Original Article

Evaluation of the Pharmacokinetics and Renal Excretion of Ma-Zi-Ren-Wan in Health Subjects

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ABSTRACT

Background: Ma-Zi-Ren-Wan (MZRW) is a classic Chinese formula for treating human constipation. It is comprised of six herbs. Our previous studies have shown its great therapeutic effect. The absorbed compounds had been studied in rat, while there was no study about its components in human body.

Objectives: To observe the components of MZRW absorbed in health subjects and study the pharmacokinetics of major compounds. At the same time, to observe the renal excretion of MZRW in health subjects based on the quantification of major compounds.

Methods: Health adults were randomly assigned to three dose groups (5g, 7.5g and 10g q.d.) of MZRW. Blood samples were collected from the medial cubital vein just before and at 0.25, 0.5, 1, 2, 4, 8 and 12 h after administration. Urine samples were collected at 0 to 3 h, 3 to 6 h, 6 to 9 h and 9 to 12 h after MZRW administration, with the urine volume recorded for each time segment. Plasma and urine samples were analyzed by optimized LC-MSMS (Liquid chromatography-tandem mass spectrometry) method for pharmacokinetics and renal excretion study of MZRW. **Results:** Ten compounds of MZRW were observed in 23 health subjects. Due to the low concentration in plasma at the current dose, only four compounds (Albiflorin, paeoniflorin, magnolol and rhein) were quantified in the plasma sample. Honokiol, aloe emodin and emodin could only meet the LLOQ at some time points of the high dose group. Hesperidin, naringin and amygdalin could not be detected in plasma sample. While seven compounds (Amygdalin, albiflorin, paeoniflorin, magnolol, honokiol, rhein and aloe emodin) could be quantified in urine, the renal excretion was well studied.

Conclusion: MZRW was safe and well tolerated in this clinical study. Albiflorin, paeoniflorin, magnolol and rhein was well quantified in plasma. The renal excretion of paeoniflorin, albiflorin and rhein were dose dependent for doses ranging between 5 and 10g.

Key words: Ma-Zi-Ren-Wan, LC-MS/MS, Pharmacokinetics, Human plasma, Renal Excretion Received 10 November 2016; Accept 13 June 2017

Introduction

Constipation is a very common disease frequently encountered in daily out-patient service affecting the patient's quality of life. Based on recent studies, about 30% of the global population suffered from the problems during life time^[1,2]. Although the morbidity is high, only one fourth of constipation patients will seek for the medical treatment yet^[3]. Laxatives are one category of conventional option for treatment of constipation. Currently, several new drugs including lubiprostone, linaclotide and prucalopride have been approved for the treatment. Nevertheless, there still remains a significant unmet need especially among those who do not obtain satisfactory efficacy from current interventions^[4,5]. Therefore, a number of patients would ask for help from traditional Chinese medicine (TCM), generally by applying Chinese herbal medicine (CHM)^[2].

Ma-Zi-Ren-Wan (MZRW) is a classic Chinese formula which was firstly recorded in a classic TCM book, *Treatise*

on Cold Damage Disease (Shang Han Lun)^[6]. It has been frequently utilized for constipation of the excess syndrome throughout Asia since the Han Dynasty (AD 200). Based on Chinese Pharmacopeia, MZRW contains six herbs: Semen Cannabis Sativae, Semen Pruni Armeniacae, Radix Paeoniae, Fructus Immaturus Citri Aurantii, Cortex Magnoliae and Radix et Rhizoma Rhei^[7]. The available researches have demonstrated the significant therapeutic effect of MZRW. One of our clinical studies proved that the optimal dose of MZRW for constipation was 7.5g b.i.d.^[8]. In addition, another randomized, double-blind, and placebo-controlled trial has illustrated that MZRW (7.5 g b.i.d.) was safe and effective for relieving functional constipation for subjects with constipation of excess syndrome^[9].

Despite the great therapeutic effect, the active compounds of MZRW remain unclear. In order to make a clear understanding of the compounds in MZRW and facilitate the later systematic study of this formula, our group identified a comprehensive list of compounds in two dosage forms of MZRW by an ultra-performance liquid chromatographyquadrupole/time-of-flight mass spectrometry (UPLC-QTOF-MS/MS)-based method^[10]. Studies have shown that rhein, emodin and aloe emodin in *Radix et Rhizoma Rhei*, Naringin and hesperidin in *Fructus Immaturus Citri Aurantii*, paeoniflorin and albiflorin in *Paeoniae Radix Alba*, magnolol and honokiol in *Magnolia officinalis* have linkage with effects on constipation^[11-14].

