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Comparative pharmacokinetics of aloe-emodin, rhein and emodin determined by liquid chromatography–mass spectrometry after oral administration of a rhubarb peony decoction and rhubarb extract to rats

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This study aimed to clarify the rationality of herbaceous compatibility of a rhubarb peony decoction (*Da-Huang-Mu-Dan-Tang,* RPD) by comparing the pharmacokinetics of aloe-emodin, rhein and emodin in rats' plasma after oral administration of RPD and rhubarb extract. A rapid, sensitive LC-MS method was developed and validated for the determination of the plasma concentrations of the three analytes after oral administration RPD and rhubarb extract. The developed method was successfully applied to a pharmacokinetic study of aloe-emodin, rhein and emodin in rats' plasma after oral administration. Compared with administration of single rhubarb, the C_{max} of rhein in RPD was decreased significantly ($p < 0.05$). Meanwhile, the $T_{1/2}$ of aloe-emodin and emodin were increased significantly ($p < 0.05$) after administration of RPD. In addition, the T_{max} of rhein and emodin were also increased significantly ($p < 0.05$) in RPD. These results indicated that the absorption of rhein in rats was suppressed after oral administration RPD. Moreover, The time for rhein and emodin to reach the peak concentration was delayed and the elimination of aloe-emodin and emodin was also postponed in RPD. This study could provide a meaningful basis for evaluating the clinical application of traditional Chinese medicine in terms of pharmacokinetics.

1. Introduction

Most of Traditional Chinese Medicines (TCM) are combinations to obtain a synergistic effect or antagonistic action. In general, one herb may produce an impact on another in pharmacokinetics among the prescription, which usually brings about interaction in pharmacodynamics (Zhu et al. 2010; Yan et al. 2011; Wu et al. 2009). Therefore, the research of possible pharmacokinetic interaction of representative ingredients in single herbs and the whole prescription will help us to know not only the real pharmacokinetic properties of compounds in complex prescriptions, but also the compatibility of complex prescriptions.

Rhubarb Peony Decoction (RPD) composed of five herbs including *Radix et Rhizoma Rhei palmati*, *Cortex Moutan Radicis, Semen Persicae*, *Semen Benincasa*e and *Natrii sulfas*, was first described in Jin Gui Yao Lve, which was a treatise on endogenous miscellaneous diseases written by the famous Chinese physician Zhang Zhongjing (150 to 219 A.D. in the Chinese Eastern Han Dynasty). It is famous in the clinical practice of TCM for its excellent treatment for acute appendicitis and Interior-Heat type constipation (Huang et al. 2008; Dong et al. 2006). In addition, it was also found that RPD has a good curative effect on multi-organ abscess and acute pancreatitis (Gao et al. 2006; Liang et al. 2005).

Radix et Rhizoma Rhei palmati is a well-known TCM used as a ministerial ingredient in RPD for treating inflammation, atherosclerosis, diabetes and cancer (Fang et al. 2007; Liu et al. 2007; Gu et al. 2003). Aloe-emodin, rhein and emodin are characteristic anthraquinones isolated from *Radix et rhizoma Rhei Palmati*. It has been reported that these anthraquinones have purgative (Zheng et al. 2002), antiviral (Andersen et al. 1991; Barnard et al. 1992), bacteriostatic (Jong et al. 1987), anti-inflammatory (Liu et al. 2008; Moon et al. 2006) and antioxidative (Matsuda et al. 2001; Lizuka et al. 2004) activities, and could also protect effect in rats with chronic renal failure (Wang et al. 2009).

The pharmacokinetic profiles of aloe-emodin, rhein and emodin in rhubarb and its prescriptions have been determined using HPLC and liquid chromatography–mass spectrometry (LC–MS). Shia et al. (2011) investigated the pharmacokinetics and tissue distribution of anthraquinones of rhubarb in rats using HPLC. Xu et al. (2010) compared the pharmacokinetics of aloe-emodin, rhein and emodin between Da-Cheng-Qi decoction and rhubarb extract in rats after oral administration using LC-MS/MS. Yan et al. (2009) studied the pharmacokinetics of anthraquinones in rat plasma following oral administration of Xiexin Decoction and rhubarb extract using HPLC. Moreover, several methods for detection and quantification of anthraquinones in RPD have been described using high performance liquid chromatography (HPLC) with ultra-violet detection (Xi et al. 1998; Cui et al. 2009). Liu et al. (2010) developed a rapid method for the simultaneous determination

of three analytes of RPD using HPLC. However, there are few data on the pharmacokinetics of aloe-emodin, rhein or emodin following oral administration of RPD.
In this study, we developed a LC-MS method for the simultaneous determination of aloe-emodin, rhein and emodin in rat plasma and applied it to the pharmacokinetic comparison of these three anthraquinones after oral administration of RPD and rhubarb extract.

