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Evaluating the effect of Ganoderma lucidum polysaccharides on five cytochrome P450 isozymes with cocktail probe drugs in rats by LC–MS/MS

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Abstract

Ganoderma lucidum polysaccharides (GLPs) are commonly used as health-promoting medicine and dietary supplement due to the positive effects in immune modulation, antitumor and antioxidant activities. However, whether GLPs executes other uncharacterized effects is largely unclear. The rats were pre-primed with GLPs and then administrated with canonical "cocktail probes" of cytochrome P450 (CYP450) isozymes including caffeine, tolbutamide, dextromethorphan, omeprazole, and midazolam. The plasma concentrations of probes at each indicated time point were simultaneously detected using the designed high-performance liquid chromatography-tandem mass spectrometric (LC-MS/MS) method. The results suggested that GLPs could increase the accumulated levels of caffeine, tolbutamide and midazolam in plasma as compared to control group. Besides, GLPs reduced the concentration of dextromethorphan in blood at high dose, while elevated it at low dose. GLPs could inhibit the activities of CYP1A2, and CYP3A4, additionally; GLPs at low dose suppressed the activity of CYP2D6, which demonstrated that drugs co-administrated with GLPs might require strictly evaluating the dose relation.

Keywords: LC-MS/MS, GLPs, cocktail probe drug, drug-drug interaction, CYP450

Introduction

Ganoderma lucidum (GL), as a famous herbal medicine in China and Japan more than 2000 years, has been prized for its medicinal vitality such as promoting longevity and benefiting for general health [1, 2]. Especially, GL polysaccharides (GLPs) are a major bioactive ingredient and has been identified that it has regulatory abilities in most of physiological progress, including antitumor effects, immune modulation, significant antioxidant activity, and hypoglycaemic [3-8]. Recently, the GLPs has been extracted and purified to be applied in health-promoting as medications or dietary supplements [9]. At present, the combination of synthetic drugs and traditional Chinese medicines (TCMs) is widely used in clinical treatment in China [10]. However, some case reports have highlighted the existence of drug-drug interactions (DDIs) between chemical drugs and herb, animals' investigations also indicated that several herbal ingredients or compound formulas of TCMs could bring significant impact on cytochromes P450 isozymes [11-13]. Several reports exhibited that TCMs have CYP inhibition or induction effect when administered together with conventional drug by altering pharmacokinetic profile, thereby causes less bioavailability or toxicity [14]. Therefore, GLPs as one of few polysaccharide-based modern medicines, its research data are still lacked. The potentially interactive risk of GLPs should be identified to reduce side effects and toxicity.

Cytochrome P450 (CYP450), is a hemoprotein super family of monooxygenases, plays a major role in the biotransformation of vast array of endogenous and exogenous compounds [15, 16]. CYP450 enzymatic system constitutes the most important pathway for drug metabolism and elimination in human, responsible for metabolizing approximately 75% of commonly used drugs [17, 18]. The primary xenobiotic-metabolizing hepatic CYP450 isoforms are CYP1A2, CYP2C9/19, CYP2D6, CYP2E1, and CYP3A4, accounting for approximately 90% of CYP450-mediated drug metabolism [19, 20]. Inhibition of CYP450enzymes may lead to increase plasma levels of a concomitantly administered drug, prolong/reduce the pharmacological effects, and elevate incidence of drug-induced toxicity or less therapeutic effect [21, 22]. At present, multidrug combination therapy is very common for the treatment of various diseases [23]. So, drug-drug and herb-drug interaction study is very significant for effective treatment [24]. But the effect of GLPs on CYP450 activities remains unknown.

Phenotyping measures could reflect the real-time activities of CYP450 and provide clinically relevant information on the combination of factors such as genetic, DDI, environmental and endogenous factors [25]. Thus, a phenotyping measure using selective CYP450 probes is a valuable tool to determine in vivo CYP450 activities. A cocktail approach is commonly performed to characterize subject's phenotype following administration of multiple probe drugs simultaneously in vivo [26]. This method can provide information on several CYP450 activities in a single experiment [27]. Caffeine, tolbutamide,

omeprazole, dextromethorphan and midazolam are often used as substrates in "cocktail probes" of cytochrome CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively [28].

In recent years, liquid chromatography-tandem mass spectrometry (LC–MS/MS) has become a preferred approach to determine analytes in biological samples because of its high selectivity and sensitivity. The object of our study was to develop and validate a LC-MS/MS method that allowing simultaneously detection of five major CYP450 isoforms activities based on cocktail approach in plasma. Moreover, this method will be further applied to assess the interactions between five probe drugs and different doses of GLPs in rats, contributing to better understand chemical and pharmacological properties of GLPs and to minimize risk of side effects.