However, there were only reports on pharmacokinetics study of one or several compounds of the above active compounds in other Chinese formula or single herbs^[15-25], which could not represent the real pharmacokinetic of these compounds in MZRW, since the complex matrix and drug-drug interaction could make great influence on the pharmacokinetics of one compound. So our research team began to use rat for absorbed compounds study of MZRW, and acquired the pharmacokinetics of these ten compounds in rat plasma after MZRW administration^[26].

Due to the species difference between human and animal, it is compulsory to conduct clinical trials to observe the pharmacokinetics of this formula in human, explore its tolerability and obtain a consistent picture of its mechanism of action. Our clinical study was approved by Hong Kong Baptist University Ethics Committee on the Use of Human Subjects for Teaching and Research (Approval No. HASC/13-14/0017) and was registered with an identifier (NCT02359396) in ClinicalTrial.gov. The protocol for this trial has been published on *European Journal of Integrative Medicine*^[27].

Up to now, no study has been reported on the pharmacokinetic study or renal excretion of MZRW in human. There were only several reports on the pharmacokinetics study of rhein^[28,29], amygdalin and paeoniflorin^[30]. However, in consideration of the possibility of interaction between components in an herbal formula and the pharmacokinetic variation of one single compound in different formulas, amultiple-component pharmacokinetic study would be more likely to illustrate the pharmacokinetic profile of MZRW precisely. Currently, LC-MS has been applied in human pharmacokinetics study^[31-35]. The best approach for simultaneous determination of multiple components in a complex biological matrix is, currently, ultraperformance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (UPLC-MS/MS) with multiple reactions monitoring (MRM). In our previous pharmacokinetics study in rat, we have established the quantification method for the above active compounds in rat plasma and the method validation has shown that this optimized LC-MS/ MS method could be well utilized in the pharmacokinetics study of these ten compounds^[26]. Thus, we conducted this phase I clinical study based on the previous method.

Materials and Methods

1. Chemical and Reagents

MZRW granula was manufactured and qualified by Pura Pharm International (H.K.) Limited (Hong Kong, China). The entire manufacturing process, from authenticating the raw materials to the final products, is in strict compliance with the standards of Good Manufactory Practice (GMP) and Chinese Pharmacopoeia.

The reference standards of amygdalin, paeoniflorin, hesperidin, naringin, magnolol, honokiol, aloe-emodin and rhein were purchased from Shanghai YuanYe Bio-Technology Co., Ltd (Shanghai, China). Emodin, albiflorin, and two internal standards (I.S.) (geniposide and liquiritin) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of all these reference standards was \geq 98.0% (HPLC). HPLC grade of acetonitrile, methanol and formic acid were purchased from Merck (Darmstadt, Germany). Deionized water was purified by the Millipore water purification system (Millipore, Milford, Massachusetts, United States). All other reagents used were of analytical grade.

2. Study Design

This is a randomized, open-labelled, three-arm clinical trial. Participants were recruited through master email of the School of Chinese Medicine, Hong Kong Baptist University. The participants were screened through telephone and eligible subjects were randomized in a ratio of 1:1:1 to receive 5.0 g, 7.5 g and 10 g q.d. of MZRW. Randomization was carried out in 1:1:1 ratio according to the sequence generated with Random Allocation Software (Version 1.0.0), Isfahan, Iran. The participants were fasted overnight for more than 8 hours and refrained from foods with Semen Cannabis Sativae, Semen Pruni Armeniacae, Radix Paeoniae, Fructus Immaturus Citri Aurantii, Cortex Magnoliae, Radix et Rhizoma Rhei (as well as the congener plants of these six herbs) for 3 days before the study until completion of the study. The study was performed at Mr. & Mrs. Chan Hon Yin Chinese Medicine Specialty Clinic and Good Clinical Practice Centre, Hong Kong Baptist University (Hong Kong, China). On the day of study, single oral dose of MZRW was administrated to the participants by site personnel at 8 am. Regular standard meals were provided to each participant at 1 h, 4 h and 8 h. They remained in the clinic under supervision for the subsequent 12 hours after drug administration.