2. Investigations and results

2.1. Optimization of chromatography

To achieve symmetric peak shape as well as a short run time for the simultaneous analysis of the three compounds, the chromatographic conditions were optimized through trials and errors. The mobile phase systems of acetonitrile–water and methanol–water in various proportions were tested. The responses of analytes were higher with methanol–water as the mobile phase than those with acetonitrile–water. After we investigated the response of analytes with the mobile phase systems of methanol-0.1% formic acid, methanol- 0.1% acetic acid and methanol-3 mmol/L ammonium acetate, it was found that when methanol-3 mmol/L ammonium acetate was used in the mobile phase, better response protonated molecular ions of aloe-emodin, rhein and emodin was obtained. Meawhile, a methanol proportion in the mobile phase from 70% to 85% was considered in the followed experiment. In view of the separation, retention times and peak shapes of each analytes and I.S, 75% methanol was the best. Under the developed chromatographic conditions for simultaneous determination of the three compounds of RPD and rhubarb extract, all analytes were eluted rapidly within 11 min (Fig. 1B and C).

2.2. Optimization of mass spectrometry

The mass spectra and chemical structures of aloe-emodin, rhein, emodin and 1,8-dihydroxyanthraquinone in (internal standard) were shown in Fig. 2. A standard solution of the analytes and I.S were directly infused along with the mobile phase into the mass spectrometer with electrospray ionization (ESI) as the ionization source. The response observed in negative ionization mode was higher than that in positive ionization mode. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M\text{-}H]^-$ m/z 268.85, 282.8, 268.85, 239.95 for aloe-emodin, rhein, emodin and I.S, respectively. The main parameters of electrospray ionization including desolvation temperature, ESI source temperature, the flow rate of drying gas and voltage of the fragmentor in SIM were optimized by using infusion with a syringe pump.

2.3. Optimization of the extraction procedure

Sample preparation is a critical step for accurate and reliable LC–MS assays. In order to extract all the analytes and the IS with high recovery and no endogenous interference at the retention time, different methods of plasma sample preparation were investigated: protein precipitation and liquid–liquid extraction. For protein precipitation, two types of reagents (methanol, acetonitrile) were tried, but none of them could completely eliminate the interferences from the sample matrix. Consequently, liquid–liquid extraction was employed as it offered purer sample and low matrix effect. Different extraction solvents were investigated (acetic ether, diethyl ether, dichloromethane, methyl tert-butyl ether). Only ethyl acetate gave the best extraction efficiency for all the analytes and I.S.

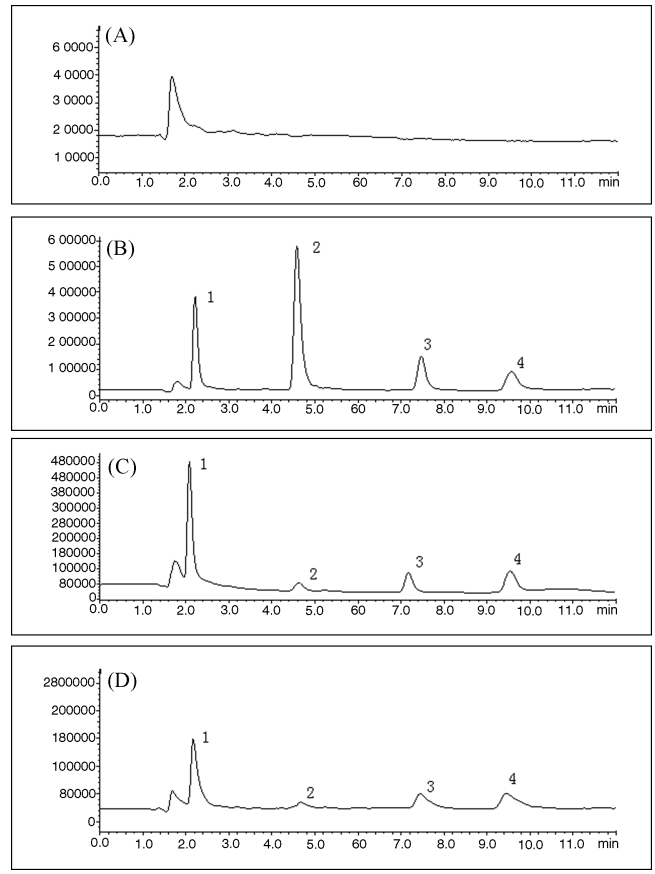

Fig. 1: Representative chromatograms of the three analytes in blank plasma (A), spiked standard solution in blank plasma (B), rat plasma sample after oral administration of Rhubarb extract (C) and rat plasma sample after oral administration of Rhubarb Peony Decoction (D). (1) rhein; (2) aloe-emodin; (3) emodin; (4) 1,8-dihydroxyanthraquinone (I.S.)