Experimental

Materials and reagents

Standard of caffeine (purity>98%), tolbutamide (purity>99%), dextromethorphan (purity>99%), omeprazole (purity>99%) and gliclazide (purity>95%) were purchased from Dalian Meilun biological technology co., Ltd (Dalian, China). Midazolam injection (2 mL: 10 mg) was purchased from Jiangsu Nhwa Pharmaceutical Co., Ltd. (Xuzhou, China). Ganoderma lucidum polysaccharides (>50%) were manufactured by Xi'an Weizhen biological technology co., Ltd. (Xi'an, China). Methanol and ethyl acetate were chromatographically pure grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (96%) was obtained from Tedia Company, Inc. (Ohio, USA). All other chemicals and solvents (analytical reagent grade) used were purchased from Kermel (Tianjin, China).

Animals

The pharmacokinetic study was carried out in healthy male rats. The study protocol was approved by the Animal Ethics Committee of Sichuan University (Chengdu, Sichuan, China). Male Sprague-Dawley rats (200-240 g) were purchased from the Experimental Animal Center of Sichuan Province (production license: SCXK (Chuan)-2015-030). Prior to the start of the experiments, all animals were allowed at least one week acclimation period under air conditioning (25 ± 1) °C and an automatically controlled photoperiod of 12h light daily. The rats were fed with standard laboratory food and water ad libitum, and 12h before administration; they were fasted with free access to water.

Liquid chromatographic and mass spectrometric conditions

High Performance Liquid Chromatography (HPLC) was performed on an Agilent 1100 Series (Agilent, USA), consisted of a binary gradient pump, an online degasser, a thermostatic column compartment and an auto sampler. HPLC-MS/MS conditions were optimized for the analytical column, mobile phase and sample detection. Chromatographic separation was achieved on an Inertsil ODS-2 (5 µm, 4.6 × 150 mm) column. The mobile phase consisted of 0.1% formic acid in water (A) and methanol (B). A gradient elution was used at a flow rate of 0.8 $mLmin^{-1}$ with the split ratio of 1:3. The column maintained at 25°C. The initial mobile phase composition was 75% B, which was maintained for 2 min then linearly changed to 25% B in 3 min and was then back to the initial condition in 1 min (hold for 2 min, for re-equilibration). The analysis run time was 8 min and injection volume was 5 µL.

Mass spectrometric detection was performed on a triple quadrupole tandem mass spectrometer (AB Sciex-API3000, USA) equipped with an electro spray ionization source. The main source parameters were optimized as follows: nebulizer gas, 12 $Lmin^{-1}$; curtain gas, 10 $Lmin^{-1}$; collision activated dissociation (CAD) gas, 4 $Lmin^{-1}$; source temperature, 450 °C and turbo ion spray, 5000V. Multiple reaction monitoring (MRM) was employed using nitrogen as the auxiliary, nebulizer, collision and curtain gas with a dwell time of 100 ms for each transition. Electro spray ionization (ESI) was performed in positive ion mode, m/z 195.2→138.3 for caffeine, m/z 271.3→155.2 for tolbutamide, m/z 272.2→147.3 for dextromethorphan, m/z 327.3→292.2 for midazolam, m/z 346.3→198.1 for omeprazole, m/z 324.4→127.2 for gliclazide, respectively. Data acquisition and processing were powered by Analyst1.4.1 software.

Preparation of standard solutions and quality control (QC) samples

The primary stock solutions of five probes and gliclazide were prepared at 1 μgmL^{-1} in methanol, respectively. The working solutions of each analyte were prepared by diluting the stock solution with methanol. Gliclazide was used as an internal standard (IS). The QC samples involved high-quality control (QC-H), medium-quality control (QC-M) and low-quality control (QC-L), were similarly prepared at concentration of combined working solutions with blank rat plasma. All the stock solutions, standards and QC samples were stored at -20.

Bio-samples pre-treatment

A dual liquid–liquid extraction method was used to extract analytes from the plasma samples. 10 µL of IS solution (1000 $ngmL^{-1}$) was added to a 100 µLaliquot of plasma sample, the sample added 100 µL 0.2M ammonium acetate solution and mixed for 2 min on a Vortex mixer (IKA, Germany). Then, 1 mL ethyl acetate was added to the samples, vortexed for 5 min and followed by centrifugation at 12,000 g for 10 min. The 0.8 mL organic layer was separated and transferred into another tube, concentrated to dryness under nitrogen gas. The residue was re dissolved in 100 µL of 75% methanol in water and vortexed for 5 min, centrifuged at 12,000 g for 5 min. Finally, 60µLof supernatant was immediately transferred to injection vial for analysis.