Blood samples (5 ml each) were collected from the medial cubital vein into evacuated tubes containing heparin just before and at 0.25, 0.5, 1, 2, 4, 8 and 12 h after administration by research nurses and were immediately centrifuged at 5000 rpm, 10 min to separate plasma (Centrifuge 5810 R, Eppendorf, Hamburg, Germany). The participants recorded the volume of urine output for each time segment within 12 h. Urine samples (50 ml each) were collected at 0 to 3 h, 3 to 6 h, 6 to 9 h and 9 to 12 h after MZRW administration. Plasma and urine fractions were stored at -20°C until analysis. All study procedures were conducted in accordance with the ethical principles of the Declaration of Helsinki, consistent with Good Clinical Practice guidelines, and approved by Hong Kong Baptist University Ethics Committee on the Use of Human Subjects for Teaching and Research (Approval No. HASC/13-14/0017). Subjects were given their written informed consent before participating in the study.

3. Preparation of Plasma and Urine Samples

Plasma sample (450 µl) and 10 µl of mixed I.S. solution (liquritin and geniposide, 5 μ g/ml each) were added to a 2.0 ml Eppendorf tube. Then extraction was performed by adding 1000 µl of methanol to precipitate protein and extract analytes from the plasma. The samples were vortexed for 2 min (Vortex-Genie 2, Scientific Industries, Inc., New York, United States), and then centrifuged for 10 min at $13000 \times g$ (Centrifuge 5424 R, Eppendorf, Hamburg, Germany). The supernatant (1400 µl) was separated and then dried under Vacuum Centrifugal Concentrator (SPD111VP1 SpeedVac, Thermo Fisher Scientific, Massachusetts, United States). The residue was re-constituted in 100 µl methanol solution, and centrifuged (13000 × g for 10 min) (Centrifuge 5424 R, Eppendorf, Hamburg, Germany). The supernatant was transferred to an auto-sampler vial and an aliquot of 3 µl was injected into the UPLC-MS/MS system (Agilent 1290 UPLC tandem Agilent 6460 Triple Quadropole, California, United States) for analysis.

Urine sample (90 μ l)) and 10 μ l of mixed I.S. solution (liquritin and geniposide, 5 μ g/ml each) were added to a 2.0 ml Eppendorf tube. The samples were directly centrifuged for 10 min at 13000 × g (Centrifuge 5424 R, Eppendorf, Hamburg, Germany). The supernatant was transferred to an auto-sampler vial and an aliquot of 3 μ l was injected into the UPLC-MS/MS system for analysis.

4. Analysis of Plasma and Urine Samples

The concentrations of ten ingredients were determined according to our reported method^[26] with modification. The analysis was performed using LC-MS/MS in MRM mode. Negative ionization mode was selected as the ionization of all analytes and I.S. had much higher relative intensity in negative ionization mode than in positive ionization mode. Pure standards were individually injected into (-)-ESI (electrospray ionization) source to optimize MS parameters. Abundant deprotonated molecular ions and two most abundant product ions were selected for each analyte. The MRM transitions and energy parameters including collision energy and fragmentor voltage of all the analytes and I.S. were optimized, and detailed information can be find in the previous paper^[26].

Liquid chromatographic analysis was performed on an Agilent 1290 UPLC system, consisting of a 1290 binary pump solvent management system, a 1290 TCC, and a 1290 auto-sampler (Agilent, California, United States). A Waters ACQUITY BEH C18 column (100 mm \times 2.1 mm, 1.7 μ m) (Waters Corporation, Massachusetts, United States) was employed for the separation of samples, and the column temperature was maintained at 40°C. The mobile phase was composed of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) using a gradient elution of 12-25% B at 0-6 min, 25-45% B at 6-8 min, 45-80% B at 8-11 min, 80-100% B at 11-11.1 min and then returned to the initial condition with a flow rate set at 0.40 ml/min. The auto-sampler was conditioned at 4°C and the injection volume was 3 μ l.