2.4. Method validation

2.4.1. Selectivity and specificity

The specificity was assessed by analyzing six different blank plasma samples whether there were interferences at their retention time for analytes and IS. The representative chromatograms in SIM of aloe-emodin, rhein, emodin and I.S are shown in Fig. 1. The retention time of aloe-emodin, rhein, emodin and I.S were found to be approximately 4.52, 2.18, 7.54, 9.50 min, respectively, indicating good resolution of the three compounds and the I.S. The results showed that there wsa no interference at the retention time of aloe-emodin, rhein, emodin and I.S.

2.4.2. Linearity and calibration curve

The regression equation, correlation coefficients and linearity ranges for the three analytes are shown in Table 1. The results showed that there was excellent correlation between the ratio of peak area and concentration for each compound within the test ranges. The LLOQs for aloe-emodin, rhein, emodin were 2.9 ng/mL, 24 ng/mL, 0.5 ng/mL, indicating that this method is sensitive for the quantitative evaluation of the three compounds. The residuals (difference between the back-calculated concentration of the calibration standard and its nominal concentration) were no more than $\pm$ 15% at all concentrations except at the LLOQ level with no more than 20% which demonstrated that the values were all within the acceptable range.

2.4.3. Precision and accuracy

The intra-day and inter-day precisions of this method were wihin 11%, and the accuracy ranged from -8.5 to 6.9%. All results are shown in Table 2.

Table 1: Regression equation, linear ranges and LLOQs of aloe-emodin, rhein, emodin

Analytes	Linear regression equation	Correlation coefficient (r)	Linear range (ng/mL)	LLOQ (ng/mL)
Aloe-emodin	$y = 0.0019x + 0.0131$	0.9901	2.9 – 2900	2.9
Rhein	$y = 0.0006x - 0.0059$	0.9981	24 – 24000	24
Emodin	$y = 0.035x - 0.00177$	0.9928	0.5 – 500	0.5

2.4.4. Recovery and matrix effect

The extraction recoveries and matrix effects of all analytes under the liquid–liquid extraction conditions with ethyl acetate from rat plasma were summarized in Table 3. The recovery of the QC samples of three concentration was in the range of 71.2–88.8% and IS was 82%.The matrix effects derived from QC samples was between 70.5% and 95.8% and IS was 85.7%. The analytes and IS did not exhibit any matrix effects in rat plasma.

2.4.5. Sample stability

The analytes remained stable and the concentrations were still within 15% deviation of the initial values. These results are shown in Table 4.

2.5. Pharmacokinetic study

The developed method was employed for pharmacokinetic investigation of aloe-emodin, rhein and emodin in rat plasma after oral administration of RPD and rhubarb extract. The mean plasma concentration–time profiles ($n = 6$) are presented in Fig. 3. The caculated pharmacokinetic parameters are shown in Table 5. On the whole, the concentration–time curves of each constituent were obviously different after administration of RPD and rhubarb extract, which implied that drug interactions occurred in this compound prescription formula. Although the main pharmacokinetic parameters after oral administration of rhubarb extract have been reported by Shia et al. (2009) this study provided pharmacokinetic parameters of aloe-emodin, rhein and emodin in the plasma from SD rats which were treated with RPD and rhubarb extract, respectively.

In comparison with rhubarb extract given alone, many pharmacokinetic parameters of aloe-emodin, rhein and emodin, including T_{max}, C_{max} and $T_{1/2}$ showed significant differences ($p < 0.05$) by unpaired Student's t-test after administration of RPD. After RPD, it took more time for rhein and emodin to reach the peak concentration of 2888.7 ± 678.78 ($p < 0.05$) ng/mL and 14.7 ± 1.59 ng/mL with the maximum time of 11.8 ± 1.40 ($p < 0.05$) min and 26.3 ± 8.87 ($p < 0.05$) min, $T_{1/2}$ of aloe-emodin and emodin was increased remarkably ($p < 0.01$). The $AUC_{0\text{-}t}$ of aloe-emodin, rhein and emodin has no significant changes between two decoctions (WPT and rhubarb extract), but compared to the $AUC_{0\text{-}t}$ value after oral administration of rhubarb extract, a smaller $AUC_{0\text{-}t}$ value (156597.4 ± 27331.57 ng min/ml) after oral administration of RPD were obtained.

