A RAPID UPLC-MS/MS METHOD FOR SIMULTANEOUS DETERMINATION OF FIVE ANTHRAQUINONES IN RAT PLASMA: APPLICATION TO A PHARMACOKINETIC STUDY

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ABSTRACT

Introduction*: A rapid and simple UPLC-MS/MS method was established and validated for simultaneous determination of aloe-emodin, rhein, emodin, chrysophanol and physcion in rat plasma.*

Materials and methods*: After sample preparation by a simple protein precipitation, the mass detection was conducted in multiple reaction monitoring using an API 6500 triple quadruple in negative-ion mode with an electrospray ionization interface. Gradient elution was performed with a mobile phase composed of acetonitrile and water containing 0.1% formic acid at a flow rate of 0.6 mL/min.*

Results*: The retention time of emodin, aloe-emodin, chrysophanol, physcion, rhein and IS was at 1.54, 1.04, 1.67, 1.69, 1.09 and 1.59 min, respectively.*

Conclusion*: This method exhibited acceptable precision, accuracy, recoveries, matrix effect and carry-over effect, which was superior to previous methods in analysis time and sample throughput.*

Keywords*: Rhubarb anthraquinones; rat plasma; pharmacokinetics; UPLC-MS/MS.*

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Introduction

As the main active constituents of rhubarb, anthraquinones (AQs), including aloe-emodin, rhein, emodin, chrysophanol, physcion and their glycosides, exhibit various biological and pharmacological activities. In our previous study, a reliable high performance liquid chromatography (HPLC) method was established and validated for simultaneous determination of rhein, aloe-emodin, emodin, physcion and chrysophanol in rat plasma[(1)]. Unfortunately, this HPLC method was rather time-consuming and insensitive, with a total run time over 30 min, and only four AQs except physcion was determined in rat plasma, as well as some time points of chrysophanol were undetectable in plasma. Therefore, a rapid and sensitive method that allows simultaneous and accurate quantification of low concentrations of five AQs in biological samples is of great value in their pharmacokinetic study.

To date, abundant studies about determining all or some of rhubarb AQs in biological fluids after orally administered rhubarb, rhubarb extract or its compound formula have been reported. Most frequently used methods determining rhubarb AQs were HPLC and liquid chromatography tandem mass spectrometry[(2-15)].

However, these previously existing methods suffered from a long running time[(2-4)], to our best knowledge, the shortest retention time of determination of aloe-emodine, rhein, emodin, chryso-

phanol and physcion was at 1.6 min(5), 1.8 min(5), 2.4 min(6), 4.01 min(7) and 3.76 min(7), respectively. And these methods also had a time-consuming and expensive extraction procedures that were used with liquid-liquid extraction[8-10] and solid-phase extraction[11], low detection sensitivity[11-13] and required large volumes of plasma[14,15].

In present study, we developed a rapid, simple and highly sensitive method to simultaneously determine all five rhubarb AQs in rat plasma using UPLC-MS/MS. This method was more rapid and convenient with retention time less than 1.7 min and demonstrated a higher sensitivity with the limit of quantification (LOQ) of 1 ng/mL for aloe-emodin, rhein, chrysophanol, physcion and 0.3 ng/mL for emodin. To our best knowledge, 1.7 min was shorter than the methods have been published concerning the retention time of simultaneous determination of five AQs in plasma. Also a simple protein precipitation procedure with acetonitrile was employed to extract the analytes from plasma, and the supernatant was immediately injected into UPLC-MS/MS. So the pre-processing of rat plasma presented shorter time-consuming and less matrix interference than the methods in references. This validated method was successfully applied to study the preclinical pharmacokinetic characteristics of five AQs in rats.

Materials and methods

Chemicals and reagents

The standard chemicals include aloe-emodin, rhein, emodin, chrysophanol, physcion and danthron (internal standard, IS) with the purity of over 98% by HPLC were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile of HPLC-grade was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid of HPLC was supplied by Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Other chemicals and solvents were of analytical reagent grade and obtained from Tianjin Chemical Reagent Company (Tianjin, China).