Method validation

Selectivity and specificity

To investigate whether endogenous matrix constituents would interfere with the assay, the specificity and selectivity of the method were evaluated by analyzing blank plasma, blank plasma extracted by ethyl acetate that spiked with analytes and IS, a plasma sample collected at 30 min after oral administration of the probe drugs to rats, respectively.

Linearity and lower limit of quantification (LLOQ)

Calibration curves were constructed used nine concentrations spiked with plasma samples and were treated in accordance with the "bio-sample pre-treatment". The LLOQ, defined as the lowest concentration on the calibration curve, was measured in six replicates on one validation day and had to meet the requirement that signal-noise ratio(S/N) was at least 10:1.

Accuracy and precision

The accuracy and precision of inter-day/intra-day measurements of the method were evaluated by analyzing the QC samples at three concentration levels (QC-L, QC-M and QC-H) in six replicates

during a single day and on three consecutive days. Samples were quantified using calibration curves constructed during the same batch. The precisions were expressed as relative standard deviation (RSD), which should be within ±15%. The accuracies were assessed by calculating the percentage of measured concentrations to the nominal concentrations of each analytes in QC samples and should be within ±15%.

Extraction recovery and matrix effect

The matrix effect and extraction recovery experiments were performed in six replicates at three different QC concentrations (QC-L, QC-M and QC-H). The extraction recovery was investigated by comparing the chromatographic peak areas response of extracted analytes in pre-extract spiked samples (Set A) with those of post-isolation and fortified quality control samples (representing 100% recovery, Set B). The matrix effect was determined by comparing the Set B with analytes in the reconstitution solution at the same concentration dissolved (Set D), respectively. The results of extraction recovery and matrix effect should be stable and repeatable.

Stability

Stability experiments were performed to demonstrate whether all the compounds were stable under different storage and typical process conditions. The stability experiments of analytes in stock solutions and plasma samples were investigated by back calculating the concentration of stability samples against freshly prepared calibration curves. In this assay, freeze-thaw (-20 °C, three cycles), long term (-20 °C, 14 days), room temperature (25 °C, 6 h) and auto sampler (25 °C, 24 h) stabilities were determined at six replicates at each QC concentration (QC-L, QC-M and QC-H). Samples were considered stable if the deviation inside of ±15% of the nominal concentration.

Pharmacokinetic applications

A total of fifteen male Sprague-Dawley rats were randomly divided into three groups: the GLPs-treated group (low dose, n = 5), GLPs-treated group (high dose, n = 5) and the control group (n = 5). GLPs was dissolved in 0.5% Carboxyl Methyl Cellulose-Na (CMC-Na) solution, and then was administered (i.g.) at 50 mg•kg^{-1}•day-1in low dose group and 400 mg•kg^{-1}•day^{-1} in high dose group for consecutive10 days. During this period, the control group received physiological saline. In the 11th morning, all rats were administered an intragastric probe substrates mixed solution containing caffeine (3mg•kg^{-1}), tolbutamide (2mg•kg^{-1}), dextromethorphan (5mg•kg^{-1}), omeprazole (10mg•kg^{-1}) and midazolam (3mg•kg^{-1}). Blood samples (0.3 mL) were collected from fossa orbital is vein at 0, 0.083, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h following the experiment. The blood samples were put into heparinized micro-centrifuge tubes and followed by centrifuging at approximately 8000 × g for 5 min. The 100 µL of plasma supernatant was separated and stored at -20 °C until analysis performed with the procedure.

Statistical analysis

Statistical analysis of pharmacokinetic results was performed using Graph Pad Prism 6.0 (Graph Pad Software, Inc. San Diego, CA). All the quantitative results of assays are expressed as the Mean ± standard deviation (SD). Student T-test was utilized to evaluate differences between 2 groups. P values of less than 0.05 were considered as a statistically significant difference.

Results and Discussion

LC–MS/MS method development and optimization

In order to obtain better sensitivity and chromatographic resolution in a short run time, a reliable LC-MS/MS method was developed and validated to simultaneously detect five probe drugs in a single biological sample. The two columns Intersil ODS-2 (5 µm, 4.6 × 150 mm) and Intersil ODS-3 (5 µm, 2.1 × 100 mm) were compared for optimization of chromatographic conditions, and the different flow rates (0.3, 0.5 and 0.8 mL•min^{-1}) were also tested for the HPLC performance. In general, the gradient elution by mobile phase of methanol-0.1% formic acid in water was finally established for analysis, delivered with a 0.8 mL•min^{-1} (split ration 1:3) flowed on an Inertsil ODS-2 column (5 µm, 4.6 ×150 mm). The chromatographic separation of the targeted analytes was carried out by a single 8 min LC-MS/MS run.