The pharmacokinetic parameters of aloe-emodin, rhein and emodin summarized in Table 5 show that there were statistically significant differences in parameters including the T_{max}, C_{max}, $T_{1/2}$ between RPD and rhubarb extract. As shown in Table 5, the similar pharmacokinetic behavior of aloe-emodin and emodin could be tentatively explained based on their similar structures. After oral administration of RPD, a relatively long T_{max} of rhein and emodin and $T_{1/2}$ of aloe-emodin and emodin was obtained, indicating that it could postpone the absorption of rhein and emodin and elimination of aloe-emodin and emodin in rat plasma and might prolong the potency of them *in vivo*. In addition, the reduction of C_{max} of rhein suggested that RPD possibly prohibited the absorption of rhein. The compounds in *Natrii sulfas* might play an important role in the absorption and metabolism of rhein. Many case reports have shown (Wang et al. 2007; Fang et al. 2011; Xing et al., 2011; Wang et al. 2009) that rhubarb anthraquinone derivatives (AQs) have both therapeutic and toxic effect on liver and kidney. Moreover, rhein was one of the main possibly toxic ingredients (Fang et al. 2011; Wang et al. 2009). Thus, the pharmacokinetic parameters of aloe-emodin, rhein and emodin determined in the present study may imply that compound TCM prescriptions may improve therapeutic effect and reduce toxicity.

3. Discussion

RPD is a famous and widely used prescription documented in the Chinese Pharmacopoeia. In our study, most of calculated pharmacokinetic parameters of aloe-emodin, rhein and emodin exhibited statistically significant differences between RPD and rhubarb extract after oral administration decoctions in rats, respectively. This implied that the influence of the drug–drug interaction on the pharmacokinetics of aloe-emodin, rhein and emodin in RPD should be considered. In addition, the huge number of active ingredients in RPD might affect multiple targets and obtain synergistic effects, which enable TCM to treat compli-

Table 2: Precision and accuracy of analytes in rat plasma ($n = 6$)

Analytes	Spiked concentration	Intra-day			Inter-day		
	(ng/mL)	Measured (ng/mL)	RSD(%)	RE(%)	Measured (ng/mL)	RSD(%)	RE(%)
Aloe-emodin	5.8	6.1	6.6	5.2	6.2	8	6.9
	58.0	55.7	2.8	−4.0	56.0	5.9	−3.4
	1450	1373	2.6	−5.3	1358	3.8	−6.3
Rhein	48.0	50.0	7.5	4.2	50.2	10.5	4.6
	480.0	501.2	5.6	4.4	506.9	3.8	5.6
	12000	11424	4.9	−4.8	11593	3.0	−3.4
Emodin	1.00	1.02	5.9	2.0	1.06	7.6	6.0
	10.00	9.93	6.8	−0.70	9.15	8.8	−8.50
	250.0	246.5	4.7	−1.4	257.7	3.5	3.1

Table 3: Recovery and matrix effects of aloe-emodin, rhein, emodin and I.S ($n = 6$)

Analytes	Spiked concentration	Recovery		Matrix effect	
	(ng/mL)	Average (%)	RSD (%)	Average (%)	RSD (%)
Aloe-emodin	5.8	71.2	10.7	70.5	11.2
	58	73.1	8.1	75.6	5.3
	1450	80.3	5.7	88.1	7.1
Rhein	48	73.6	10.4	71.5	11.8
	480	74.5	11.7	74.1	8.5
	12000	88.8	5.2	95.8	6.7
Emodin	1	71.5	9.7	86.7	8.1
	10	75.2	8.4	73.3	9.2
	250	82.2	7.1	79.6	6.5
I.S.	16100	82	5.2	85.7	3.4

Table 4: Stability of aloe-emodin, rhein, emodin in rat plasma

Analytes	Theoretical concentration	Long-term stability storage		Room temperature		Freeze–thaw cycles	
	(ng/mL)	Measured (ng/mL)	RE(%)	Measured (ng/mL)	RE(%)	Measured (ng/mL)	RE(%)
Aloe-emodin	5.8	5.5	−5.2	5.3	−8.6	5.6	−3.4
	58.0	55.4	−4.5	53.9	−7.1	55.5	−4.3
	1450.0	1551.5	7.0	1584.9	9.3	1555.9	7.3
Rhein	48.0	48.9	1.9	42.9	−10.6	46.8	−2.5
	480.0	449.8	−6.3	451.2	−6	447.4	−6.8
	12000	12528	4.4	13428	11.9	12684	5.7
Emodin	1.00	1.07	7.0	0.90	−10.0	0.95	−5.0
	10.0	9.4	−6.0	10.5	5.0	9.5	−5.0
	250.0	262.3	4.9	234.8	−6.1	268.8	7.5

cated diseases. Therefore, this experiment has demonstrated that RPD could exhibit profit to pharmacological potency in terms of pharmacokinetics, indicating the reasonable compatibility of this recipe.

