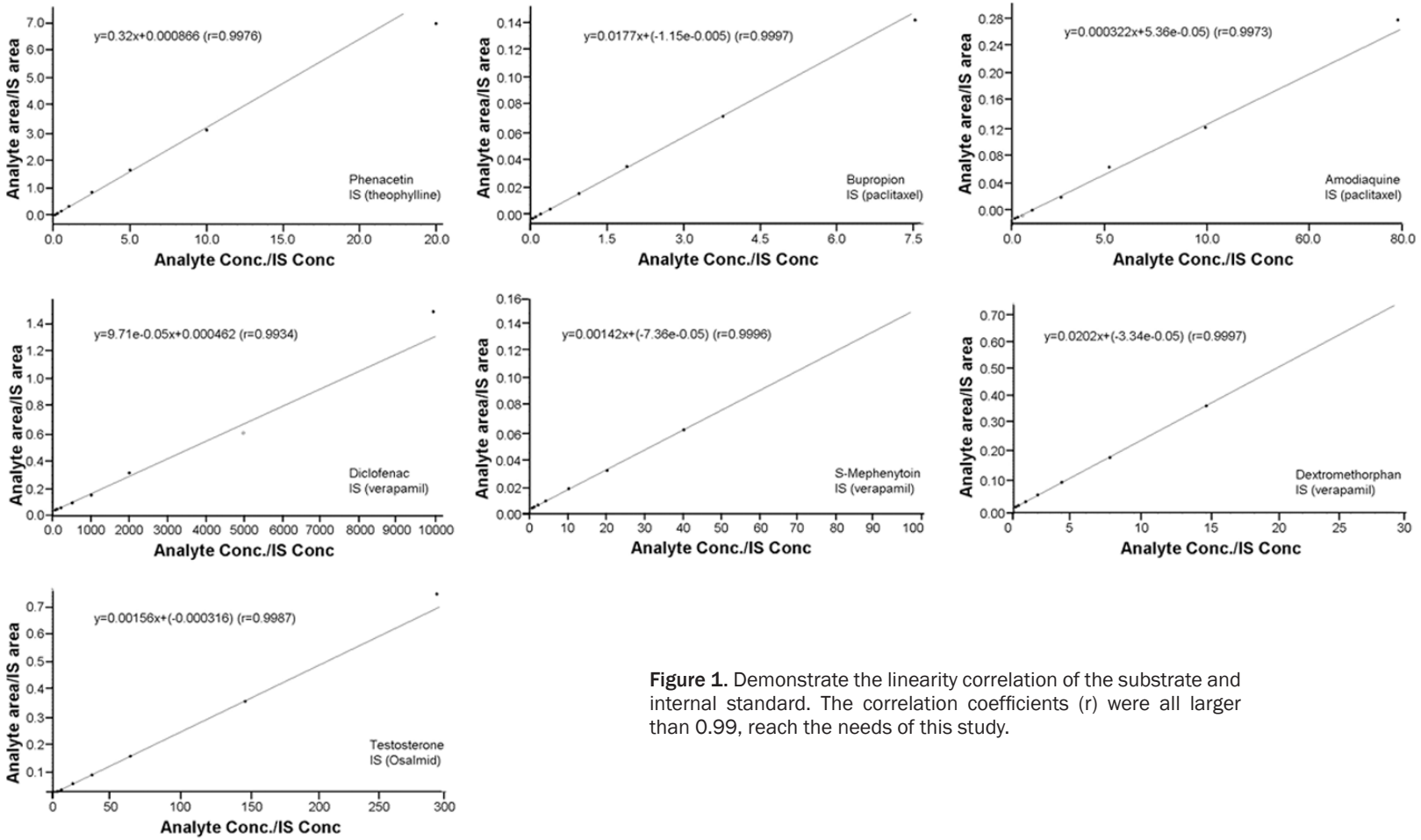

Figure 1. Demonstrate the linearity correlation of the substrate and internal standard. The correlation coefficients (r) were all larger than 0.99, reach the needs of this study.