Development of sample pretreatment method

Because of the extremely high sensitivity of LC-MS/MS for each analyte, it was essential to establish a method for extracting the analytes from relatively large complex endogenous biological matrices. In this study, the optimization of pretreatment was investigated by comparing protein precipitation with liquid-liquid extraction. It was showed that protein precipitation performed significant endogenous impurities interference; low response of analytes and unstable results of determination, while liquid-liquid extraction generated a narrow and symmetric chromatographic peak with an optimal resolution, the extraction recovery is better and more stable. Taking into the lipid solubility of analytes and environmental friendly extractive solvents, ethyl acetate was used as the extract ant instead of dichloromethane and chloroform. We found that the addition of ammonium acetate was contributed to obtain high extraction recoveries and improve peak shape in all analytes as compared to sodium carbonate or sodium hydroxide.

Method validation

All the analytes in this study produced a prominent, protonated precursor molecular ions $[M+H]^+$ in positive ionization mode. The most abundant fragment ions were selected for MRM (Supporting Information). The MRM transitions and optimized, collision-induced dissociation conditions were described in Table 1. The most intensive precursor→fragment transitions were shown in Figure 1.Typical MRM chromatograms for blank plasma spiked with QC-L levels of analytes and plasma collected from 30 min after oral administration of mixed probe drugs were shown in Figure2. No significant interference was observed in drug-free plasma sample at the retention times of the target drugs and IS.

Calibration curves of analytes in bio-samples were obtained by plotting the peak-area ratio of analytes/IS versus the theoretical concentration with a 1/X weighting factor. The calibration curves for each analyte were linear with correlation coefficients (R^2) greater than 0.9976 (Table 2).The precision and accuracy of intra-/inter-day were within the acceptable limit for five analytes. The intra-/inter-day data both were accurate and reproducible with acceptable accuracies (RE %) ranged and precisions (RSD%), the statistical results were shown in Table 3. Results summarized in Table 4 showed that extraction recoveries and matrix effect for five probe drugs. Due to the significant differences in polarity of five substrates and solvent transferred imperfectly, recovery loss existed in process of sample pre-treatment, leading to the extraction recoveries for all analytes ranged between 65.05% to 87.55%. The average matrix factor values

ranged from 86.18% to 105.64%, except caffeine, which arrived at 129%, approximately. No significant endogenous interference was observed near the retention times between the lots of different assays. Stability had been tested for bench-top (25 °C, 6 h), autos ampler (25 °C, 24 h), freeze-thaw (-20 °C, three cycles) and long-term (-20 °C, 14 days) stabilities. The summary of stability evaluation of each analyte was given in Table 5, which indicated that each analyte in bio-samples was of good stability.

Table 1: MRM transitions and fragmentation parameters for probe drugs and internal standards (DP: declustering potential; CE: collision energy; CXP: collision cell exit potential)

Analyte	Polarity	Precursor (m/z)	Product (m/z)	DP(V)	CE (eV)	CXP(V)
caffeine	+	195.2	138.3	48.1	27	12
tolbutamide	+	271.3	155.2	50.7	24	9.1
dextrometho rphan	+	272.2	147.3	64.9	44.3	13.6
midazolam	+	327.3	292.2	58.9	39.1	7.9
omeprazole	+	346.3	198.1	46.8	17.6	13
gliclazide (IS)	+	324.4	127.2	60.13	27.24	7.13

Table 2: Summary of calibration range, correlation coefficient (mean±SD), LLOQ

Analyte	Retention time	Calibration range ($ng•mL^{-1}$)	Correlation coefficient (R^2)	LLOQ ($ng•mL^{-1}$)
caffeine	2.47	2.5-4000	0.9987±0.0011	2.5
tolbutamide	3.43	5.0-8000	0.9990±0.0005	5
dextromethorphan	1.42	1.0-1600	0.9981±0.0002	1
midazolam	1.75	1.0-1600	0.9976±0.0008	1
omeprazole	2.64	0.8-1280	0.9999±0.0001	0.8

Table 3: Summary of intra-day (six replicates per concentration) and inter-day (three individuals runs) precision and accuracy of quality control samples for probe drugs in plasma. Results are expressed as concentration mean±SD; RSD, relative standard deviation; RE, relative error

