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Original Article

Shenmai-Yin decreased the clearance of nifedipine in rats: The involvement of time-dependent inhibition of nifedipine oxidation

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ABSTRACT

The traditional Chinese herbal formula Shenmai-Yin (SY) and nifedipine have both been used to treat patients with cardiovascular disorders. Nifedipine is primarily oxidized by cytochrome P450 (CYP) 3A. The oxidation and pharmacokinetics of nifedipine were studied in rats *in vitro* and *in vivo* to illustrate the interaction of SY with nifedipine. Schisandrol A, schisandrin A and schisandrin B were identified as the main lignans in SY. In the study *in vitro*, the ethanolic extract of SY was used due to the solubility and the extract inhibited nifedipine oxidation (NFO) activity in a time-dependent manner. Among lignans, schisandrin B caused the most potent inhibition. According to the time-dependent inhibition behavior, rats were treated with SY 1 h before nifedipine administration. After oral treatment with 1.9 g/kg SY, nifedipine clearance decreased by 34% and half-life increased by 142%. SY treatment decreased hepatic NFO activity by 49%. Compared to the change caused by ketoconazole, the SY-mediated reduction of nifedipine clearance was moderate. These findings demonstrate that SY causes a time-dependent inhibition of NFO and schisandrin B contributes to the inhibition. The decreased nifedipine clearance by SY in rats warrants further human study to examine the clinical impact of this decrease.

Abbreviations: AUC, area under the plasma concentration versus time curve; CYP, cytochrome P450; h, hour; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NFO, nifedipine oxidation; PEG, polyethylene glycol; SI, Shenmai injection; SY, Shenmai-Yin; UGT, UDP-glucuronosyltransferase; UPLC, ultra-performance liquid chromatography.

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1. Introduction

Herb-drug interactions that occur via the modulation of cytochrome P450 (CYP)-catalyzed drug oxidation are of increasing concern during the use of herbal remedies [1,2]. The calcium channel blocker, nifedipine, is currently used for the treatment of various cardiovascular disorders, including hypertension and angina. Exposure to a high level of nifedipine may cause cardiovascular side effects, such as headache and the elongation of the QT interval during the electrical cycle of the heart. *Ginkgo biloba* leaf extract increased the mean maximal plasma concentration (C_{max}) of nifedipine by 27% and the duration and severity of nifedipine-induced headache in participants [3]. Nifedipine is rapidly and almost completely absorbed from the gastrointestinal tract, but undergoes extensive hepatic first-pass metabolism, resulting in an absolute bioavailability of 40–50% in humans [4]. Nifedipine is primarily oxidized by human CYP3A4 and its rat homolog, CYP3A1, to generate the inactive pyridine metabolite, dehydronifedipine [5,6]. The hydroxylated metabolites of dehydronifedipine can be further metabolized by UDP-glucuronosyltransferase (UGT) to form glucuronides [7,8]. CYP3A is the main hepatic CYP subfamily in humans and experimental rodents [9]. In addition to nifedipine, CYP3A catalyzes structurally diverse substrates including erythromycin, midazolam and testosterone. Differences have been found in the responses of human CYP3A4-catalyzed oxidations to the effectors, such as α-naphthoflavone [10]. In recombinant CYP3A4 system, α-naphthoflavone stimulated 17β-estradiol 2-hydroxylation, but inhibited testosterone 6β-hydroxylation.

The oral decoction, Shenmai-Yin (SY), is a prescription commonly used for the treatment of coronary atherosclerosis in traditional Chinese medical care in Asia [11,12]. SY is prepared from three herbal medicines, Radix ginseng (*Panax ginseng*), Radix ophiopogonis (*Ophiopogon japonicus*), and the ripe fruits of *Schisandra chinensis*. The injectable Shenmai ("Shenmai injection", SI), which is developed from SY or Shendong Yin, is prepared from extracts of Radix ginseng and Radix ophiopogonis [12–14]. SI reduced midazolam 4-hydroxylation activity, but stimulated midazolam 1′-hydroxylation activity in assay systems employing recombinant human CYP3A4 and rat and human liver microsomes [12]. SI inhibited midazolam 4-hydroxylation activity with a mixed type of competitive pattern [14]. Intraperitoneal administration of SI to rats decreased the clearance of midazolam, while the area under the plasma concentration versus time curve (AUC) for 4-hydroxymidazolam was decreased and the AUC for 1′-hydroxymidazolam was increased [12]. These reports suggested the bifunctional influence of SI on midazolam hydroxylations [15]. It was unclear whether SI affected the metabolism of other important CYP3A drug substrates, such as nifedipine. The oral effects of SY on the metabolism of CYP3A substrates have not been explored previously.

Time-dependent CYP inhibitors are oxidized by a CYP to generate an active metabolite, which irreversibly binds to the apoprotein or heme of CYP, leading to functional inactivation [16]. The inhibition of CYP activity shows time-dependence on the NADPH-fortified oxidation of the inhibitor. Unlike the competitive inhibition of midazolam 4-hydroxylation by SI [14], our findings first demonstrate that SY extract inhibits NFO activity in a time-dependent manner in this report. Guo et al. [17] reported that the extract of *Angelica dahurica* inhibited human liver microsomal testosterone 6β-hydroxylation activity (CYP3A activity) in a time-dependent manner. In rats, a 1-h (h) pre-treatment with *A. dahurica* extract increased the C_{max} of a CYP3A substrate diazepam whereas the change in clearance remained unclear [18]. Thus, to examine the herb-drug interaction, rats were treated with SY for 1 h prior to nifedipine administration in the pharmacokinetic study. Ketoconazole is a CYP3A substrate and a competitive inhibitor of human CYP3A4 [19] and UGT [20], and was therefore used as a control for comparison.

2. Materials and methods

2.1. SY, chemicals and solvents

The pharmaceutical product SY (powdered remedy) was purchased as powdered remedy from Sun Ten pharmaceutical company (Sun Ten Pharmaceutical Co., Ltd., New Taipei City, Taiwan) that use Good Manufacturing Practices. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate ($NADP^+$) sodium salt and nifedipine were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Schisandrol A, schisandrin A, and schisandrin B were purchased from SunHank Technology Co., Ltd. (Tainan, Taiwan) and their purities were >98%. Dehydronifedipine was synthesized and generously provided by Dr. F. Peter Guengerich (Nashville, TN, USA) [5]. Dimethyl sulfoxide (DMSO), methanol and dichloromethane were purchased from Merck KGaA (Darmstadt, Germany). Ethanol (95%) used for the preparation of SY extract was purchased from Taiwan Sugar Corporation (Tainan City, Taiwan).