Table 2. Effect of SBP on activity of CYP450s-relative activity compared with NC and PC (n = 3)

Test Group	Relative Activity (% of NC)													
	CYP1A2		CYP2B6		CYP2C8		CYP2C9		CYP2C19		CYP2D6		CYP3A4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NC	100	4.69	100	6.78	100	7.15	100	11.6	100	5.75	100.0	9.73	100	11.1
SBP 0.1 μg/mL	106	46.8	76.1	8.18	125	16.6	100	22.7	121	31.0	139	12.6	81.3	14.7
SBP 0.5 μg/mL	100	39.3	71.7	7.22	135	28.9	90.9	8.04	144	22.5	159	3.58	96.9	8.58
SBP 1 μg/mL	99.5	39.6	71.2	10.4	139	34.0	100	29.2	148	33.8	157	6.69	105	6.14
SBP 5 μg/mL	97.3	35.8	70.8	5.25	134	38.6	85.3	5.85	139	19.7	155	11.2	102	4.92
SBP 10 μg/mL	92.5	33.3	69.1	4.20	136	17.7	92.2	23.4	136	23.7	148	10.6	105	5.70
SBP 50 μg/mL	86.1	31.4	60.8	5.24	133	21.0	79.9	8.09	125	23.7	140	13.6	103	2.66
SBP 100 μg/mL	73.5	25.8	52.3	2.70	121	13.3	84.8	3.05	101	17.1	128	8.29	107	9.26
SBP 500 μg/mL	51.8	17.6	25.2	1.50	102	15.9	75.1	6.10	53.0	9.03	106	12.4	75.1	2.04
PC	12.1	0.82	20.8	0.94	22.1	2.00	14.1	0.96	23.0	3.51	5.24	0.52	1.83	1.13
IC 50	NA		260.4 μg/mL		NA		NA		302.2 μg/mL		NA		NA	
95% Confidence	NA		187.7 to 361.1 μg/mL		NA		NA		157.6 to 579.7 μg/mL		NA		NA	

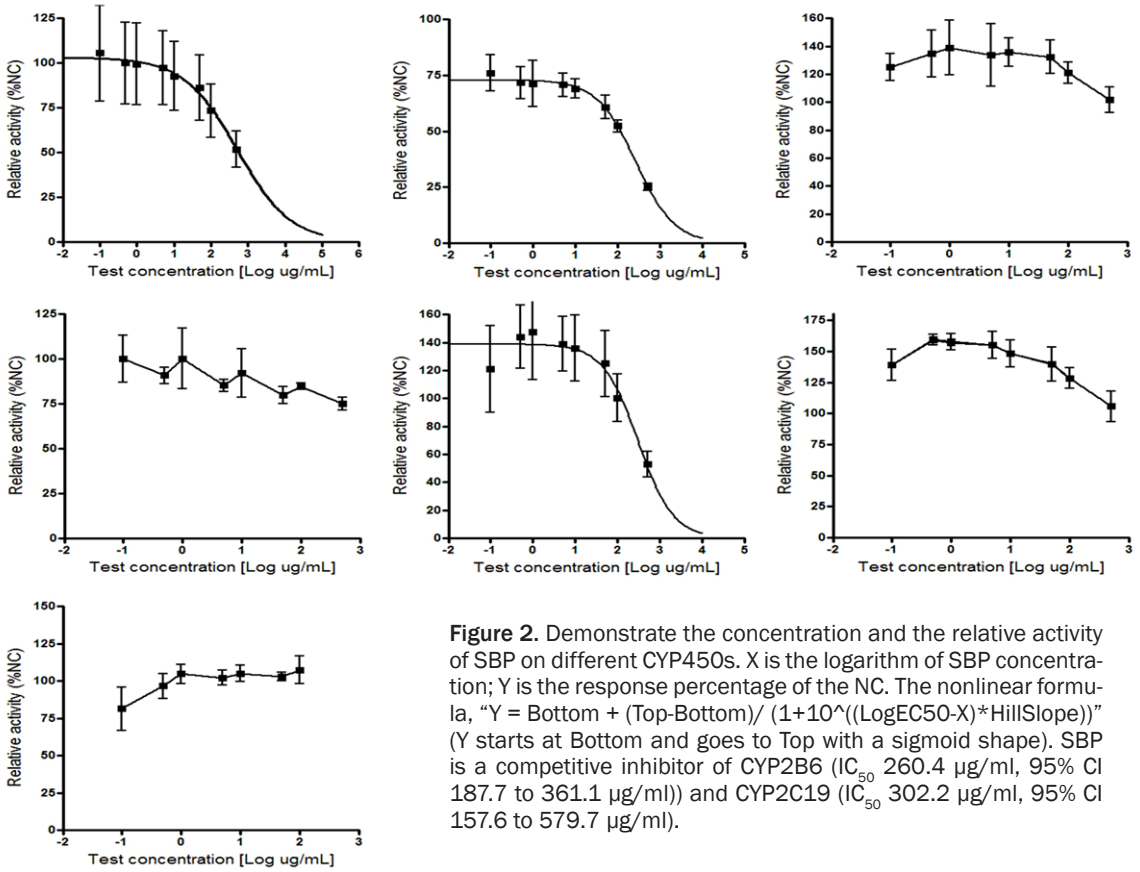

Figure 2. Demonstrate the concentration and the relative activity of SBP on different CYP450s. X is the logarithm of SBP concentration; Y is the response percentage of the NC. The nonlinear formula, "Y = Bottom + (Top-Bottom)/ (1+10^((LogEC50-X)*HillSlope))" (Y starts at Bottom and goes to Top with a sigmoid shape). SBP is a competitive inhibitor of CYP2B6 (IC_{50} 260.4 μg/ml, 95% CI 187.7 to 361.1 μg/ml)) and CYP2C19 (IC_{50} 302.2 μg/ml, 95% CI 157.6 to 579.7 μg/ml).