Analyte	Concentration ($ng•mL^{-1}$)	Intra-day (n = 6)			Inter-day (n = 18)		
		Mean ± SD (ng•mL-1)	RSD (%)	RE (%)	Mean±SD (ng•mL-1)	RSD (%)	RE (%)
caffeine	5	5.00±0.35	7.04	0.13	4.96±0.38	7.61	-0.71
	125	129.33±6.59	5.10	3.47	129.78±9.48	7.30	3.82
	2000	2060.00±166.85	8.10	3.00	2088.89±149.62	7.16	4.44
tolbutamide	10	10.08±1.12	11.15	0.78	9.95±0.96	9.71	-0.52
	250	263.83±15.32	5.81	5.53	268.61±13.30	4.95	7.44
	4000	4255.00±115.02	2.70	6.38	4145.00±313.19	7.56	3.63
dextromethorphan	2	2.03±0.18	8.78	1.40	2.02±0.15	7.67	0.78
	50	53.67±2.33	4.34	7.33	54.61±2.09	3.82	9.22
	800	806.50±61.29	7.60	0.81	811.39±64.42	7.94	1.42
midazolam	2	1.64±0.16	9.57	2.75	2.01±0.18	9.03	0.36
	50	43.03±3.31	7.69	7.58	51.69±3.66	7.09	3.38
	800	559.50±32.87	4.91	4.61	798.28±90.66	11.36	-0.22
omeprazole	1.6	2.15±0.09	4.20	7.42	1.56±0.15	9.67	-2.57
	40	51.42±4.08	7.93	2.83	41.81±2.85	6.82	4.53
	640	792.33±89.61	11.31	-0.96	659.22±50.84	7.71	3.00

Table 4: Summary of matrix effect in rat plasma for probe drugs. Results are expressed as percent nominal±SD, n = 6.

Analyte	Concentration (ng•mL^{1})	Extraction recovery		Matrix effect	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
caffeine	5	81.55±5.21	6.38	129.51±5.66	4.37
	125	64.22±5.03	7.84	102.86±5.24	5.24
	2000	69.37±4.74	6.83	89.51±6.16	6.88
tolbutamide	10	68.86±3.60	5.23	102.83±5.86	5.70
	250	65.05±5.05	7.77	97.05±11.05	11.38
	4000	74.55±5.25	7.04	98.53±3.17	3.21
dextromethorphan	2	79.83±4.93	6.18	104.05±10.01	9.62
	50	71.89±6.80	9.46	101.52±13.39	13.19
	800	82.84±5.98	7.22	92.14±6.73	7.30
midazolam	2	86.09±5.23	4.86	88.18±7.39	8.38
	50	77.22±8.00	10.36	91.37±8.65	9.47
	800	71.30±2.90	4.07	86.18±5.51	6.39
omeprazole	1.6	86.75±9.00	10.37	105.64±7.38	6.99
	40	78.61±5.64	7.17	97.66±8.23	8.43
	640	87.55±4.50	5.13	88.12±6.19	7.02

Table 5: Freeze and thaw (4 cycles), short-term (24 h) and long-term (40 days) stability results for CAF, TOL, DM, MDZ, and OME in plasma. Results are expressed as percent nominal±SD, n = 6

Analyte	Concentration (ng•mL^{-1})	Bench-top (ambient temperature, 6 h)	Autosampler (25, 24 h)	Freeze/Thaw (-20 three cycles)	Long-term (-20 14 days)
caffeine	5	102.82±3.33	96.80±6.54	95.87±7.74	100.82±5.61
	125	111.50±1.76	106.33±4.93	110.33±3.98	111.33±4.13
	2000	110.45±7.86	110.20±7.89	97.73±6.76	104.55±7.25
tolbutamide	10	105.12±4.49	102.13±8.70	94.70±9.22	105.65±4.99
	250	93.97±9.76	107.08±6.04	105.97±5.74	109.33±1.86
	4000	95.27±6.73	105.58±6.54	95.45±6.29	91.73±5.25
dextromethorphan	2	94.62±8.13	89.95±3.68	95.57±3.11	95.57±9.64
	50	105.25±7.02	105.54±5.67	111.67±3.39	110.50±2.74
	800	102.13±6.35	102.12±12.05	98.75±6.73	94.65±11.22
midazolam	2	111.30±41.10	96.92±7.40	96.45±6.18	104.07±8.38
	50	105.17±2.48	101.67±9.54	98.82±6.67	104.92±7.61
	800	107.50±3.08	106.98±5.65	100.28±6.37	105.68±4.65
omeprazole	1.6	103.30±5.20	93.64±6.32	93.70±6.63	106.57±7.77
	40	98.38±4.02	107.12±8.93	106.63±9.23	109.00±4.29
	640	95.25±7.11	106.67±6.25	93.92±5.39	97.67±6.20