In summary, an LC-MS method was developed for the simultaneous pharmacokinetic determination of aloe-emodin, rhein and emodin in rat plasma. This method is sensitive, highly accurate, simple and appropriate to the bioanalytical requirements. Meanwhile, there were statistically significant differences in pharmacokinetic parameters of aloe-emodin, rhein and emodin including T_{max}, C_{max} and $T_{1/2}$ after oral administration of RPD and rhubarb extract, indicating that competition or inhibition between the chemical constituents in RPD could lead to prolonging in the elimination of aloe-emodin and emodin and the absorption of rhein and emodin. The C_{max} of rhein were decreased, suggesting that rhein during absorption and metabolism was possibly occurred interaction with the compounds of *Natrii sulfas* in RPD during absorption and metabolism, which resulted in diminishing of toxicity. The obtained knowledge can be used to evaluate impact of these differences on the efficiency and safety of Chinese compound prescriptions and further profound research is in process.

4. Experimental

4.1. Materials and reagents

Rhubarb, *Cortex Moutan*, *Semen Persicae*, *Benincasa Hispida Kernal*, and *Natrii sulfas* were purchased from Nanjing Municipal Hospital of Traditional Chinese Medicine (Nanjing, China) and authenticated by Prof. Liu from the Department of Pharmacognosy, the Nanjing University of Chinese Medicine (Nanjing, China). Standards of aloe-emodin (> 98%) and rhein (> 98%) and emodin (> 98%) and 1,8-dihydroxyanthraquinone (> 98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products as the internal standard (Beijing,China). Methanol, ethylacetate, acetic acid (HPLC grade) were obtained from Tedia Co.Ltd (Fairfield, OH, USA), and ammoniumacetate, sodium acetate, L(+)-Ascorbic acid, hydrochloric acid (analysis grade) were obtained from Nanjing Chemical Reagent Limited Company (Nanjing, China). Purified water was used in the experiments.

4.2. Instruments and analytical conditions

4.2.1. Instruments

The HPLC system consisted of a Shimadzu LC-20AD pump, an SIL-20A auto sampler, a CTO-20A column oven, a DGU-20A_3 degasser, and an SPD-M20A diode-array detector (Shimadzu, Kyoto, Japan). The mass spectrometer is an LC/MS-2020 single quadrupole equipped with an electrospray ionization (ESI) source interface (Shimadzu). A Speed Vacplus model vac-

Table 5: Pharmacokinetic parameters of aloe-emodin, rhein and emodin in male SD rats following oral administration of RPD and rhubarb extract

Parameter	Aloe-emodin		Rhein		Emodin	
	RPD	Rhubarb extract	RPD	Rhubarb extract	RPD	Rhubarb extract
T_{max}(min)	14.8 ± 10.31	12.0 ± 4.21	11.8 ± 1.40**	6.5 ± 2.41	26.3 ± 8.87*	11.2 ± 1.21
C_{max}(ng/ml)	34.5 ± 11.41	45.9 ± 12.58	2888.7 ± 678.78*	5486.9 ± 1371.44	14.7 ± 1.59	21.1 ± 4.03
$T_{1/2}$(min)	88.8 ± 29.70*	34.9 ± 7.42	25.9 ± 8.81	17.3 ± 5.37	142.7 ± 41.62*	67.2 ± 30.91
$AUC_{(0\text{-}t)}$(ng·min/ml)	2663.4 ± 666.18	3082 ± 1259.38	145463.7 ± 29258.82	238062.1 ± 29808.39	1443.9 ± 446.49	2299.3 ± 768.28

Mean ± S.D., $n = 6$. * $p < 0.05$, ** $p < 0.01$ versus oral administration of rhubarb extract