UPLC-MS/MS conditions

The separation was carried out on a BEH C18 column (2.1×50 mm, 1.7 μm; Waters Co., Ltd., Milford, MA, USA). The column temperature was maintained at 60°C. Gradient elution was performed with a mobile phase composed of acetonitrile and water containing 0.1% formic acid at a flow rate of 0.6 mL/min. Concretely, the initial mobile phase composition was 20% acetonitrile during 0-0.2 min, and a linear gradient was applied to reach a composition of 70% after 0.6 min, and maintained for 0.3 min, and reached 90% after 0.05 min, and maintained 0.65 min then returned to initial conditions within 0.01 min and balanced for 0.69 min. The total analysis time was 2.5 min. Five microliters of sample solution or standard solution was injected into system for analysis.

The mass analysis and detection was conducted using an API 6500 triple quadruple (AB Sciex, Foster city, California, USA) with an electrospray ionization (ESI) interface. The ESI source was operated in the negative ionization mode. The optimum MS parameters were as following: ionspray voltage: -4500 V; temperature: 550°C; ion source gas 1: 60 psi; ion source gas 2: 60 psi; curtain gas: 30 psi; declustering potential (DP) and collision energy (CE) showed in Table 1. Quantitative analysis was performed by monitoring [M-H]- for analytes in multiple reaction monitoring (MRM) mode. The fragmentation transitions were m/z 269.2→225.0 for emodin, m/z 269.2→239.8 for aloe-emodine, m/z 253.1→225.0 for chrysophanol, m/z 283.2→239.8 for physcion, m/z 283.2→238.8 for rhein and m/z 239.0→210.8 for IS, respectively. The retention time of emodin, aloe-emodin, chrysophanol, physcion, rhein and IS was at 1.54, 1.04, 1.67, 1.69, 1.09 and 1.59 min, respectively.

Analytes	Ion sourse	Monitoring ion	Parent ion	Daughter ion	DP (V)	CE (V)	t_R (min)
Emodin	ESI-	[M-H]-	269.2	225	-100	-37	1.54
Aloe-emodin	ESI-	[M-H]-	269.2	239.8	-75	-31	1.04
Chrysophanol	ESI-	[M-H]-	253.1	225	-103	-38	1.67
Physcion	ESI-	[M-H]-	283.2	239.8	-81	-37	1.69
Rhein	ESI-	[M-H]-	283.2	238.8	-48	-19	1.09
IS	ESI-	[M-H]-	239	210.8	-88	-39	1.59

Table 1: summary of optimized MRM parameters of the five analytes and IS.

Standard solutions, calibration standards and quality control (QC) sample

Stock solutions (1.00 mg/mL) of aloe-emodin, rhein, emodin, chrysophanol, physcion and IS were prepared in methanol. Stock solutions of the analytes were then mixed and serially diluted with methanol to yield final concentrations of 0.01-0.64 μg/mL for aloe-emodin, 0.01-10 μg/mL for rhein, 0.01-2.5 μg/mL for chrysophanol, 0.01-0.64 μg/mL

for physcion and 0.003-0.32 μg/mL for emodin. A 10 μg/mL IS working solution was also prepared by dilution of the stock solution with methanol. All the solutions were kept at 4°C and brought to room temperature before use.

Mixed calibration standards were prepared by spiking 90 μL aliquots of blank plasma with 10 μL of the appropriate standard solutions to obtain final concentrations of 1, 2, 4, 8, 16, 32 and 64 ng/mL for aloe-emodin; 1, 2, 10, 50, 100, 500 and 1000 ng/mL for rhein; 1, 2, 5, 25, 50, 100 and 250 ng/mL for chrysophanol; 1, 2, 4, 8, 16, 32 and 64 ng/mL for physcion and 0.3, 1, 2, 4, 8, 16 and 32 ng/mL for emodin. The low, medium and high quality control (QC) samples (2, 8 and 16 ng/mL for aloe-emodin; 2, 100 and 500 ng/mL for rhein; 2, 25 and 100 ng/mL for chrysophanol; 2, 8 and 16 ng/mL for physcion and 1, 8 and 16 ng/mL for emodin) were prepared independently in the same way.