5. Statistical Analysis

The concentration of the analytes in human plasma and urine samples were calculated by establishing standard curves of each analyte in blank plasma and urine matrix. Liquritin was selected as the I.S. for each analyte. The plasma C-T (concentration-time) curves of each analyte were established for the three dose groups. The maximum plasma concentration (C_{max}) and time to reach the maximum concentrations (T_{max}) were obtained directly from the curve.

Results and Discussion

1. Materials and Methods

Twenty-Eight participants were enrolled and randomized. Twenty-five participants completed the study with no protocol deviations. Two participants were withdrawn, one was because of hypoglycemia, and the other was because of too thin blood vessels for drawing blood. Because of these withdrawals, 23 participants were randomized and received MZRW (5 g, 7.5 g or 10 g). All of these 23 participants were included in the pharmacokinetic and renal excretion analysis (Table 1). During the study, no participant took or received concomitant therapy or food which might contain similar core compounds that were judged to have the potential to affect pharmacokinetic parameters.

2. Pharmacokinetic Analysis

The optimized LC-MS/MS method was applied to the detection of all the analytes in plasma and urine samples of all participants. The result showed that in plasma samples, only albiflorin, paeoniflorin, magnolol and rhein could meet the demand of LLOQ (lower limit of quantification) at the current dose, even though the plasma samples were concentrated for 4.5 times. The other analytes, such as honokiol, aloe emodin and emodin could only meet the LLOQ at some time points of the high dose group, that we couldn't make the entire C-T curve. Different with the tendency in rat plasma after MZRW administration reported before, hesperidin, naringin and amygdalin could not be detected in human plasma post-dosing, showed the obvious species difference between human and rat. Emodin, aloe-emodin showed poor peak shape in the current LC-MS/ MS method, while the peaks were good in stock solution and

Table 1. Participant characteristics.

Characteristics	Number (%) or mean and range	
Age, years	29	
Range	23-35	
Male [n (%)]	11 (47.8)	
Female [n (%)]	12 (52.2)	
Weight (kg)	56.2	
Range	45.2-67.5	
Body mass index (kg/m ²)	s index (kg/m ²) 22.5	
Range	18.9-23.6	



Figure 1. The concentration-time curves of rhein, albiforin, paeoniflorin and magnolol of three dose groups in human plasma after MZRW administration.

in rat plasma studied before. So we believe that the matrix in human plasma had more obvious influence on the detection and separation of the two compounds. The other reason may be that there were metabolites of the two compounds generated in human plasma, which own the same MRM fragments with the analytes. These metabolites may also have similar polarity with the analytes that they existed for a close retention time with the analytes. Hence the quantification of the two compounds was not studied in this paper. The concentration of rhein, albiflorin, paeoniflorin and magnolol in plasma were calculated using the established calibration curves in human plasma. The C-T curves of rhein, albiflorin, paeoniflorin and magnolol of three dose groups in human plasma were shown in Figure 1. The pharmacokinetic parameters (C_{max} and T_{max}) of rhein, albiflorin, paeoniflorin and magnolol in human plasma were shown in Table 2. Comparing with previous rat pharmacokinetic study result, we can find that all these ten compounds were well quantified in rat plasma samples, because the dose to rat in the previous study was much higher than the biological equivalent dose. As a result, most compounds was much easier for quantification in rat plasma than in human plasma. The C-T curve of albiflorin and paeoniflorin in human plasma were quite similar, this phenomenon was consistent with previous rat pharmacokinetic result, which can be attributed to the similar structure of paeoniflorin and albiflorin. The T_{max} of albiflorin (1 h in 3 dose groups) and paeoniflorin (1 h for 5 g and 7.5 g dose group, 0.5 h for 10 g dose group) were quite close

Table 2. The pharmacokinetic parameters of rhein, albiforin,
paeoniflorin and magnolol in human plasma after MZRW
administration.