A previous study suggested that realgar-*Indigo natural* is (RIF) could inhibit CYP1A2 enzyme activity and induce CYP2C11 activity [29]. Compared with their HPLC method, our method had better sensitivity and accuracy as well as greatly improved efficiency. Besides, due to the limitation of LOD (limit of detection), HPLC required more plasma sample and larger dosage of intragastric administration. However, overweight dose of probe drugs in rats might cause tolerability and toxicity. The dosage of administration current study was calculated by data conversion according to commonly used dose in human, even lower than their clinical usage to ensure the probes specificity to the corresponding P450 enzyme, which rendered our result with more clinical significance. Besides, the method required a small volume of plasma and the sample preparation technique was efficiency and inexpensive. The low LLOQs and wide range of linearity confirm our method selective and credibly acceptable. The sample preparation procedure and rapid analysis render the method valuably and easily applicable.

Effects of GLPs on the CYP450 activities in rats

The developed method was successfully applied to simultaneously determine concentrations of the target drugs in rat plasma. The

pharmacokinetic parameters of analytes were calculated using Phenix Winnolin 6.3 (friendly provided by XP iscoric) and summarized in Table 6.

Table 6: Effects of GLPs at different levels on the pharmacokinetics of five probe drugs (n = 5). *P<0.05, **P<0.01 vs. control group of probe substrate

Parameters/Unit		caffeine	tolbutamide	dextromethorphan	midazolam	omeprazole
Cmax (ng mL^{-1})	Control	2590.00	5372.00	36.1	766.80	120.66
	Low dose	3668.00**	6446.00	48.2	1422.80	105.94
	High dose	3744.00**	7052.00	31.3	1402.00*	117.88
T_{max} (h)	Control	0.23	2.12	0.56	0.12	0.23
	Low dose	0.15	1.30	0.50	0.08	0.08
	High dose	0.08	1.32	0.40	0.08	0.08
$t_{1/2}$ (h)	Control	0.89	4.29	1.59	2.29	0.47
	Low dose	0.85	4.07	0.998*	3.17	0.37
	High dose	1.29	4.26	1.10	3.26	0.36
AUC_{0-t} (h*ng mL^{-1})	Control	4329.89	43590.82	66.32	314.47	59.17
	Low dose	5658.04	44780.09	100.5	587.91	62.41
	High dose	6114.99*	51492.44	56.39	762.94*	59.36
$AUC_{0-\infty}$ (h*ng mL^{-1})	Control	4333.44	45018.54	71.14	344.50	60.73
	Low dose	5670.56	45931.09	1.53	607.40*	63.15
	High dose	6119.38*	52984.28	64.44	777.01*	60.40
MRT_{0-t} (h)	Control	1.36	5.87	1.60	1.77	0.54
	Low dose	1.25	5.19	1.35	1.83	0.52
	High dose	1.51	5.46	1.28	1.11	0.46
$MRT_{0-\infty}$ (h)	Control	1.37	6.57	2.15	3.10	0.60
	Low dose	1.27	5.80	1.59*	2.49	0.55
	High dose	1.53	6.11	1.74	1.49	0.50
Vz/F (Lkg^{-1})	Control	9.11E-04	3.61E-04	0.227	0.031	0.13
	Low dose	6.53E-04	2.58E-04	0.109	0.026	0.10
	High dose	9.32E-04	2.66E-04	0.143	0.020	0.096
CL/F (L*h-1kg^{-1})	Control	7.11E-04	5.92E-05	0.108	0.0096	0.22
	Low dose	5.32E-04	4.45E-05	0.0866	0.0055*	0.20
	High dose	4.94E-04*	4.41E-05	0.100	0.0042*	0.19

For caffeine (Figure 3A), GLPs treatment groups (low and high dose) both significantly elevated maximum concentration (C_{max}) compared with control group (P<0.01). Moreover, GLPs in high dosage increased the exposure levels (AUC) and accelerated the elimination (CL) in rat plasma as compared to control group (P<0.05), which manifested a good dose-dependent manner. Therefore, the pharmacokinetic behaviors of caffeine indicated that GLPs might suppress the CYP1A2 activity, especially at high dosage.

For tolbutamide (Figure 3B), although the results had no significant difference (P>0.05), we found that C_{max} in GLPs treatment groups were obviously high compared with control group. Thus, it was worth noting that GLPs was co-administration with the drugs metabolized by CYP2C9.

For dextromethorphan (Figure 3C), except $t_{1/2}$ and MRT(0-∞) of dextromethorphan in the GLPs-low dose group were lower than in control group (P<0.05), the differences of the other parameters were relatively small in three groups. These evidences demonstrated that low dosage GLPs might inhibit the metabolism of dextromethorphan, whereas high dosage GLPs had no obvious effect on the metabolism of dextromethorphan. It was inferred that the dose of GLPs might be involved in the CYP2D6 activity, but the dose relationship required validating in further studies.