2.2. Preparation and chromatographic analysis of SY ethanolic extract

Due to the presence of excipient, the pharmaceutical product of SY had a low solubility in DMSO and water. Ethanolic extract of SY was prepared for the *in vitro* study. SY powdered remedy (45 g) were extracted with 300 ml of distilled ethanol for 48 h on an orbital shaker (75 rpm, room temperature), filtered and concentrated under reduced pressure at 40 °C using a rotary evaporator (BÜCHI Labortechnik GmbH, Essen, Germany). The ethanolic extract was lyophilized (extraction yield: 19% (w/w)) and stored at −20 °C before being used in the inhibition study *in vitro*. To establish the method of chromatographic analysis, the SY powdered remedy (1 g) was extracted with 20 ml of 50% methanol (sonication for 30 min) and the filtrate was analyzed using an ultra-performance liquid chromatography (UPLC) system (Acquity, Waters Co., MA, USA) equipped with a photodiode array detector and a Thermo Syncronis C18 column (100 × 2.1 mm, 1.7 μm). Separation was performed at a column temperature of 35 °C and a stepwise gradient of acetonitrile (A) and water (B) was conducted as follows: 0–3 min: 2–40% A; 3–6 min: 40–55% A; 55% A for 4 min; 10–12 min: 55–70% A; 12–14 min: 70–80% A;

14–16 min: 80–100% A. Absorbance at 254 nm was detected. Lignans were identified based on their retention time and spectra, which were compared with the standards. The SY ethanolic extract was dissolved in methanol and subjected to UPLC analysis. Contents of lignans in the SY methanolic and ethanolic extracts were determined.

2.3. Animal treatment, microsomal preparation and activity determination

Male Sprague–Dawley rats (6 weeks old, weight 225–250 g) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the National Research Institute of Chinese Medicine. Before experimentation, rats were allowed at least a 1-week acclimation period in the institutional animal quarters [21]. In Taiwan, the SY powdered remedy is taken with water (in suspension) in patients. SY remedy (ground and suspended in water) was administered to rats using a gastrogavage and livers were removed after 1-h SY treatment. Control group was treated with the same dose of water. Livers were immersed in 1.15% potassium chloride. After several changes of the potassium chloride solution for reducing the blood content, liver wet weight was measured and microsomes were prepared as described previously [21]. Microsomal protein concentration, CYP content, and activities of reduced $NADP^+$ (NADPH)-CYP reductase, NFO and UGT were determined [21]. To examine the influence of NADPH-dependent microsomal oxidation on the SY extract (dissolved in DMSO)-mediated inhibition, microsomes were pre-incubated with the extract at 37 °C for the indicated time-period in the presence of a NADPH-generating system [22]. The final DMSO concentration was 0.5%. The reaction was then initiated by the addition of nifedipine and an additional NADPH-generating system was added to maintain the NADPH level during the NFO assay.

2.4. Pharmacokinetic study

In the kinetic study, experimental protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taipei. Before experimentation, rats were allowed at least a 1-week acclimation period in the institutional animal quarters. The human-equivalent dose of SY in rats was estimated to be 1.2–2.0 g/kg/day, based on a 50–60-kg person (daily dose: 12–16 g) and a body surface area ratio of 6.2 (Table 3 in the Guidance for Industry, Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, 2005). All the treatment groups *in vivo* received nifedipine at the same dose of 3 mg/kg with a vehicle of 50% polyethylene glycol (PEG) 400 (v/v). In the SY treated groups, rats were pre-dosed with 0.95 or 1.9 g/kg SY using a gastrogavage 1 h prior to nifedipine administration. Due to the CYP3A inhibition, ketoconazole was acted as a positive control [19] and orally co-administered with nifedipine to the rats at the dose of 30 mg/kg. The rats were anesthetized by isoflurane and cannulated with a tail vein catheter for blood sampling. Blood samples (0.25 ml in heparinized tubes) were collected at time 0 (pre-dose; blank), 5, 15, 30, 60, 90, 120, 180, 240, and 360 min after nifedipine administration and 0.25 ml of 0.9% saline was infused back into the rats to maintain their body fluid volume. Plasma samples were prepared, protected from light, and stored at −80 °C until analysis. Livers were removed after blood sampling and microsomal activity was determined as described above.

2.5. Plasma sample preparation

A simple liquid-liquid extraction was applied for nifedipine and dehydronifedipine extraction from plasma samples. A 100-μl aliquot of rat plasma was spiked with 50 μl of 3 μg/ml diazepam (internal standard) and then completely mixed with 2 ml of n-hexane:dichloromethane (7:3). After centrifuging at 3000× *g* for 10 min, the upper organic phase was collected and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in 150 μl of 50% acetonitrile and 3 μl of the sample solution was subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

2.6. Determination of plasma nifedipine and dehydronifedipine concentrations

Plasma concentrations of nifedipine and dehydronifedipine were determined using a LC-MS/MS system (Agilent Technologies 1200 series high performance liquid chromatography (Boeblingen, Germany) equipped with a Biosystems-Sciex API 3000 series triple-quadrupole mass spectrometer (Foster City, CA, USA) and an electronspray ionization (ESI) interface). Chromatographic separation was carried out on a C18 column (Waters Symmetry C18, 4.6 × 100 mm, 3.5 μm) using a mobile phase composed of 20% A (2 mM ammonium formate and 0.1% formic acid in water) and 80% B (2 mM ammonium formate and 0.1% formic acid in acetonitrile) at a flow rate of 0.35 ml/min. The column and autosampler were conditioned at 40 °C and 10 °C, respectively. Acquisition for mass spectrometry was performed in positive electrospray ionization mode and the transitions for precursors to the fragmentation ion product are listed in Table 1. The ion-source temperature, the ion spray voltage, and the dwell time were set at 450 °C, 5.5 kV, and 200 ms per channel, respectively. Data were processed using Analyst 1.4.2 software (Applied Biosystems-Sciex, Foster City, CA, USA). Control plasma spiked with nifedipine and dehydronifedipine at the concentrations from 0.005 to 5 μg/ml were analyzed to establish the standard curve for quantification. Assay validation including specificity, selectivity, linearity, stability, recovery and matrix effects of both analytes were conducted according to the Food and Drug Administration (FDA) Guidance for Bioanalytical Method Validation (2001). The lower limit of quantification (LLOQ) was 3.0 ng/ml in plasma.