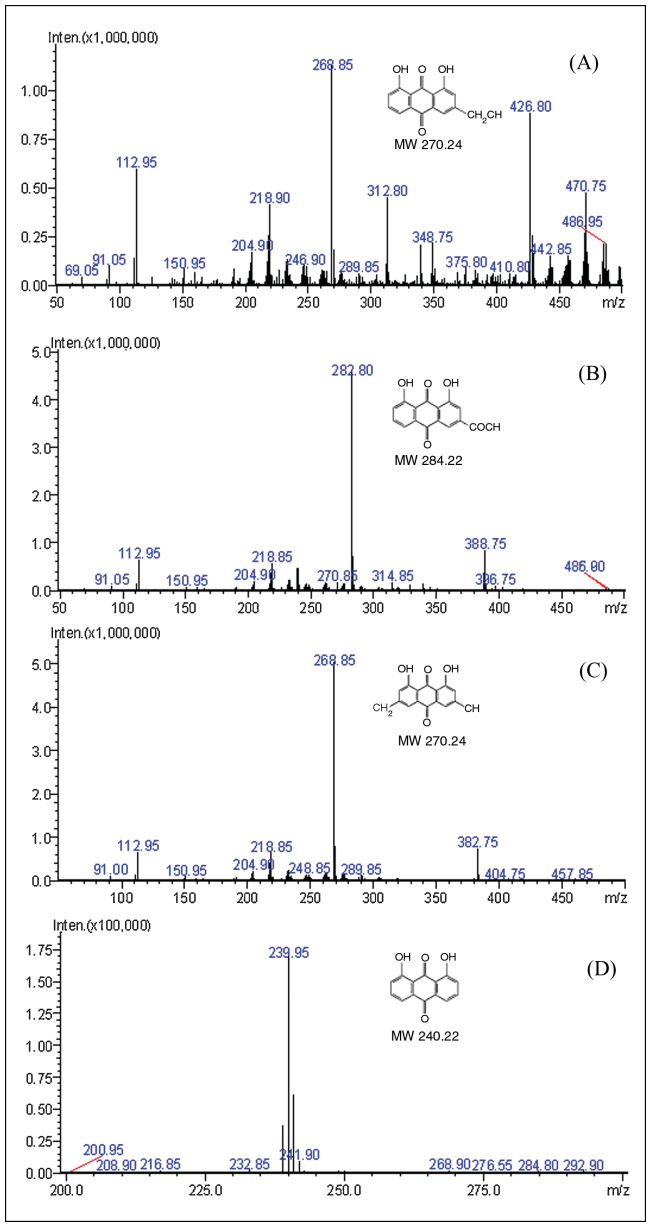

Fig. 2: Protonated molecular ions in mass spectra for aloe-emodin (A), rhein (B), emodin (C) and internal standard 1,8-dihydroxyanthraquinone (D)

uum drier (Buchi, Sweden) was used in the preparation of samples. Data were processed using LC/MS Solution software (Shimadzu, Kyoto, Japan).

4.2.2. *Chromatographic conditions*

Separation was performed on a kromsail C_{18} column (150mm × 4.6 mm, i.d.: 5 μm) with 3 mmol/L ammonium acetate – methanol (25:75, v/v) as the mobile phase. The column temperature was 40 °C at a flow rate of 1 mL min^{-1} and the injection volume was 20 μL. Detection wavelength was set at 254 nm.

4.2.3. *Mass spectrometry conditions*

The mass spectrometer was operated using an electrospray source configured to the negative ion mode and quantification was performed using select-ion-monitoring (SIM) mode. The aloe-emodin, rhein, emodin and 1,8-dihydroxyanthraquinone ions were recorded as m/z 268.85, m/z 282.8, m/z 268.85, m/z 239.95 for quantification respectively. The typical ion source parameters were as follows: ESI probe temperature 350 °C, CDL temperature 250 °C, heat block temperature 200 °C, ESI probe voltage 4.5 kV, detector voltage 1.3 kV, DL voltage 50 V, Q-array DC voltage 50 V, Q-array RF voltage 120 V, drying gas flow 8 L/min and nebulizing gas flow 1.5 L/min. Tuning of the mass spectrometer was accomplished with the help of the autotuning function of LCMS Solution software using the tuning standard solution (polypropylene glycol). Optimization and calibration of the mass spectrometer were achieved with autotuning.