For midazolam (Figure 3D), C_{max} in high GLPs treatment group was relatively high compared with control group (P<0.05). Note worthily, there were no statistical significances between low dose group and control group for C_{max}, but C_{max} in low dosage was equivalent to high dose group, which might be caused by existence of individual difference in low dose group. In addition, both GLPs treatment groups elevated the exposure levels (AUC) and decreased elimination (CL) (P<0.05). Thus, we speculated that the simulative effect of GLPs is not only on midazolam, but also on the biological transformation of other products that metabolized by CYP3A4.

GLPs had few influences in metabolism of omeprazole (Figure 3E). And there were no significant influences on T_{max}, $t_{1/2}$ and MRT

between GLPs treatment groups and control group for all analytes (P>0.05).

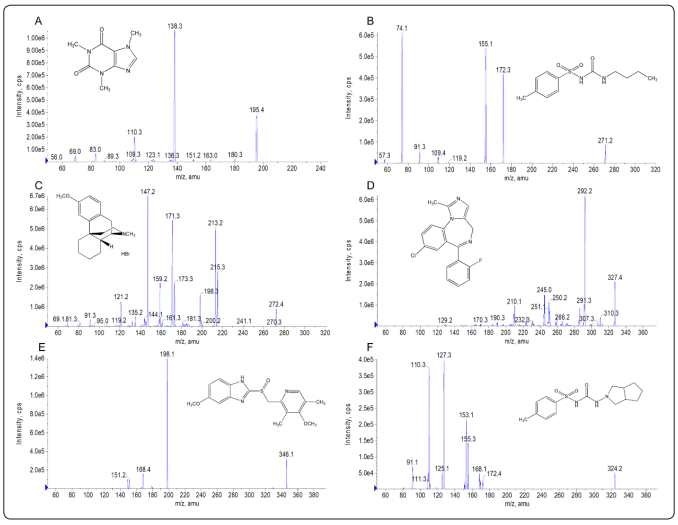

Figure 1: MS/MS spectra and chemical structures of caffeine (A), tolbutamide (B), dextromethorphan (C), midazolam (D), omeprazole (E) and IS (F)

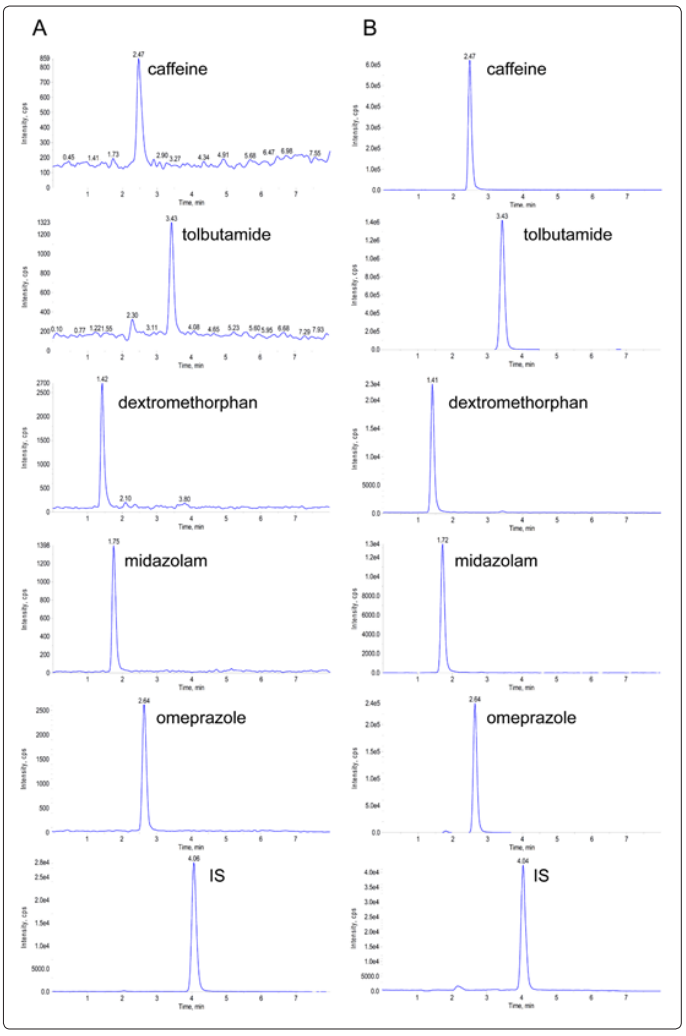

Figure 2: MRM chromatograms of IS and five probe drugs in plasma samples spiked with QC-L concentrations (A) and plasma samples collected from a rat 0.5 h after dosing (B).