2.7. Data and statistical analyses

The concentrations of inhibitors required for a 50% inhibition of activity (IC_{50}) were calculated by curve fitting (Grafit, Erithacus Software Ltd., Staines, UK). The IC_{50} values for the

Table 1 – Multiple reaction monitoring transitions and fragmentation parameters for nifedipine, dehydronifedipine and diazepam.

Analyte	Precursor (*m*/z)	Product (*m*/z)	DP (V)	FP (V)	CEP (V)	CE (eV)	CXP (V)	RT (min)
Nifedipine	347.0	315.2	36	150	10	36	10	3.40
Dehydronifedipine	345.2	284.3	50	200	10	39	9	3.45
Diazepam	285.1	154.0	55	250	5	36	10	4.27

Diazepam was used as the internal standard for the chromatographic analysis. Chromatographic separation was carried out as described in the methods. DP: declustering potential; FP: focusing potential; CEP: cell entrance potential; CE: collision energy; CXP: cell exit potential; RT: retention time.

Table 2 – Precision and accuracy of intra-day validation of quantification of nifedipine and dehydronifedipine.

Expected concentration (μg/ml)	Nifedipine			Dehydronifedipine		
	Observed (μg/ml)	RSD (%)	Accuracy (%)	Observed (μg/ml)	RSD (%)	Accuracy (%)
0.005	0.0053 ± 0.0001	1.9	106.0	0.0053 ± 0.0001	1.9	106.0
0.010	0.0094 ± 0.0004	4.2	94.0	0.0092 ± 0.0002	2.2	92.0
0.025	0.0238 ± 0.0009	3.8	95.2	0.0237 ± 0.0018	7.6	94.8
0.05	0.045 ± 0.001	2.2	90.0	0.046 ± 0.003	6.5	92.0
0.10	0.097 ± 0.002	2.1	97.0	0.095 ± 0.004	4.2	95.0
0.50	0.511 ± 0.013	2.5	102.2	0.496 ± 0.014	2.8	99.2
1.00	1.004 ± 0.094	9.4	100.4	1.053 ± 0.093	8.8	105.3
1.50	1.556 ± 0.025	1.6	103.7	1.556 ± 0.017	1.1	103.7
3.00	3.136 ± 0.097	3.1	104.5	3.116 ± 0.104	3.3	103.9
5.00	5.385 ± 0.184	3.4	107.7	5.408 ± 0.237	4.4	108.2

Rat plasma was spiked with 0.005–5.00 μg/ml nifedipine and dehydronifedipine. The peak area normalized by the area of internal standard had good linear relationship with the concentrations of nifedipine and dehydronifedipine with the coefficients (r) of 0.9998 and 0.9999, respectively. Data represent the mean ± SD of 5 determinations.

Table 3 – Precision and accuracy of inter-day validation of quantification of nifedipine and dehydronifedipine.

Expected concentration (μg/ml)	Nifedipine			Dehydronifedipine		
	Observed (μg/ml)	RSD (%)	Accuracy (%)	Observed (μg/ml)	RSD (%)	Accuracy (%)
0.005	0.0052 ± 0.0001	1.9	104.0	0.0051 ± 0.0001	1.0	102.0
0.010	0.0097 ± 0.0001	1.0	97.0	0.0097 ± 0.0004	4.1	97.0
0.025	0.0236 ± 0.0011	4.7	94.4	0.0248 ± 0.0017	6.9	99.2
0.05	0.048 ± 0.003	6.3	96.0	0.047 ± 0.002	4.3	94.0
0.10	0.098 ± 0.002	2.0	98.0	0.096 ± 0.002	2.1	96.0
0.50	0.506 ± 0.026	5.1	101.2	0.499 ± 0.012	2.4	99.8
1.00	1.027 ± 0.028	2.7	102.7	0.996 ± 0.027	2.7	99.6
1.50	1.561 ± 0.034	2.2	104.1	1.560 ± 0.025	1.6	104.0
3.00	3.093 ± 0.151	4.9	103.1	3.140 ± 0.066	2.1	104.7
5.00	5.029 ± 0.199	4.0	100.6	5.153 ± 0.092	1.8	103.1

Data represent the mean ± SD of 5 determinations.

extract-mediated inhibition was divided by the extraction yield to generate the value expressed as mg SY remedy/ml. The difference in microsomal CYP content after NADPH-fortified pre-incubation with the ethanolic extract of SY remedy was analyzed using a paired *t*-test. A non-compartmental model (WinNonlin version 5.3, Pharsight Corporation, Mountain View, CA, USA) was used for pharmacokinetic analysis. The C_{max}, time taken to reach the C_{max} (T_{max}), AUC, half-life ($t_{1/2}$), apparent volume of distribution (V_d/F), and clearance (CL/F) were estimated. Differences between >2 sets of data were analyzed by one-way analysis of variance followed by a post-hoc Dunnett's test (for the comparison with the control) using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). The differences in metabolic ratios between the control and ketoconazole-treated rats were analyzed using a Student's *t*-test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. *Chromatographic analyses of SY extracts*

The method for chromatographic analysis of SY has been established using the filtrate obtained from the methanol extraction of SY remedy. The chromatograms identified lignans, schisandrol A, schisandrin A and schisandrin B, in this methanolic extract (Fig. 1A), with spectral characteristics

(Fig. 1B, left panel) comparable to those of the purified standards (Fig. 1B, right panel). The ethanolic extract showed a similar chromatographic profile (Fig. 1C). The linear ranges of schisandrol A, schisandrin A and schisandrin B were 1.0–200 ($r^2 = 0.9999$), 1.0–200 ($r^2 = 0.9999$) and 0.5–200 ($r^2 = 0.9996$) μg/ml, respectively. By using this chromatographic method, quantitative determination of the lignans in SY remedy showed that the lignan contents determined using the ethanolic extraction (0.561 ± 0.006 mg/g schisandrol A, 0.101 ± 0.001 mg/g schisandrin A, and 0.200 ± 0.003 mg/g schisandrin B) were higher than those using methanol for extraction (0.124 ± 0.005 mg/g schisandrol A, 0.0213 ± 0.0002 mg/g schisandrin A, and 0.0254 ± 0.0016 mg/g schisandrin B). In addition, SY remedy was insoluble in either water or DMSO. Thus, the SY remedy and its ethanolic extract were used in the following *in vivo* and *in vitro* studies, respectively.