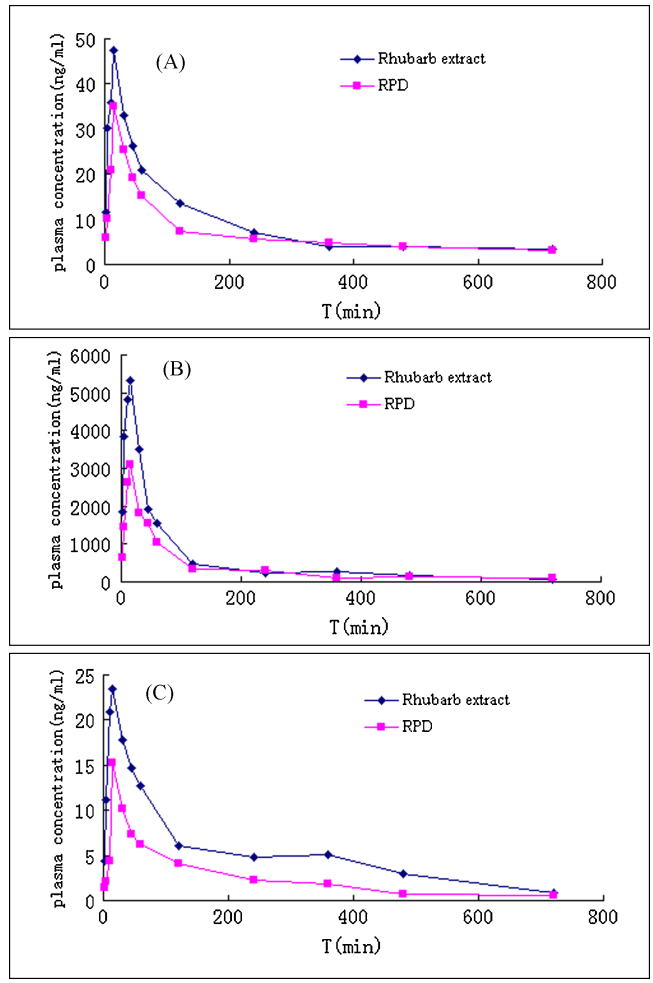

Fig. 3: Mean concentration–time curves in rat plasma after oral administration of RPD and Rhubarb extract. (A) aloe-emodin; (B) rhein and (C) emodin

4.3. *Preparation of RPD and Rhubarb extract*

Rhubarb 12 g, *Cortex Moutan* 9 g, *Semen Persicae* 12 g, *Benincasa Hispida Kernal* 30 g, and *Natrii sulfas* 9 g were mixed and immersed in 600 mL distilled water for 30 min and then decocted twice with water (400 mL and 200 mL) for 1 h. The extracted solution was concentrated to 100 mL to obtain RPD. Rhubarb (54 g) was also immersed in 600 mL distilled water for 30 min and then decocted with water (600 mL and 300 mL) for 1 h and the extracted solution was concentrated to 300 mL to obtain Rhubarb extract.

To calculate the dose to be administered, the contents of aloe-emodin, rhein and emodin in RPD and rhubarb extract were determined by HPLC with external standard method. About 1 mL of extracts were diluted 10 times with methanol. After centrifuging at 4500 rpm for 10 min, 20 μL supernatant was injected into the HPLC system after filtration through a 0.45 μm Millipore filter. Aloe-emodin, rhein and emodin were separated using a kromsail C_{18} column (250 mm × 4.6 mm, i.d.: 5 μm). The mobile phase consisted of 0.1% aqueous acetic acid (A) and acetonitrile (B) using an gradient elution of 30–75% (v/v) B within 0–30 min and then maintaining 75% B within 30–40 min. The effluent was delivered at 1 mL min^{-1} during the gradient program. UV detection wavelength was at 254 nm and the column temperature was 30 °C.

4.4. *Preparation of calibration standards, quality control and internal standard*

Stock solutions were prepared by dissolving various accurate amounts of standards in methanol solution: 388.8 μg/mL of aloe-emodin and 240 μg/mL of rhein and 200 μg/mL of emodin. The internal standard stock solution was prepared by dissolving 1,8-dihydroxyanthraquinonein methanol to 161 μg/mL. An appropriate volume of each stock solution was mixed together. The mixture was subsequently diluted serially to prepare the reference working solutions.

The analytical standard and quality control (QC) samples were prepared as the following: 20 μL of standard working solution was evaporated to dryness by a gentle stream of nitrogen, and then 100 μL of blank rat plasma was added. The final calibration concentrations of aloe-emodin (2900, 1450, 290, 58, 11.6, 5.8, 2.9 ng/mL), rhein (24000, 12000, 2400, 480, 96, 48, 24 ng/mL) and emodin (500, 250, 50, 10, 2, 1, 0.5 ng/mL) were obtained. Quality control (QC) samples were independently prepared in the same way at 1450, 58, 5.8 ng/mL for aloe-emodin, 12000, 480, 48 ng/mL for rhein, 250, 10, 1 ng/mL for emodin. The standards and quality controls were extracted on each analysis day with he same procedures for plasma samples as described below.