solved vesicles were collected to the fluorometer plate. Add 100 μL 0.1 N H_2SO_4 to each well, and measure the fluorescence intensity of each well with Ex 428 nm and Em536 nm.

The BCRP vesicles on filters were dissolved by adding 50 μL 10% sodiumdodecyl sulfate (SDS), then centrifuged at 2500 r/min, and 2 min for twice. The filtrates were mixed and collected to the fluorometer plate. Add same volume DMSO solution to each well and the fluorescence intensity measurement was the same as MDR1 described above.

Positive control (PC): Incubation human relative vesicles with different selective inhibitors:

Table 3. Effects SBP and inducers (PC) on CYP activities in human hepatocytes (n = 3)

Test Group	Relative Activity (% of NC)					
	CYP1A2		CYP2B6		CYP3A4	
	Mean	SD	Mean	SD	Mean	SD
NC	100	33.5	100	5.53	100	14.1
PC	2315	398	643	88.3	771	86.2
SBP 5 μg/mL	86.9	15.9	61.1	8.16	97.4	20.4
SBP 50 μg/mL	33.1	5.93	24.5	1.10	45.3	3.14
SBP 500 μg/mL	NA	NA	8.93	0.84	33.8	1.55

MDR1 (Ketoconazole, 100 μM), BCRP (novobiocin, 100 μM) and NMQ (50 μM) or LFY (10 μM) at 37°C for 2 min in the presence of ATP or AMP (4 mM) then measure the fluorescence intensity.

Negative control (NC): Human relative vesicles without SBP and incubate with NMQ (50 μM) or LFY (10 μM) at 37°C for 2 min in the presence of ATP or AMP and repeat the measurement as described above.

Evaluation the inhibition effect of SBP on human MDR1 and BCRP

Uptake amount of NMQ (pmole) = (Fluorescence of NMQ inside of MDR1 vesicles/Total fluorescence in incubation media without MDR1) × Amount of NMQ in the incubation media ATP-depended uptake activity of MDR1 vesicles (pmole/min/mg) = (Uptake amount of NMQ with ATP-Uptake amount of NMQ with AMP)/ incubation time/MDR1 protein.

Inhibitory potential relative activity (% of NC) = (ATP-dependent uptake activity of SBP or PC samples/ATP-depended uptake activity of NC)%.

The BCRP evaluation formula is the same as MDR1 while substitute NMQ for LYF, MDR1 for BCRP.

Statistical analysis

Data are presented as means ± S.D. of three replicates. A two-sample *t*-test was used to test the effect of SBP, NC and PC on CYP450 and transporter activity. Analysis of variance was used to compare the effects on each CYP and transporter by each SBP concentration. Statistical analysis was performed on Microsoft® Excel (2007), IC_{50} and nonlinear regression analysis was calculated on GraphPad Prism 5. A *P*-value <0.05was considered to be statistically significant.

Results

Linearity

The linearity of the method was evaluated by analyzing seven calibration curves containing eight non-zero concentrations. The linearity of the calibration curve was confirmed by plotting the peak-area ratios of each analyte to the internal standard versus the plasma concentrations using a $1/x^2$ weighted linear least-squares regression model [18] and all samples correlation coefficient (r) were larger than 0.9900 (**Table 1** and **Figure 1**).

Inhibitive potential of SBP on activities of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4

The NC and PC relative activities of each CYP450 isoform were analyzed according to the FDA guidance [13]. The selective inhibitors (PC) of CYP450s inhibited 77% to 98% of relative isoform's activity, suggesting the test system was qualified for this study. SBP is a competitive inhibitor of CYP2B6, 2C9 (IC_{50}260.4 μg/ml and 302.2 μg/ml, respectively) whilst no inhibitive potentials found toward other CYP450s (**Table 2** and **Figure 2**).

Inducible potential of SBP on activities of CYP1A2, 2B6 and 3A4

The CYP inductive properties of SBP were investigated at three different concentrations, as shown in **Table 3**. All concentrations are estimated to be relevant for in vivo extrapolated biological concentrations. Main statistically significant inductive properties were not found (P>0.05, **Figure 3A-C**).