3.2. *Effects of ketoconazole, SY ethanolic extract and lignans on rat liver microsomal NFO activity*

Ketoconazole inhibited NFO activity with an IC_{50} value of 1.73 ± 0.21 μg/ml (Fig. 2A). After NADPH-fortified pre-incubation, the IC_{50} value of ketoconazole increased to 3.50 ± 0.65 μg/ml, potentially due to the degradation of ketoconazole (e.g. microsomal oxidation of ketoconazole). After the pre-incubation of microsomes with SY extract, the IC_{50} value for SY extract-mediated inhibition of NFO was reduced from >6.0 to 3.97 ± 0.46 mg powdered remedy/ml (Fig. 2B). To examine the dependence on pre-incubation time, microsomes were pre-incubated with SY extract for 5, 10, or 15 min prior to determining the NFO activity. The vehicle control retained 80%–96% activity after 5–15 min pre-incubations (Fig. 2C). Without pre-incubation, SY extract (3 mg powdered remedy/ml) caused a 27% decrease in NFO activity. After 5-, 10-, and 15-min pre-incubations, SY extract decreased NFO activity by 33 ± 10%, 41 ± 6%, and 49 ± 2% (mean ± SD), respectively. The plot of natural logarithm (ln) values of the remaining activities versus the pre-incubation time clearly showed that the absolute value of the slope was increased in the presence of SY extract. Pre-incubation with SY extract decreased the spectrally detected CYP content by 19 ± 5% (Fig. 2D). These results revealed that SY-mediated inhibition was enhanced by pre-metabolism, indicating the presence of one or more time-dependent CYP3A inhibitors.

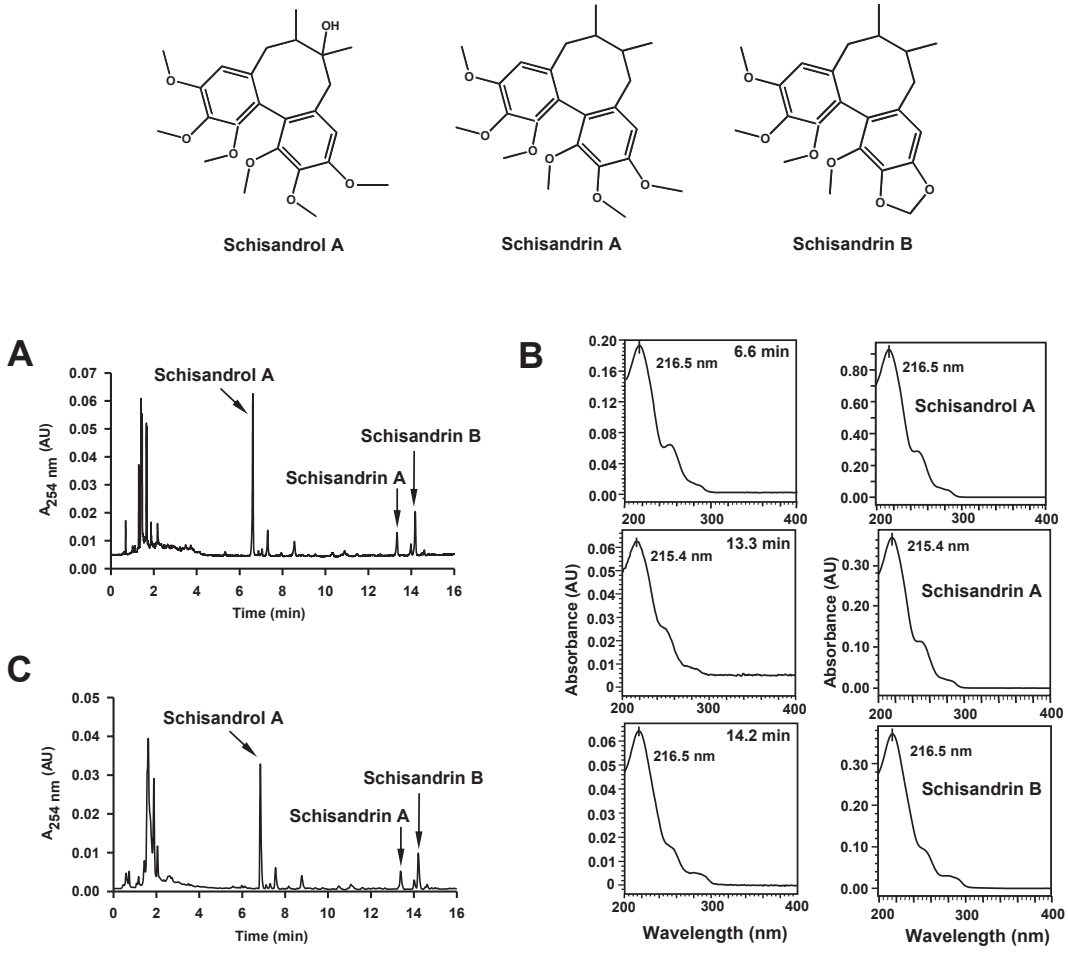

Fig. 1 – The chromatograms of UPLC analysis of SY methanolic (A) and ethanolic (C) extracts and the spectra of selected peaks identified in the chromatographic analysis of SY methanolic extract (B, left panel) and lignan standards (B, right panel). One microliter of the filtrate of methanolic extract, ethanolic extract (10 μg/ml) and the ingredient standards schisandrol A (200 μg/ml), schisandrin A (200 μg/ml), and schisandrin B (200 μg/ml), were injected into the UPLC and analyzed as described in the methods.