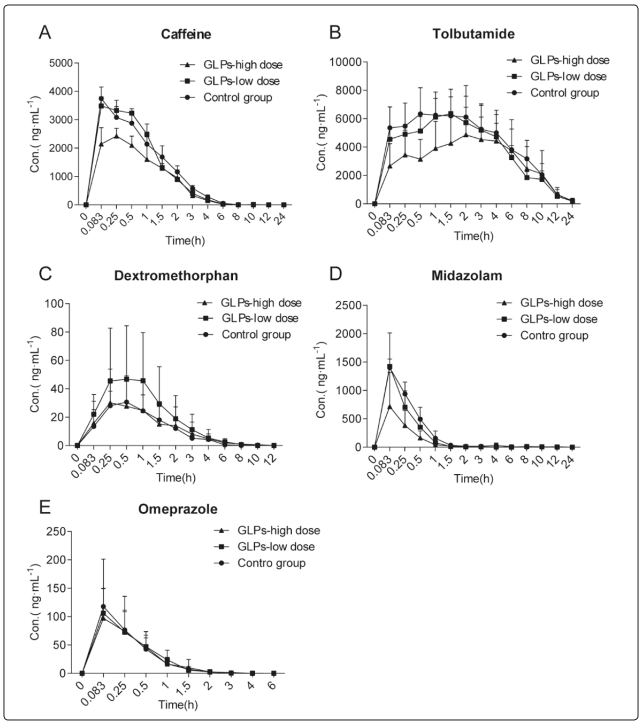

Figure 3: Plasma concentration-time curves of caffeine (A), tolbutamide (B), dextromethorphan (C), midazolam (D) and omeprazole (E) in rats. Data represent mean ± SD.

It is reported that metabolized model in rat presented better value than *in vitro* metabolite profile [30]. The purpose of the paper was to elucidate the potential influences of GLPs on the activities of CYP450 isoforms in rats. Numerous cocktails composed of several CYP450 selective probe drugs have already been described in the literature to study in vivo drug-metabolism activities [31], but they often comprise probe drugs which are not or no longer available in several countries. In our study, the substrate for each CYP isoform was selected according to the FDA guidance. Caffeine was selected as substrate in "cocktail probes" of cytochrome CYP1A2 instead of phenacetin, because phenacetin was forbade selling in most of countries, including in China. Besides, some papers used metoprolol tartrate as substrate of cytochrome CYP2D6, but later research found that metoprolol tartrate was not specifically metabolized by CYP2D6. However, caffeine, tolbutamide, dextromethorphan, omeprazole and midazolam were demonstrated to be selective substrates of human CYP1A2, CYP2C9, CYP2D6, CYP2C19 and CYP3A4 [32]. Hence, we assumed that effects of GLPs determined in rat could be speculated to human in clinical use, which still required more data to support it.

Modification of P450 enzymatic activity by inhibition, induction, or activation is linked to altered biological activity of endogenous substrates and therapeutic drugs, resulting in human diseases and harmful DDI [33]. Our study had demonstrated that GLPs could inhibit the activities of CYP1A2, CYP2C9 and CYP3A4, induce the activity of CYP2D6 at high dosage but inhibit it at low dosage. The results showed that during the concomitant use of GLPs with other drugs metabolized by CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 in human, great cautions should be taken by monitoring the therapeutic drug concentrations to reduce some adverse reactions

and avoid drug accumulation or the failure in treatment.

Conclusion

In summary, a LC–MS/MS method capable of quantifying five probe drugs simultaneously in rat plasma has been developed and fully validated reliable, precise and sensitive. This study reported the effects of GLPs on five probe drugs using a HPLC-MS/MS method at first time. The result demonstrated that GLPs was closely associated with activities of CYP1A2, CYP2D6 and CYP3A4. Specifically, GLPs in low and high doses could inhibit the activities of CYP1A2 and CYP3A4, the GLPs in low dose might suppress the activity of CYP2D6. Therefore, it is worth concerning strictly that co-administration of GLPs and synthesis drugs that metabolized by CYP1A2, CYP2D6 and CYP3A4 in clinical treatment. Unavoidably, there were some limitations in this study. The CYP450 isozyme in human may have few differences to in rats. Thus, further studies could compare the differences between the metabolite patterns of probe drugs co-administrated with GLPs *in vivo* of rat and *in vitro* of human liver microsomes, contributing better translation of rodent toxicity data to human.

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