Inhibitive potential of SBP on human OATP1B1

The net uptake activity of E17βG was decreased to 18.1% in HEK293-OATP1B1 cells after incubation with reference inhibitor of hOATP1B1, rifampin, at the concentration of 100 μM (**Table 4**), suggesting that test system be qualified for evaluating the inhibition on hOATP1B1. Moreover, the uptake rate was not significantly decreased in HEK293-hOATP1B1 cells after incubation with SBP at the concentrations of 0.5, 5 and 50 μg/ml, but decreased to 8.22% at 50 μg/ml. The IC_{50} of SBP on the uptake of E17βG by hOATP1B1 was 179 μg/ml (**Figure 4** and **Table 4**).

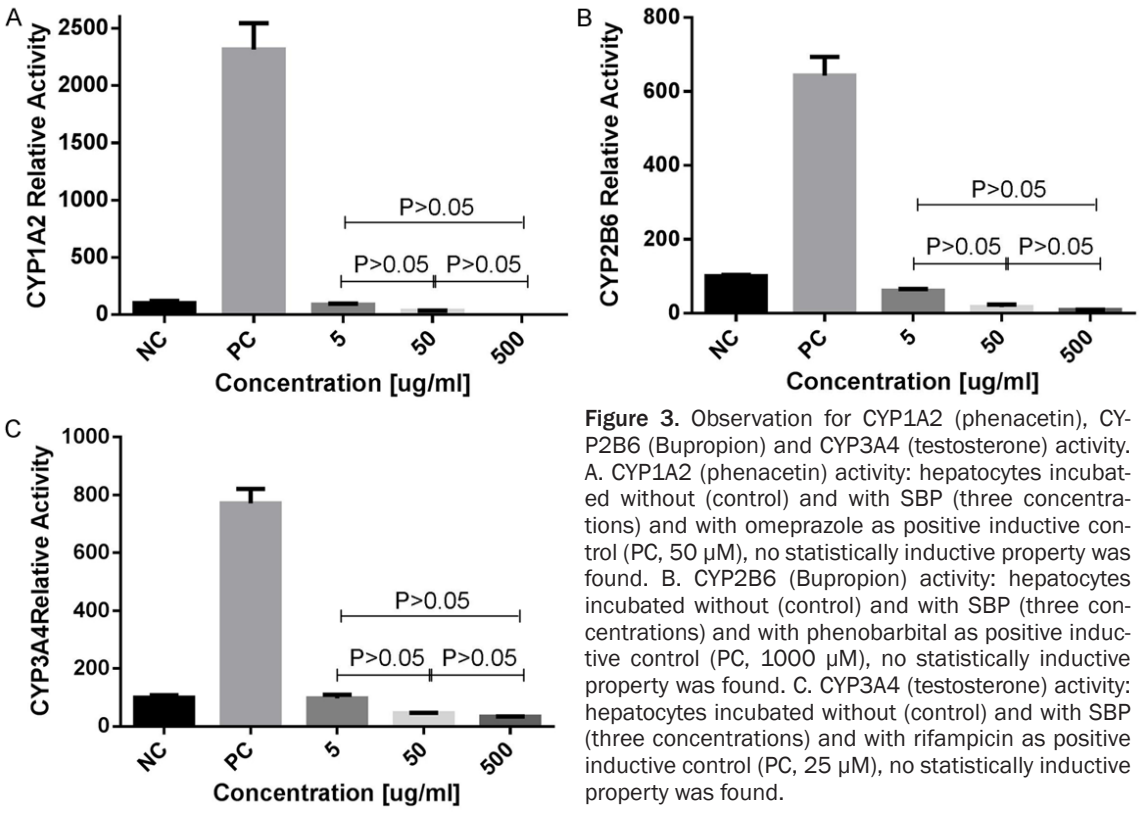

Figure 3. Observation for CYP1A2 (phenacetin), CYP2B6 (Bupropion) and CYP3A4 (testosterone) activity. A. CYP1A2 (phenacetin) activity: hepatocytes incubated without (control) and with SBP (three concentrations) and with omeprazole as positive inductive control (PC, 50 μM), no statistically inductive property was found. B. CYP2B6 (Bupropion) activity: hepatocytes incubated without (control) and with SBP (three concentrations) and with phenobarbital as positive inductive control (PC, 1000 μM), no statistically inductive property was found. C. CYP3A4 (testosterone) activity: hepatocytes incubated without (control) and with SBP (three concentrations) and with rifampicin as positive inductive control (PC, 25 μM), no statistically inductive property was found.