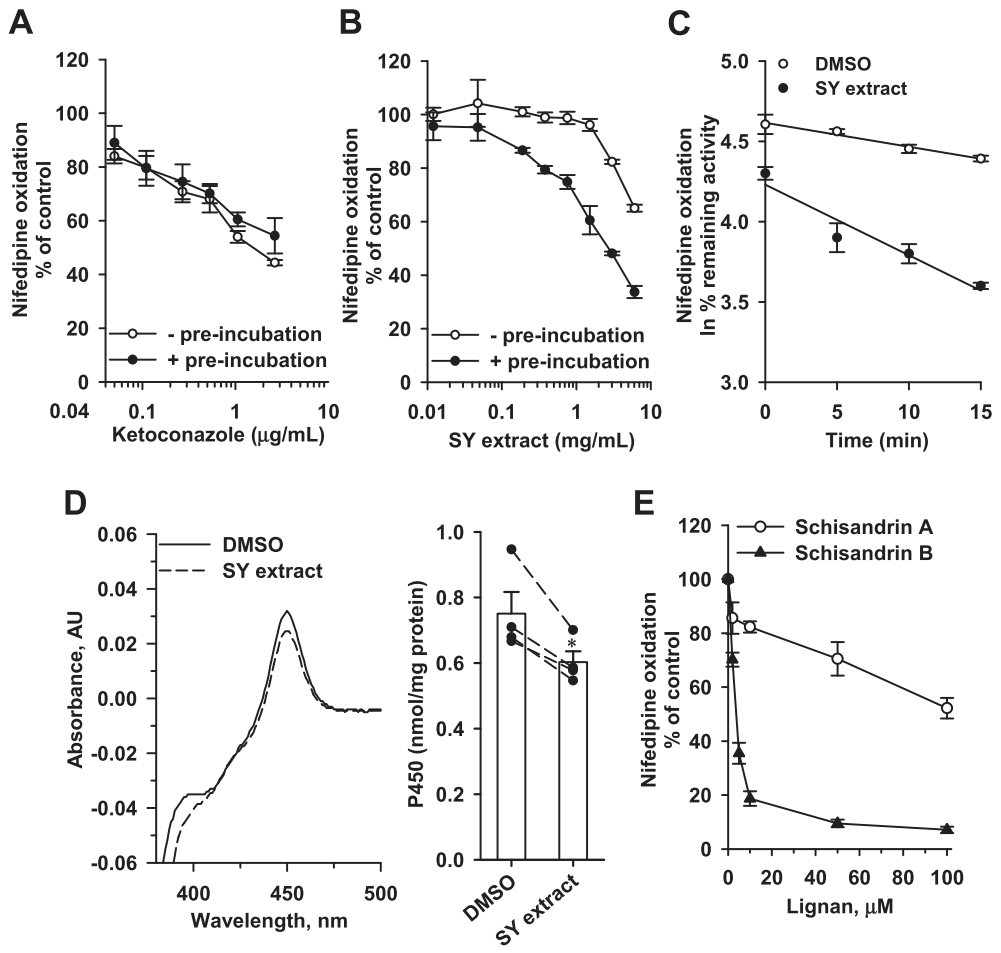

Fig. 2 – Effects of NADPH-fortified pre-incubation with ketoconazole (A), SY extract (B–D) and lignans (E) on rat liver microsomal NFO activity or CYP content *in vitro*. In panels (A) and (B), the activities were determined without (○) and with (●) a 10-min NADPH-fortified pre-incubation of microsomes with ketoconazole or SY extract. In panel (C), NFO activity was determined after the pre-incubation of microsomes with the vehicle (DMSO) or the SY extract (3 mg powdered remedy/ml) for the indicated time-periods. Data represent the mean ± standard error of the mean (SEM) of three rats. Panel (D) shows the reduction of spectrally determined CYP content in microsomes pre-incubated with or without SY ethanolic extract at a concentration corresponding to 5 mg powdered remedy/ml for 15 min. The right and left panels show the changes of representative CO-difference spectra and CYP contents (dot and dashed line) in microsomes of individual rats, respectively. The bars show the mean ± SEM of 4 rats, $^{*}p < 0.05$. In panel (E), microsomal NFO activity was determined after microsomes were pre-incubated with increasing concentrations of schisandrin A or schisandrin B in the presence of NADPH for 10 min. The results show the mean ± SEM of 3 rats.

After NADPH-fortified pre-incubation of microsomes with SY lignans for 10 min, schisandrin B appeared to cause the most potent inhibition of NFO activity with an IC_{50} of 3.48 ± 0.43 μM (Fig. 2E). The pre-incubation with schisandrin A caused a 48% decrease of NFO activity when its concentration was increased to 100 μM. Microsomal NFO activity was not decreased by schisandrol A at a concentration as high as 100 μM (data not shown). Among the main lignans identified in SY, these results indicated that schisandrin B made the main contribution to SY-mediated NFO inhibition.

3.3. *Method validation of LC-MS/MS analyses of nifedipine and dehydronifedipine in plasma*

The study yielded a mean recovery greater than 83.5% for all analytes. The 14-day (−80 °C) stabilities of nifedipine and dehydronifedipine in plasma samples were 95.1–102.1% and 99.6–100.8%, respectively. Thus, plasma samples were thawed only once and subjected to chromatographic analysis within 3 days. The standard calibration curves had good linearity over the concentration range (0.005–5 μg/ml) of nifedipine and dehydronifedipine with the coefficients (r) of 0.9998 and 0.9999, respectively. In addition to the relative matrix effects of all the analytes, which were less than 4.3%, the absolute matrix effects including the internal standard diazepam were quite limited since the peak area ratios of standards in plasma to those in methanol were always higher than 93.1%. The intra-day (Table 2) and inter-day (Table 3) relative standard deviation (RSD) of the plasma analyses spiked with increasing concentrations of nifedipine and dehydronifedipine were 1.0–9.4% and 1.0–8.8%, respectively. The accuracies of the determination of nifedipine and

dehydronifedipine were 90.0–107.7% and 92.0–108.2%, respectively.

3.4. *Effects of ketoconazole and SY treatments on the pharmacokinetics of nifedipine and dehydronifedipine in rats*

Ketoconazole co-treatment caused 3- to 6-fold increases in the nifedipine $t_{1/2}$ and AUC values (AUC_{0-t} and $AUC_{0-\infty}$), a 68% increase in the C_{max}, and an 84% decrease in clearance (Table 4, Fig. 3). Ketoconazole increased the C_{max}, AUC_{0-t}, $AUC_{0-\infty}$, and $t_{1/2}$ parameters of dehydronifedipine by 3-, 4-, 13-, and 6-fold, respectively. Ketoconazole significantly decreased the V_d/F and clearance of dehydronifedipine by 50% and 89%, respectively. The metabolic ratio of dehydronifedipine to nifedipine was significantly decreased (a 48% decrease) by ketoconazole at 5 min after nifedipine administration, but not at time-points >15 min after nifedipine administration (Fig. 3, inset). Acute treatment with 0.95 g/kg SY increased the C_{max} of nifedipine by 52% (Table 4; Fig. 3, upper panel). When the dose was elevated to 1.9 g/kg, SY increased the $t_{1/2}$ of nifedipine by 142% and decreased nifedipine clearance by 34%, whereas the change of dehydronifedipine pharmacokinetics was minimal (Fig. 3, lower panel). Results revealed that the clearance of nifedipine was significantly reduced in rats treated with a high dose of SY remedy.