Plasma sample preparation

AQs were extracted by a direct protein precipitation procedure. Firstly, an aliquot of 20 μL of plasma sample was added with 60 μL acetonitrile which contains of IS (20 ng/mL) for protein precipitation, and then the mixture was vortexed at 1,500 rpm for 2 min, and centrifuged at 5,800 rpm for 10 min. An aliquot of 5 μL of supernatant was injected into the UPLC-MS/MS system for analysis.

Method validation

The method was fully validated in accordance with the guildlines of US-FDA document with respect to specificity, linearity, precision and accuracy, recovery, matrix effect, stability and carry-over effect[16]. Specificity of the method was assessed by analyzing the chromatograms of blank rat plasma, blank rat plasma spiked with mixed standard solution containing five AQs and IS, and plasma samples which were obtained after oral administration of rhubarb on a base of MRM responses and retention times of analytes and IS. Linearity was assessed by analyzing calibration standards at seven concentration level over three consecutive days. Calibration curve was prepared by plotting the peak area ratio (y) of analytes to IS against the corresponding nominal concentration (x) by weighted (1/x2) least-squares linear regression. The limit of detection (LOD) and LOQ were determined using the signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively. The intra-day precision and accuracy were evaluated by six replicates of QC samples at the concentrations of low-, medium, high- on the same day, while the inter-day precision and accuracy were assessed on three consecutive days. The precision and accuracy were expressed as relative standard deviation (RSD) and relative error (RE), respectively, which all required to be less than 15%. Recovery was calculated by determination of QC samples at low, medium and high concentrations in six replicates. The assay recovery was expressed as the ratio of the measured value to the true value.

The extraction recovery was evaluated by samples pre-spiked with standard solutions and samples post-spiked with standard solutions. The recovery of IS was determined at a single concentration (20ng/mL) using QC samples at medium concentration. Considering the enhancement or suppression effect resulted from endogenous material in ESI-MS/MS quantification, the matrix effect should be investigated by comparing analytes standard peak areas dissolved with blank matrix extracts against those dissolved with acetonitrile at the same level in six replicates. Stability of five analytes in rat plasma was assessed in six replicates of the low, medium and high QC samples by analyzing plasma samples under the following sample processing and storage stage: 12 h at room temperature; after three freeze-thaw cycles at -80°C; after long-term storage at -80°C for 2 weeks; and the samples in autosampler vials at 4°C for 12 h. The highest concentration of sample and the blank sample were injected into the system in order, respectively. The carry-over effect is the ratio of the peak area of analytes in the blank sample and the LOQ, and the ratio was needed to be within 20%.

Application to a pharmacokinetic study

Healthy Sprague-Dawley (SD) rats weighing 180-240g were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (License No. SCXK2012-0001) used to study the pharmacokinetics of five AQs. All animal experiments were performed in strict accordance with the Guidelines for the Care and Use of Laboratory Animals, as adopted and promulgated by the Ministry of Science and Technology of China. The rats underwent a 12 h fast with water ad libitum prior to study. Two hundred microliters of plasma samples were taken into a heparinized blood collection tube at 0.250, 0.5, 1, 2, 4, 6, 12, 14, 24, 48 and 72 h after orally administered rhubarb total free anthraquinones oral colon-specific drug delivery

granules (RTFA-OCDD-GN, 0.3070 g/kg, containing 4.51 mg/kg of aloe-emodin, 9.11 mg/kg of rhein, 4.31 mg/kg of emodin, 11.25 mg/kg of chrysophanol and 1.40 mg/kg of physcion). Then the samples were immediately centrifuged separated at 3,000 rpm for 10min at 4°C, and the supernatants were decanted and preserved at -80°C until analysis. All the pharmacokinetic parameters were calculated by drug and statistics (DAS) pharmacokinetic program (version 2.0, Chinese Pharmacological Society).