Analyte	Dose group	C _{max} (ng/mL)	<i>T_{max}</i> (h)
Albiflorin	A (5 g)	71.05 ± 34.18	1.00
	B (7.5 g)	96.78 ± 26.70	1.00
	C (10 g)	79.90 ± 27.60	1.00
Paeoniflorin	A (5 g)	130.26 ± 63.63	1.00
	B (7.5 g)	166.11 ± 49.72	1.00
	C (10 g)	139.24 ± 64.34	0.50
Rhein	A (5 g)	786.17 ± 484.77	0.25
	B (7.5 g)	790.20 ± 218.41	0.50
	C (10 g)	910.89 ± 241.42	1.00
Magnolol	A (5 g)	234.34 ± 103.24	0.25
	B (7.5 g)	275.36 ± 151.27	0.50
	C (10 g)	335.87 ± 185.75	0.25

 $C_{\rm max}$ the maximum plasma concentration; ${\cal T}_{\rm max}$ time to reach the maximum concentrations.

to the T_{max} of this two compounds in rat plasma, which was both 0.75 h. The T_{max} of rhein went larger as the dose grew from 5 g to 10 g, indicating the potential saturation in absorption. Magnolol exhibited similar T_{max} (0.25 h to 0.5 h) with the data in rat sample (0.58 h).

3. Renal Excretion Analysis

Urine samples of all participants at 0~3 h, 3~6 h, 6~9 h and 9~12 h were analyzed using the established LC-MS/MS method. The result showed that only trace amount of hesperidin and naringin could be detected in urine sample, indicating that the main excretion pathways of the two compounds were not through urine excretion of prototype drug. Emodin showed very low amount and poor peak shape in the urine samples by the current LC-MS/MS method, while there was a big peak in the retention time of 2.6 minutes before emodin, which owned the same MRM fragments with emodin, and may be a metabolite of emodin with a larger polarity. This peak will be identified and quantified in the future study. Albiflorin, amygdalin, aloe emodin, rhein, magnolol, honokiol and paeoniflorin could be quantified in urine samples. The calibration curves of the above compounds in urine matrix were established and validated. The result showed that all these curves exhibited satisfactory linearity ($r^2 > 0.98$). The concentrations of the compounds in urine at four time segments of three dose groups were calculated, and the amounts of urine excretion of the prototype compounds of these analytes were calculated by multiplying the concentration with the corresponding urine volume. The result was shown in Figure 2. The dose proportionality of renal excretion amount of albiflorin, amygdalin, aloe emodin, rhein, magnolol, honokiol and paeoniflorin were graphically displayed in Figure 3. The squared correlation coefficients of paeoniflorin, albiflorin and rhein were

close to 1 for doses ranging between 5 and 10 g of MZRW, indicating that the renal excretion amount was proportional to the dose within the current dose range. Amygdalin and aloe emodin showed bad dose proportionality, which might be attributed to their quite lower concentration and worse peak shape. Honokiol and magnolol exhibited quite stranger dose proportionality. The dose (7.5 g) exhibited the highest total renal excretion amount. The bad MS response of the two compounds might be one reason, in addition, the absorption of magnolol and honokiol might have saturation that when the dosage went higher, no more amount was absorbed into blood, thus the renal excretion was not increased. Besides, different dose might cause different metabolism.

The limitation of this study includes that the dosages were only divided into three levels due to the small sample size; and it is merely a study on single-dose pharmacokinetic and renal excretion, which can not reflect multi-dose features. The study is only carried out in 18-65 year old healthy subjects, but we know that pharmacokinetics, tolerability and adverse effects may be different in medium or relatively weak people, such as elder or weak patients. Nevertheless, the current study in health subjects can provide further consolidated evidence for the safety, tolerability and pharmacokinetics of MZRW in healthy volunteers, which can facilitate the future multi-dose study in patients; additionally, this will also provide a reference for other herbal medicine interventions researchers.



Figure 2. Amount of urine excretion of albiflorin, amygdalin, aloe emodin, rhein, magnolol, honokiol and paeoniflorin in three dose groups after MZRW administration.





Figure 2. (continued).





Summary

MZRW was safe and well tolerated in this clinical study. No Serious Adverse Events (SAEs) occurred, and all subjects were in good compliance. The C-T curve of rhein, albiflorin, magnolol and paeoniflorin in plasma were established. Renal excretion of MZRW in health subjects were studied, and no hesperidin or naringin was detected. The renal excretion amount of albiflorin, amygdalin, aloe emodin, rhein, magnolol, honokiol and paeoniflorin were calculated in urine. On the basis of this study in healthy subjects, MZRW is worthy of further investigation for treating constipation.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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