3.5. *Effect of SY treatment on hepatic activities of CYP3A and UGT in rats*

Oral administration of 1.9 g/kg SY remedy for 1 h did not cause a significant change of the ratio of liver to body weight (Fig. 4). Consistent with the decrease in CYP content after pre-incubation of microsomes with SY extract *in vitro* (Fig. 2D), oral administration of SY remedy to rats decreased the spectrally (CO-difference spectrum) detected hepatic CYP content in rats by 20% (Fig. 4). However, results of immunoblotting analysis of microsomal CYP3A showed that acute SY treatment did not decrease the immunoreactive protein level (supporting information). The mean degradation half-life of rat CYP3A2 was estimated to be 12–27 h [23]. The inactivated CYP might keep immunoreactivity but was not spectrally detected. The SY treatment caused a 49% decrease in hepatic NFO activity without affecting the activities of NADPH-CYP reductase (microsomal electron-transfer partner of CYP) and UGT in rats. Results showed that hepatic CYP content and NFO activity were decreased in rats treated with SY remedy.

4. Discussion

The aqueous extract of Schisandra fruit and a minor lignan in *S. chinensis*, schisantherin A (gomisin C), caused a time-dependent inhibition of human liver microsomal erythromycin N-demethylation activity (mainly catalyzed by CYP3A4) [24]. The main *S. chinensis* lignans, including schisandrin A and schisandrin B inhibited rat liver microsomal oxidation activity toward midazolam in a time-dependent manner [25]. Although lignans generally had low water solubility, lignans including schisandrins were identified in plasma samples of rats treated with the pharmaceutical product or extract of *S. Chinese* [26,27] and humans taking Wuzhi tablet [28]. The oral bioavailability of schisandrol A in herbal extract (38–78%) was higher than purified schisandrol A (16%) in rats [27]. Oral administration of 8 and 16 mg/kg schisandrin B to rats decreased midazolam 1′-hydroxylation activity [29]. Schisandrins A and B increased the AUC_{0-24} and $AUC_{0-\infty}$ values of the CYP3A substrate tacrolimus in rats [30]. Results of chromatographic analysis revealed that schisandrins A and B were retained after preparation of SY decoction, from which the powdered remedy was made. Our findings provided the first evidence that schisandrin B strongly inhibited rat liver microsomal NFO activity with an IC_{50} value comparable to or less than those for inhibiting human liver microsomal oxidation activities toward testosterone and erythromycin [24] when 10-min NADPH-fortified pre-incubation was

Table 4 – Effects of ketoconazole and SY remedy on the pharmacokinetic parameters of nifedipine and dehydronifedipine in rats.

Parameters	Control	Ketoconazole (30 mg/kg)	SY (0.95 g/kg)	SY (1.9 g/kg)
Nifedipine				
C_{max} (μg/ml)	2.30 ± 0.19	3.69 ± 0.40*	3.50 ± 0.34*	2.48 ± 0.11
T_{max} (h)	0.42 ± 0.05	0.85 ± 0.54	0.25 ± 0.00	0.33 ± 0.05
AUC_{0-t} (h·μg/ml)	4.3 ± 0.3	13.5 ± 1.6*	5.2 ± 0.36	5.3 ± 0.5
$AUC_{0-\infty}$ (h·μg/ml)	4.6 ± 0.3	29.5 ± 4.2*	5.5 ± 0.34	7.1 ± 0.7
$t_{1/2}$ (h)	1.2 ± 0.2	7.1 ± 1.6*	1.7 ± 0.2	2.9 ± 0.5*
V_d/F (l/kg)	1.19 ± 0.21	1.02 ± 0.13	1.32 ± 0.19	1.73 ± 0.26
CL/F (l/h/kg)	0.68 ± 0.05	0.11 ± 0.02*	0.56 ± 0.04	0.45 ± 0.06*
Dehydronifedipine				
C_{max} (μg/ml)	0.04 ± 0.01	0.10 ± 0.02*	0.05 ± 0.01	0.04 ± 0.00
T_{max} (h)	0.70 ± 0.12	1.80 ± 0.55*	0.29 ± 0.05	0.58 ± 0.08
AUC_{0-t} (h·μg/ml)	0.12 ± 0.02	0.46 ± 0.11*	0.11 ± 0.01	0.13 ± 0.02
$AUC_{0-\infty}$ (h·μg/ml)	0.14 ± 0.02	1.77 ± 0.53*	0.13 ± 0.02	0.24 ± 0.04
$t_{1/2}$ (h)	2.0 ± 0.1	12.3 ± 3.9*	1.9 ± 0.5	4.8 ± 0.8
V_d/F (l/kg)	68.9 ± 9.0	34.3 ± 7.4*	61.0 ± 13.3	89.7 ± 14.4
CL/F (l/h/kg)	23.7 ± 2.9	2.6 ± 1.0*	24.3 ± 3.2	15.2 ± 3.3

Rats were orally treated with 3 mg/kg nifedipine together with ketoconazole or 1 h after SY treatment. Results represent the mean ± SEM of 5 rats in the ketoconazole-treated group and 6 rats in control and SY-treated groups. CL/F: apparent clearance. *, $p < 0.05$.