Table 4. The relative net uptake activity of E17βG by hOATP1B1-expressing cells (n = 3)

Test Group	Relative net uptake activity (% of NC)			Mean	SD
NC	108	101	91.5	100	8.10
PC	19.5	22.7	12.0	18.1	5.51
SBP 0.5 μg/ml	80.1	83.4	94.5	86.0	7.54
SBP 5 μg/ml	89.4	119	108	106	15.1
SBP 50 μg/ml	106	111	68.5	95.1	23.1
SBP 500 μg/ml	4.13	3.35	17.2	8.22	7.76
IC_{50}	179 μg/L				
95% CI	62.6-510 μg/L				

Inhibitive potential of SBP on human MDR1 and BCRP

Inhibitive potential of SBP on MDR1: Ketoconazole was selected as the positive control, which is the selective inhibitor of MDR1, and can inhibit more than 45% of transportation of NMQ after pre-incubation with Human MDR1 vesicles at the concentration of 100 μM (**Table 5**). The above results suggest that the testing system is qualified for evaluating inhibitory potential of test article on Human MDR1. The mean relative uptake activity of NMQ was not significantly decreased after incubation with SBP at the concentrations of 0.5, 5, 50, 500 μg/ml, respectively (P>0.05, **Figure 5A**).

Inhibitive potential of SBP on BCRP: Novobiocin was selected as the positive control, which is the selective inhibitor of BCRP, and can inhibit more than 100% of transportation of LFY after pre-incubation with Human BCRP vesicles at the concentration of 100 μM. The above results suggest that the testing system be qualified for evaluating inhibitory potential of test article on Human BCRP (**Table 6**). The mean relative uptake activity of LFY was not significantly decreased after incubation with SBP at the concentrations of 0.5, 5, 50 μg/ml, respectively. While only high concentration of SBP (500 μg/ml) could significantly decrease LFY's uptake activity and inhibit the activity of BCRP (P>0.05, **Figure 5B**).

Discussion

The combination of herbs and drugs for the treatment of diseases has been a common practice in China for decades and most of the clinical observation proved its efficacy and satisfactory outcome. Moreover, such practice has

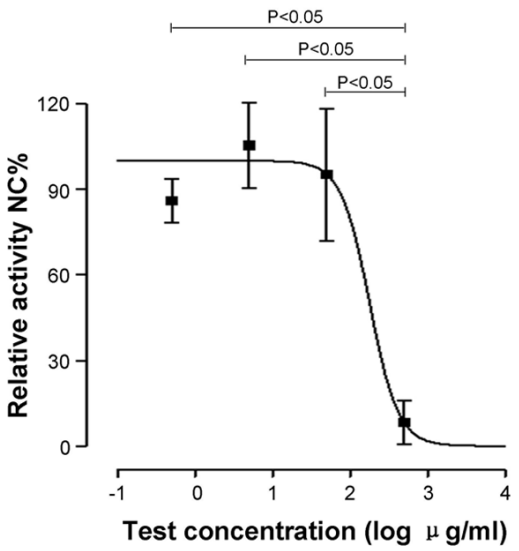

Figure 4. The uptake rate in HEK293-hOATP1B1 cells after incubation with SBP at the concentrations of 0.5, 5, 50 and 500 μg/ml. The IC_{50} of SBP on the uptake of E17βG by hOATP1B1 was 179 μg/ml, 95% CI 62.6-510 μg/ml.

Table 5. Effect of SBP on the activity of human MDR1-relative activity (n = 3)

Test Concentrations	Relative Activity (% of NC)			Mean	SD
NC	125	80.9	93.6	100	23.0
PC (Ketoconazole)	62.4	46.3	55.8	54.9	8.09
SBP 0.5 μg/ml	146	112	166	141	27.2
SBP 5 μg/ml	125	159	137	140	17.3
SBP 50 μg/ml	104	81.2	131	106	25.2
SBP 500 μg/ml	140	86.0	78.1	101	33.5

now spread to developed countries, such as the United States, in the form of health supplements [19]. Conventionally, people all over the world take herbs or natural medicine as health products considering that they are always safe and stable. But unfortunately, that is so far from the case. As it is mentioned above, many plant extracts and their isolated compounds from various areas will affect the CYP450s or transporters with either inhibitory or inductive effect. The increasing use of herbs along with drugs suggests that adverse herb-drug interactions may be of significant public health concern [20].

Currently, there is very little report on herb-drug interactions while the use of herbs and drugs is progressively increasing. The lack of evidence guiding the proper combination of herbs and drugs will probably trigger serious problems since certain herbal supplements can cause potentially dangerous side effects when taken with drugs. Also, the number of cases reported for the emerging herb-drug interactions are already on the rise [21].