Results

Method validation

All five ingredients exhibited good linearity with r≥0.9992 over the concentration range of 1-64 ng/mL for aloe-emodin, 1-1000 ng/mL for rhein, 1-250 ng/mL for chrysophanol, 1-64 ng/mL for physcion and 0.3-32 ng/mL for emodin. The LOQ was 0.3 ng/mL for emodin and 1ng/mL for other four AQs. Intra- and inter-day precision were less than 8.4%, and accuracy was within ±7.6%, indicating the overall reproducibility of the method. The extraction recoveries were all above 89.5% for five AQs and IS with all RSD values less than 15%. The matrix effects of all analytes ranged from 89.1% to 97.1% at the three QC concentrations. The results indicated that ion suppression or enhancement from blank plasma matrix was negligible under the above-described conditions. Results of stability studies under the various storage conditions tested were summarized in (Table 2), which demonstrated that all analytes were stable in rat plasma. The carry-over effect was 2.86%, 2.89% and 3.45% in the three parallel experiments, respectively, and there was no residue for IS.

Application in a pharmacokinetic study

This UPLC-MS/MS method was successfully applied to a pharmacokinetic study of aloe-emodin, rhein, emodin, chrysophanol and physcion after a single oral dose of RTFA-OCDD-GN. The mean plasma concentration-time profiles were shown in Figure 1. The main pharmacokinetic parameters of rhubarb AQs were calculated using non-compartment mode and presented in Table 3.

Discussion

Optimization of UPLC-MS/MS conditions

The response values of five AQs and IS were significantly higher in negative-ion mode than in positive-ion mode. Through continuous adjustment of the chromatographic separation conditions, it was found that acetonitrile gave better resolution, lower back pressure and shorter analysis time than methanol. And the detection sensitivity, peak symmetry and ionization efficiency of analytes were significantly improved by adding 0.1% formic acid in aqueous phase. Meanwhile, gradient elution was adopted to produce higher ionization efficiency, better peak shape, exerted fast elution and shortest possible running time. The resolution and tailing factor obtained for emodin and physcion showed some improvement with the increasing of column temperature. Therefore, the column temperature of 60°C was preferred. At this column temperature and flow rate of 0.6 mL/min, a satisfactory and rapid separation of multicomponents was completed within only 1.69 min per sample with symmetric peak shape and high sensitivity. This method meets the requirement for a relatively higher sample throughput in bioanalysis.

Optimization of sample preparation

Sample preparation is an important step for accurate and reliable UPLC-MS/MS analysis. Although the high performance of SPE, it was time-consuming and expensive for the preparation of abundant biological samples. Meanwhile, LLE with various organic solvents were initially tested, which gave rise to severe matrix effects for rhein and emodin. And it was observed that the extraction recoveries of physcion were significantly decreased. Hence, direct protein precipitation with acetonitrile was selected to carry out the quantitative analysis in this work, which has the advantages of simplicity, convenience and time-saving.

Conclusion

A rapid, sensitive and simple UPLC-MS/MS method has been developed, optimized and validated for the simultaneous determination of five AQs in rat plasma in this study. Compared with the existing analytical methods, this method proved to be superior with respect to single run time and sample preparation. The time required for detecting individual sample containing five AQs was only 1.7 min, which meant that the method meets the requirement of high sample throughput in bioanalysis. The simplified sample preparation procedure using acetonitrile extraction was time-saving and

Analytes	Concentration	25°C for 12h		Three freeze-thaw cycles at -80°C		-80°C for 14 days		In autosampler vials for 12h	
	(ng/mL)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)
Aloe-emodin	2	-0.6	5.6	4.4	1.8	0.8	5.4	-5.1	4.5
	8	-1.4	2.6	2.4	-3.9	-6.9	3.4	-6.5	2.1
	16	1.5	2.1	2.3	6.1	2.4	2.5	4.5	1.9
Rhein	2	-0.4	5.4	4.2	1.6	0.6	5.4	-5.9	4.3
	100	-1.2	2.4	2.2	-3.8	-6.8	3.3	-5.4	2.3
	500	1.3	1.8	2.1	5.9	2.2	2.4	4.3	2.2
Emodin	1	6.3	3.8	-2.1	3.7	5.7	5.1	6.8	0.4
	8	3.9	5.1	2.5	2.7	1.9	2.5	-0.7	0.7
	16	-1.6	4.3	-5.1	1.6	-4.9	0.7	5.4	1.5
Chrysophanol	2	-0.4	5.5	4.3	1.7	0.7	5.4	-5.6	4.1
	25	-1.4	2.5	2.3	-3.9	-6.9	3.3	-5.5	2.1
	100	1.4	1.9	2.2	5.9	2.3	2.3	4.1	2.2
Physcion	2	-0.2	4.8	3.4	1.2	0.4	4.4	-5.2	3.7
	8	-1.1	2.1	2.2	-3.2	-5.8	3.1	-4.4	2.2
	16	1.1	1.4	1.9	5.1	1.8	1.9	3.7	1.9