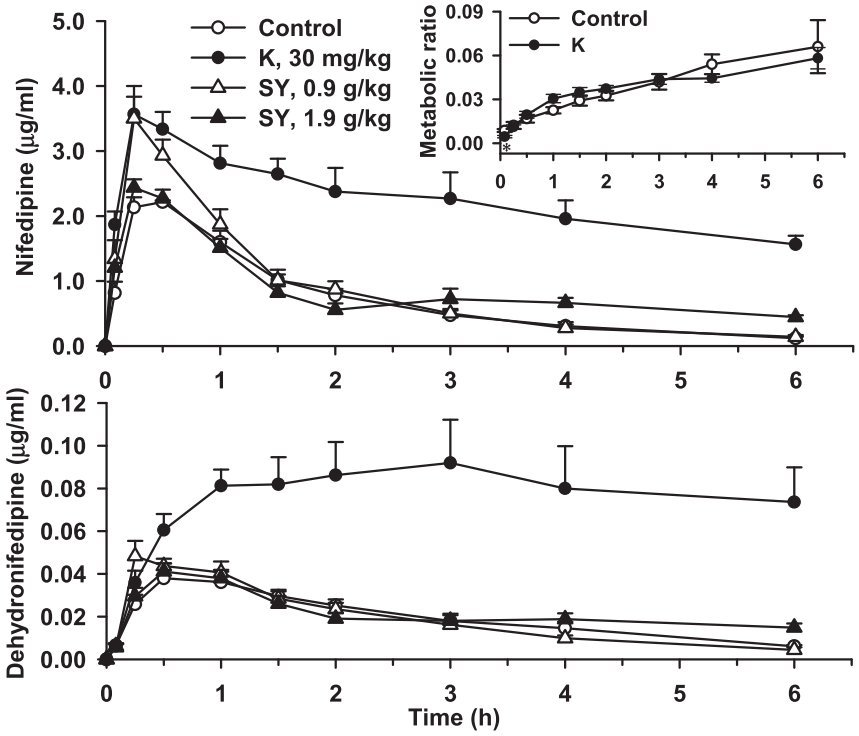

Fig. 3 – Effects of ketoconazole and SY remedy on the pharmacokinetics of nifedipine and dehydronifedipine in rats. Nifedipine (3 mg/kg) was administrated to rats together with ketoconazole (K, 30 mg/kg) or 1 h after receiving SY remedy. Plasma nifedipine (upper panel) and dehydronifedipine (lower panel) concentrations were determined. The metabolic ratio of plasma dehydronifedipine to nifedipine in control and ketoconazole-treated group are shown in the inset ($^{*}p < 0.05$). The results show the mean ± SEM of 5 rats in the ketoconazole-treated group and 6 rats in the control and SY-treated groups.

performed. However, rat NFO activity was not inhibited by 100 μM schisandrol A and the inhibition by schisandrin A was moderate. According to the content in SY and the potent NFO inhibition, schisandrin B plays a primary role in the SY-mediated NFO inhibition. In addition to schisandrin B, other ingredients, including schisantherin A [24], flavonoids and ophiopogonone A (in Radix ophiopogonis) [12], may also be involved in NFO inhibition by SY. The interactions between constituents of the component herbs did not eliminate the inhibitory effect of SY on NFO.

Nifedipine is a poorly water-soluble drug with a water solubility of 5.9 μg/ml at 25 °C [31]. Thus, PEG was commonly used in the preparation of nifedipine solution for rat treatment in pharmacokinetic studies. Results of our determination showed that control rats had a nifedipine half-life of 1.2 h, which was similar to previous reports that the half-life of nifedipine after oral dosing was ranged from 0.5 to 1.2 h when nifedipine was prepared using PEG [32–34]. The T_{max} was 0.25–0.47 h. However, compared to the results in rats treated with nifedipine in PEG-containing solution, the terminal half-life (9.7 h) and T_{max} (0.71 h) of nifedipine were longer when rats were treated with nifedipine suspended in water (10 mg/ml) at an oral dose of 10 mg/kg [35]. Even that the rat dose was 3.3-fold higher than the dose we used, the C_{max} and AUC_{inf} of nifedipine were 49% and 129% of our determinations, respectively. To our best knowledge, there were four reports discussing the pharmacokinetics of dehydronifedipine. In three of these reports, the AUC ratio of the dehydronifedipine to nifedipine was 38% in rats treated with nifedipine suspension (in water) [35–37] However, in the other one report published by Han et al. [6], the AUC_{0-t} ratio of dehydronifedipine to nifedipine was 2.7 ± 0.3%, which was identical to our result of 2.7 ± 0.5% in rats. When the nifedipine suspension was administered to rats, the half-life of dehydronifedipine was surprisingly long (15.7 h) [35]. This long half-life may come from that the plasma concentration slowly declined in the terminal elimination phase due to the slow release and the prolonged absorption of nifedipine. Consequently, the elimination half-life of dehydronifedipine they predicted was longer than our observation (2.0 h) and the AUC_{inf} of dehydronifedipine might be overestimated [35,37], leading to the high AUC ratio of dehydronifedipine to nifedipine. In 1.9 g/kg SY-treated group, hepatic NFO activity decreased, leading to the decreased clearance and prolonged half-life of nifedipine. The pronounced change of plasma nifedipine concentration primarily occurred in the elimination phase. Although the $AUC_{0-\infty}$ could be overestimated due to that plasma nifedipine level decreased slowly and remained high at 6 h after nifedipine administration, the mean AUC_{0-t} was increased by 23% without a statistical significance. The mean AUC_{0-t} ratio of nifedipine to nifedipine was decreased by 11–25% after 0.95 and 1.9 g/kg SY treatments. Plasma nifedipine levels in this high dose group were significantly higher than the respective levels in control rats at 4 and 6 h (elimination phase) after nifedipine administration ($p < 0.05$).