4.5. Sample preparation

Rat plasma (100 μL) and 10 μL of IS solution (1,8-dihydroxyanthraquinone, 161 μg/mL) were placed in an eppendorf tube, followed by the addition of 50 μL of pH 5 acetate buffer and 50 μL of ascorbic acid (100 mg/ml). The mixture was acidified with 50 μL of 0.1 N HCl. A volume of 800 μL ethyl acetate was then added and the samples were extracted by vortex-mixing for 3 min. The aqueous and the organic layers were separated by centrifugation at 4000 rpm for 5 min, and the organic layer was transferred to another Eppendorf tube and dried under a flow of nitrogen gas at 37 °C. The residue was reconstituted in 100 μL of mobile phase, and centrifuged (12,000 rpm for 5 min). The supernatant was transferred to an auto-sampler vial with a vial insert (LVI, 150 μL, Waters) and a 10 μL aliquot was injected onto the LC–MS system for analysis.

4.6. Method validation

The method was validated in terms of specificity, calibration curve, sensitivity, matrix effect, accuracy, precision and stability according to the USA Food and Drug Administration (FDA) bioanalytical method validation guidance (FDA, CDER, 2001).

4.6.1. Selectivity and specificity

The specificity of the method was tested by comparing the chromatograms of six individual blank rat plasma samples, plasma samples spiked with the analytes and IS, and plasma samples after an oral dose. Blank rat plasma samples were analysed for endogenous interference, followed by spiking with IS for the interference of IS.

4.6.2. Linearity and lower limits of quantification (LLOQ)

Calibration curves were constructed with the peak area ratios of each analyte to internal standard. Versus plasma concentrations using a $1/c^2$ weighted linear least-squares regression model. The lower limit of quantification (LLOQ) were the lowest concentrations, which could be quantitatively determined with precision (relative standarddeviation, R.S.D.) and accuracy (relative error, R.E.) were less than or equal to 20%.

4.6.3. Precision and accuracy

The accuracy and precision were assessed by analyzing QC samples in six replicates at low, medium and high concentrations on the same day and on three consecutive days, respectively. The precision was expressed as R.S.D. and the accuracy as R.E.

4.6.4. Recovery and matrix effect

The extraction efficiency of the three analytes was determined by analyzing six replicates of QC samples at three concentration levels. The extraction recovery was calculated by comparing the peak areas obtained from extracted spiked samples with those of samples spiked post-extraction at corresponding concentrations. The matrix effects were evaluated by comparing the peak areas obtained from samples where the extracted matrix was spiked with standard solutions to those obtained from the pure reference standards solutions at the same concentration.

4.6.5. Stability experiments

The stability of analytes in rat plasma was assessed by analyzing three replicates of low, mid and high QC samples during the sample storage and processing procedures: long-term stability at − 40 °C for 14 days, post-preparative stability at room temperature for 8 h, and three freeze–thaw cycles. Stability of the analytes was checked by comparing measured results with those of freshly prepared samples of the same concentration.

4.7. Pharmacokinetic study

Sprague-Dawley male rats ($n = 6$), weighing 200–230 g, were obtained from Silaike Lab Animal Ltd. (Shanghai, China). The experimental protocols were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine. The rats were kept in a controlled environment at 23 °C and 55% relative humidity under a 12 h dark–light cycle, with free access to the standard laboratory food and water until 12 h prior to experiments. The rats were randomized into two groups, 6 in each group: animals in group A were administered orally with dose of 10 mL/kg RPD, and animals in group B were administered orally with dose of 10 mL/kg rhubarb extract. Blood samples (0.5 mL) were collected from the suborbital vein into heparinized tubes at 0, 2, 5, 10, 15, 30, 45, 60, 120, 240, 360, 480 and 720 min after drug administration. The rats had free access to water during the experiment. All blood samples were immediately centrifuged for 5 min at 12,000 rpm, and the plasma was transferred into clean tubes and stored at − 20 °C until analysis. Plasma samples were spiked with IS, and processed as described in Section 2.6 above.

Pharmacokinetic parameters were calculated using 3P97 (Practical Pharmacokinetics Program Version 1.0), the maximum plasma concentration (C_{max}) and the time to reach it (T_{max}) were obtained from the experimental data. The elimination rate constant (ke) was calculated as the slope of the linear regression of log-transformed concentration values versus time date using the terminal four points. The eliminate half-live ($T_{1/2}$) was calculated as 0.693/ke. The area under the plasma concentration–time curve from time zero to the final measurable point at 720 min (AUC_{0-720}) was estimated by linear trapezoidal rule. An unpaired Student's t-test was used to compare the differences in pharmacokinetic parameters between the two groups. All results were expressed as arithmetic mean ± standard deviation (S.D.).

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