Tab. 2: Stability of the five analytes in rat plasma under different storage conditions (n=3).

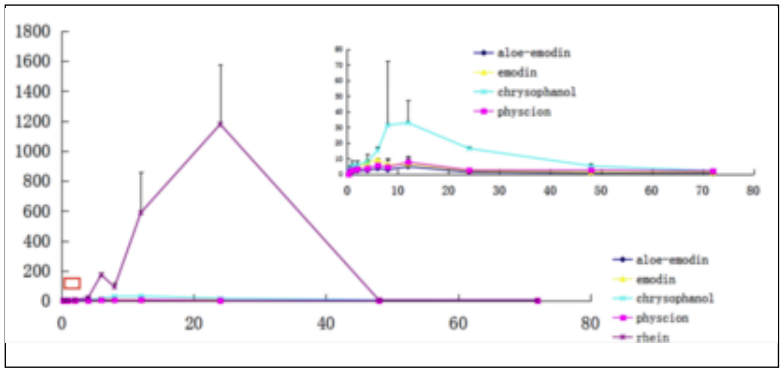

Fig. 1: Figure 1: mean plasma concentration-time profiles of five AQs after orally administered RTFA-OCDD-GN (mean ± SD,n = 6).

Parameters	Aloe-emodin	Rhein	Emodin	Chrysophanol	Physcion
AUC_0-t(μg/L*h)	119.201±34.2043	26756.7915±9172.4032	202.9435±61.4030	874.327±295.8096	245.924±40.1057
AUC_0-∞(μg/L*h)	128.902±41.5821	26791.348±9174.8986	227.2265±83.0377	932.2875±336.3035	360.748±29.7310
C_{max}(μg/L)	5.945±1.4920	1178.5±398.1011	9.513±0.4596	41.55±26.5165	7.865±3.5850
T_{max}(h)	9±4.2426	24±0	6±0	10±2.8284	12±0
$t_{1/2z}$(h)	17.452±3.7505	12.1605±1.0387	13.952±8.337	17.449±3.7576	37.2945±7.6714
$V_{z/F}$(L/kg)	897.513±100.1348	6.249±1.6306	19.612±2.8751	312.6055±47.2878	210.854±60.2413
$C_{Lz/F}$(L/h/kg)	36.918±11.9091	0.3615±0.1237	20.3425±7.4338	12.917±4.6598	3.8855±0.3203
MRT_0-t(h)	22.755±1.0027	21.625±0.0948	22.1695±0.6413	22.6195±1.9877	29.665±3.0024
MRT_0-∞(h)	27.9805±2.6311	21.7165±0.1181	29.7655±5.6788	27.0365±0.1181	60.7105±10.8944

Tab. 3: The main pharmacokinetic parameters of the five analytes in rat plasma after oral administration of rhubarb (mean±SD, n=6).

provided clean sample, which is also eco-friendly for its low consumption of organic solvents as compared to other analytical techniques. All the validation data meet the requirements, and the method was highly sensitive with LOQ of 1ng/mL for aloe-emodin, rhein, chrysophanol and physcion, and 0.3ng/mL for emodin using only 20 μL rat plasma. Overall, the validated method showed greater simplicity and efficiency for analyzing lots of biosamples, and has also been successfully applied to the pharmacokinetic study of rhubarb AQs in rats after oral administration of RTFA-OCDD-GN.

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