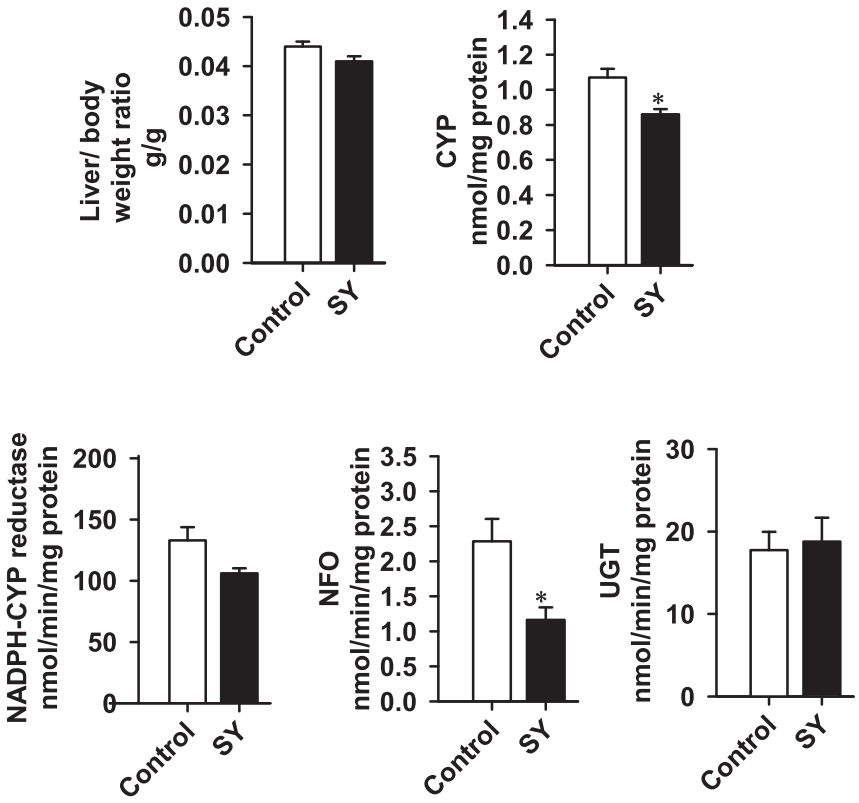

Fig. 4 – Effects of SY treatment on liver to body weight ratio, hepatic content of CYP and activities of NADPH-CYP reductase, NFO, and UGT in rats. The SY remedy (1.9 g/kg) was administrated to rats for 1 h and then microsomes were prepared for the determinations as described in the methods. The results show the mean ± SEM of 5 rats. $^{*}p < 0.05$.

The inhibition of NFO activity by SY and its lignans can contribute, at least in part, to the observed decrease in clearance. Ishihara et al. [18] reported that the observed decrease in hepatic NFO activity in 3- and 6-h *A. dahurica* extract-treated rats was recovered after 12 h. Our findings revealed that the decreased hepatic NFO activity in 1-h SY-treated rats was restored after the last blood collection (6 h) in the pharmacokinetic study of SY (1.9 g/kg)-treated rats (data not shown). Compared to the large ketoconazole-induced decrease in nifedipine clearance, the reduction caused by SY was moderate. When the SY dose was reduced to 0.95 g/kg, the nifedipine clearance was not different from that in the control group. The underlying cause of the greater increase of C_{max} in the 0.95 g/kg SY-treated group than in the 1.9 g/kg SY-treated group remains unclear. To confirm this unexpected result, we have repeated the pharmacokinetic study and obtained the same results. One of the possible causes is that the higher dose of particulate SY remedy (in suspension) may cause greater physical interference with the absorption rate of SY (or nifedipine) in the gastrointestinal tract, leading to the reduction of the quick increase in C_{max} of nifedipine in 1.9 g/kg SY-treated group. After the absorption process, SY-treatment decreased nifedipine clearance with dose-related manner. There was little information about the influence of the density or volume of granules in drug suspension on the drug-drug interaction. Differences in the time-dependent changes of plasma schisandrol A concentration have been reported in rats treated with low and high doses of *S. Chinese* remedy [27]. At each sampling time within 5–45 min after the remedy administration, the mean level of plasma schisandrol A in 3 g/kg *S. Chinese* remedy-treated rats was higher than the respective level in 10 g/kg remedy-treated group, suggesting the faster absorption after lower dose treatment and dose-independence in the absorption phase [27]. However, the mean C_{max} level of schisandrol A (T_{max}: ~ 3 h) in 10 g/kg remedy-treated rats was higher than those with 3 g/kg treatment. The T_{max} of schisandrin B was about 4 h in *S. Chinese* remedy-treated rats [26]. Although the pharmacokinetics of lignans, such as schisandrin B, remained unclear in rats treated with different doses of SY, the differential pharmacokinetic behaviors of a lignan in rats treated with low and high doses of herbal extract could be one of the factors for the significant increase in the C_{max} of nifedipine (T_{max} of nifedipine: 0.42 h) by 0.95 g/kg SY treatment and the primary change in the elimination phase (3–6 h) of nifedipine pharmacokinetics in rats treated with a high dose of 1.9 g/kg SY. Treatment of rats with SY decreased the clearance and prolonged the half-life of nifedipine in a dose-dependent manner. On the other hand, the antifungal agent posaconazole has been used in suspension and is orally administered to patients. The mean value of C_{max} of posaconazole was 2-fold higher in participants receiving oral suspension (40 mg/ml) at 200 mg 4 times daily than those at 400 mg twice daily for 7 days [38]. Although the daily dose was same, results of this report revealed that treatment with a greater volume and less frequency of posaconazole suspension generated a lower C_{max}.

The dosing process of suspension might be one of the influencing factors in the pharmacokinetic interaction of drugs and the suspension of herbal granules.

Ketoconazole co-treatment did not cause a great decrease of the metabolic ratio of dehydronifedipine to nifedipine due to that multiple enzymes (including UGT) participate in the metabolism of dehydronifedipine and the potent UGT inhibition by ketoconazole [20]. The inhibition of UGT by ketoconazole can be one of the causes for the significant increase in the AUC and $t_{1/2}$ and decrease in clearance of plasma dehydronifedipine in ketoconazole-treated rats. For a potent CYP3A and UGT inhibitor, such as ketoconazole, the plasma concentration of nifedipine can be a better marker than the metabolic ratio of dehydronifedipine/nifedipine for assessing CYP3A-mediated drug interaction. Of the compounds present in the SY component herbs, lignans of *S. Chinese* selectively inhibited the activities of human UGT isoforms [39]. The Radix ophiopogonis constituents, ophiopogonin D and D′, have also been reported to inhibit the activities of recombinant human UGTs [40]. However, acute treatment of rats with SY remedy did not change hepatic UGT activity. SY extract exhibited weak inhibition of rat liver microsomal UGT activity when *p*-nitrophenol was used as a substrate. The decrease in UGT activity was 24 ± 3% when the extract concentration was increased to a level corresponding to 6 mg SY remedy/ml in our assay. As a result, compared to the changes caused by ketoconazole, the influence of SY on the pharmacokinetics of dehydronifedipine was minimal in rats.

In conclusion, the present study is the first to demonstrate that ketoconazole decreases the clearances of both nifedipine and dehydronifedipine in rats. The time-dependent inhibition of NFO activity by SY *in vitro* might be one of the major reasons for the decreased clearance and prolonged half-life of nifedipine in rats treated with SY 1 h prior to nifedipine administration. Schisandrin B contributes at least in part to the NFO inhibition by SY. Adverse effect might happen when SY was taken 1 h before nifedipine administration. In another rat study, repeated SY treatment also decreased nifedipine clearance (unpublished results). Further human study is warranted to assess the clinical relevance of the pharmacokinetic interaction of SY with nifedipine.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jfda.2018.10.005.

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