Therefore, the present study was undertaken to evaluate the effect of representative Chinese patent medicine, SBP on metabolism and transporter based potential herb-drug interaction in vitro. The use of human hepatocytes closely resembles an in vivo situation with all co-factors and other substances present that the CYP450 enzymes normally encounter in the liver [22]. Also, the CYP enzymes can be induced in vitro in a way that correlates to an in vivo situation [23], given that the experiments are conducted at clinically and pharmacologically relevant concentrations and the study was firmly undertaken guided by FDA guideline [13]. The result showed that SBP is a competitive inhibitor of CYP2B6 (IC_{50} 260.4 μg/ml) and CYP2C19 (IC_{50} 302.2 μg/ml) while neither inhibitory nor inductive potentials toward other CYP450s were detected. CYP450s are a superfamily of heme-protein that catalyze the oxidation of drugs, mainly via a mono-oxygenase reaction [24]. The top CYP isoforms that contribute to the metabolism of small molecule drugs and the percent of each enzyme compared to the total CYP concentration in the liver are: CYP3A (15% to 58%), CYP2C (8% to 35%), CYP1A2 (2% to 28%), CYP2A6 (1% to 14%), CYP2E1 (2% to 13%), CYP2D6 (0.1% to 6%) and CYP2B6 (0.1% to 0.8%) [25]. CYP2B6, as one of the most important exogenous toxic metabolic enzymes in our body, also involved in the metabolism of approximately 7% of the commonly used drugs and endogenous hormones [26, 27]. CYP2C19 participates in the metabolism of Clopidogrel, warfarin, most of the antiepileptic drugs, sedatives and anti-depressants [28]. A membrane transport protein (or simply transporter) is a membrane protein involved in the movement of ions, small molecules, or macromolecules, such as another protein across a biological membrane. The transportation of the protein, no matter active of passive, needs the participation of ATP and AMP. As a consequence, MDR1 and BCRP vesicle kit were used to determine the ATP dependent uptake activity when incubated with SBP. However, no significant MDR1 inhibitory poten-

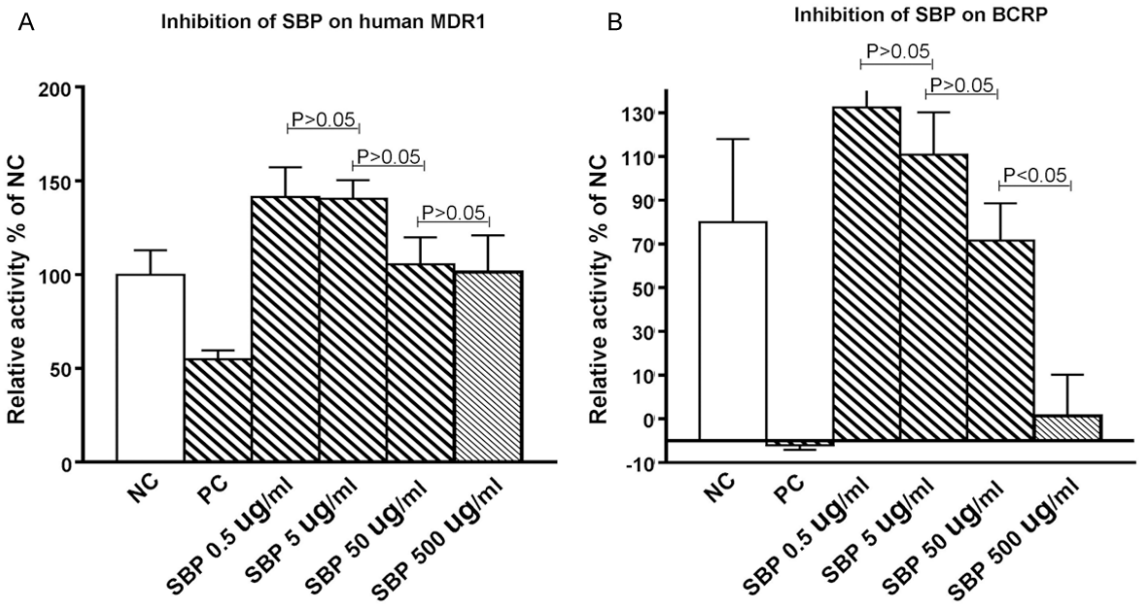

Figure 5. Effects of inhibitory potential of SBP on MDR1 and BCRP. A. The relative activity of NMQ did not decrease significantly with the gradually increased SBP concentration, suggesting SBP has no inhibitory potential on MDR1. B. The relative activity of LFY did not decrease significantly under the concentration from 0.5 to 50 μg/ml, however a great decrease of the activity was analyzed on the concentration of 500 μg/m.

Table 6. Effect of SBP on the activity of human BCRP-relative activity (n = 3)

Test Concentrations	Relative Activity (% of NC)			Mean	SD
NC	62.4	61.6	176	100	65.9
PC (novobiocin)	0.158	-0.315	-6.15	-2.10	3.51
SBP 0.5 μg/ml	158	183	117	152	33.4
SBP 5 μg/ml	170	114	109	131	33.7
SBP 50 μg/ml	89.9	122	62.9	91.5	29.5
SBP 500 μg/ml	30.2	-7.36	-18.4*	11.4	26.5

*removed cause of experimental error.

tial was estimated, while only high concentration of SBP (500 μg/ml) could inhibit the activity of BCRP. The OATP1B1 transporter, a subtype of OATP, is involved in the transportation of angiotensin receptor blocker as valsartan, Olmesartan and most of the statins [29, 30]. The study demonstrated that SBP had a moderate inhibition potential against OATP-1B1 (IC_{50} 179 μg/ml) by estimating probe substrates Estradiol-17β-glucuronide while incubated in HEK293-OATP1B1 and HEK293-MOCK cell system with different concentration of SBP.

In our study, we found that SBP had different inhibitory effect on CYP2B6, 2C19 and OATP-1B1 with IC_{50} of 260.4 μg/ml, 302.2 μg/ml and 179 μg/ml, respectively. It could be concluded that precautions should be taken for CYP and OATP-related herb-drug interactions when using SBP. According to the medicine instruction, total recommended daily intake of SBP: 2 pills (22.5 mg/pill), three times per day. Given the condition of a normal person with 60 to 70 kg, total blood concentration of SBP may never reach such IC_{50} levels. In other words, it could be inferred that taking SBP within daily dosage is safe and reasonable. However, in vitro studies still could not replace the actual situation in vivo. Although lots of the clinical studies on SBP proved its efficacy and safety, when co-administration of other drugs, more than 3 kinds or with substrates of CYP2B6, 2C19 and OATP1B1. Especially in the treatment of coronary artery disease, potential CYP450 or transporter-related herb-drug interaction should be considered.

The studies of herb-drug interaction are still in the gestation stage. Unlike the detailed interaction specification of drugs, the Chinese patent medicine lacks such information and should do more to supplement its potential interaction with CYP450s and transporters to avoid unexpected adverse effect. Furthermore, for

some low bioavailability drugs, we could also use definite herb to enhance its curative effect and reduce some adverse effect by herb-drug interaction to some extent.

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Disclosure of conflict of interest

None.

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References

[1] Zhu H, Luo XP, Wang LJ. Evaluation on Clinical Effect of Long-term Shexiang Baoxin Pill Administration for Treatment of Coronary Heart Disease. Zhongguo Zhong Xi Yi Jie He Za Zhi 2010; 30: 470-474.

[2] Zhang YQ. Influence of Shexiang Baoxin Pills with unstable angina pectoris onoxidative stress in patients of coronary heart disease. Chinese Journal of Experimental Traditional Medical Formulae (Chin) 2013; 19: 334-336.

[3] Peng C, Yang Y, Lv C, Jin H, Tao J, Yuan X, Huang H, Han L, Chang W, Liu R, Zhang W. Pharmacokinetic study of five ginsenosides using a sensitive and liquid chromatography-tandem mass spectrometry method following single and multiple oral administration of Shexiang Baoxin Pills to rats. Biomed Chromatogr 2015; 29: 425-436.

[4] Li YF, Cao FF, Liu F, Bai XY, Lu Y. Effects of Shexiangbaoxin pills on the expression of cardiac alpha-and beta-adrenergic receptor subtypes in rat heart with heart failure induced by myocardial infarction. Chin Med J (Engl) 2012; 125: 1556-1562.

[5] Jiang B, Cai F, Gao SH, Meng L, Liang F, Dai X, Xu W, Chen W. Induction of cytochrome P4503A by Shexiang Baoxin Pill and its main components. Chem Biol Interact 2012; 195: 105-113.

[6] Zou QG, Su JJ. Quantitative analysis of muscone in shexiang boaxin pills by gas chromatography. Zhongguo Zhong Xi Yi Jie He Za Zhi 1994; 19: 418-419.

[7] Yang XL. Case analysis on the combination of Chinese and Western medicine. Nei Mongol J Tradit Chin Med (Chin) 2014; 6: 85.

[8] Pei X. Rational use of traditional Chinese medicine injection and prevention of adverse reactions. Chin J Clin (Chin) 2014; 42: 79-80.

[9] Ouyang Y, Yan CX, Liao YF. Analysis and countermeasures on 127 cases of Chinese and Western medicine unreasonable combination. Med Innov China (Chin) 2014; 11: 123-125.

[10] Coon MJ. Cytochrome P450: nature's most versatile biologicalcatalyst. Annu Rev Pharmacol Toxicol 2005; 45: 1-25.

[11] Guengerich FP. Cytochrome p450 and chemical toxicology. Chem Res Toxicol 2008; 21: 70-83.

[12] Watanabe T, Kusuhara H, Maeda K, Kanamaru H, Saito Y, Hu Z, Sugiyama Y. Investigation of the rate-determining process in the hepaticelimination of HMG-CoA Reductase inhibitors in rats and humans. Drug Metab Dispos 2010; 38: 215-222.

[13] Drug Interaction Studies-Study Design, Data Analysis, and Implications for Dosing and Labeling. Guidance for Industry. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), 2006. (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101).

[14] Li AP. Human hepatocytes: Isolation, cryopreservation and applications in drug development. Chem Biol Interact 2007; 168: 16-29.

[15] International Transporter Consortium, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL. Membrane transporters in drug development. Nat Rev Drug Discov 2010; 9: 215-236.

[16] Xia CQ, Milton MN, Gan LS. Evaluation of drug-transporter interactions using in vitro and in vivo models. Curr Drug Metab 2007; 8: 341-363.

[17] Elsby R, Smith V, Fox L, Stresser D, Butters C, Sharma P, Surry DD. Validation of membrane vesicle-based breast cancer resistance protein and multidrug resistance protein 2 assays to assess drug transport and the potential for drug-drug interaction to support regulatory submissions. Xenobiotica 2011; 41: 764-783.

[18] Chang WL, Han L, Huang HM, Wen B, Peng C, Lv C, Zhang W, Liu R. Simultaneous determination of four volatile compounds in rat plasma after oral administration of Shexiang Baoxin Pill (SBP) by HS-SPDE-GC-MS/MS and its application to pharmacokinetic studies. J Chro-

matogr B Analyt Technol Biomed Life Sci 2014; 963: 47-53.
[19] Tripathi P, Gupta PP, Lal VK. Influence of Allium sativum extract on the hypoglycemic activity of glibenclamide: an approach to possible herb-drug interaction. Drug Metab Drug Interact 2013; 28: 225-230.
[20] Coxeter PD, McLachlan AJ, Duke CC, Roufogalis BD. Herb-drug interactions: an evidence based approach. Curr Med Chem 2004; 11: 1513-1515.
[21] Gohil KJ, Patel JA. Herb-drug interactions: a review and study based on assessment of clinical case reports in literature. Int J Pharmacol 2007; 39: 129-39.
[22] Bent HH, Zhuohan H, Odd GN. The Induction of CYP1A2, CYP2D6 and CYP3A4 by Six Trade Herbal Products in Cultured Primary Human Hepatocytes. Basic Clin Pharmacol Toxicol 2007; 100: 23-30.
[23] Kato M, Chiba K, Horikawa M, Sugiyama Y. The quantitative prediction of in vivo enzyme-induction caused by drug exposure from in vitro information on human hepatocytes. Drug Metab Pharmacokinet 2005; 20: 236-243.
[24] Irene PS, Andreas BS. Pre-systemic metabolism of orally administered drugs and strategies to overcome it. J Control Release 2014; 192: 301-309.
[25] Guengerich FP. Human cytochrome P450 enzymes. In: Ortiz de Montellano PR, editor. Cytochrome P450: structure, mechanism, and biochemistry. 3rd edition. New York: Kluwer/ Plenum Publishers; 2005. pp. 382.
[26] Hodgson E, Rose RL. The importance of cytochrome P450 2B6 in the human metabolism of environmental chemicals. Pharmacol Ther 2007; 113: 420-428.
[27] Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, Desta Z. The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. J Pharmacol Exp Ther 2003; 306: 287-300.
[28] Fukasawa T, Yasui-Furukori N, Suzuki A, Inoue Y, Tateishi T, Otani K. Pharmacokinetics and pharmacodynamics of etizolam are influenced by polymorphic CYP2C19 activity. Eur J Clin Pharmacol 2005; 61: 791-795.
[29] Yamashiro W, Maeda K, Hirouchi M, Adachi Y, Hu Z, Sugiyama Y. Involvement of transporters in the hepatic uptake and biliary excretion of valsartan, a selective antagonist of the angiotensin II AT1-receptor, in humans. Drug Metab Dispos 2006; 34: 1247-1254.
[30] Nakagomi-Hagiha R, Nakai D, Kawai K, Yoshigae Y, Tokui T, Abe T, Ikeda T. OATP1B1, OATP1B3, and mrp2 are involved in hepatobiliary transport of olmesartan, a novel angiotensin II blocker. Drug Metab Dispos 2006; 34: 862-869.