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Variation in quality of the different prepared formulation granules of rhubarb was evaluated by quantitative analysis of multicomponents with single marker

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Abstract: Rhei Radix et Rhizoma formula granule (RRFG) , Winy Rhei Radix et Rhizoma formula granule(WRFG) and Rhubarb charcoal formula granules (RCFG) are the three most popular and effective formula granules of rhubarb in China and anthraquinone components are their main active ingredients. In order to discuss the difference in anthraquinone components of these three drugs, a simultaneous quantitative analysis method of multicomponents by single-marker (QAMS) was developed. Emodin was chosed as the internal reference standard, the relative correction factors (RCFs) of aloe-emodin, rhein, chrysophanol and physcion were established and the contents of the four components were calculated based on the RCFs, respectively. Meanwhile, the contents of these components was determined by external standard method (ESM) and compared with QAMS to verify its rationality, feasibility and repeatability. The results showed that there were no significant difference between QAMS and ESM (RSDs≤2.26%). The contents of anthraquinone components showed a wide variation in these three drugs. All of 5 components were higher in RRFG than that in the others and RCFG had the lowest content. This method was successfully applied for the evaluation on difference of these drugs and the wide variation in anthraquinone components indicated there were different pharmacodynamic basis.

Keywords: QAMS, rhubarb, formulation granules, anthraquinone components.

INTRODUCTION

Traditional Chinese Medicine（TCM）formula granules, also known as Single Concentrated Chinese Herbal Tea (SCCHT) or scientific Chinese medicine, it was manufactured from decoction pieces to a certain proportion of powder or granular by using modern manufacturing process, extracted with water or ethanol, concentrated at low temperature, spray dried and then mixed with dextrin and other excipients in accordance with a certain proportion of powder or granular products made of concentrated. TCM formula granules have the characteristics of accurate dosage, hygiene, instant serviceable and convenient storage, which had changed the raw material pattern of Chinese herbal medicine mode and overcomed the problems such as dosage inaccuracy, pollution and some other inconvenience (Yin *et al.*, 2017).

In recent years, the Chinese Materia Medica (CMM) has been very popular both at home and abroad (Roy, 2015) and also the decoction pieces and TCM formula granules are being used more frequently (Rudolf, 2014). However, the quality problems of TCM formula granules are also increasingly exposed. Some manufacturers use much more excipients than need to reduce the production costs illegally. So that the content of the main components of the same formula granules produced by different manufacturers differs greatly (Tang *et al.*, 2018). Therefore, "Rules for the Administration of Tradintional Chinese Medicine Granules (Draft for Comment)" issued by the State Food and Drug Administration (SFDA) of China in December 2015, had aroused widespread concern in the pharmaceutical industry. Meanwhile, the pharmaceutical quality is getting more and more attention, because reliable quality standards can ensure them to be adopted for the euramerican and other regional pharmacopoeia.

Rhubarb (Dahuang in Chinese), officially listed in Chinese Pharmacopoeia (Vol. I) (National Pharmacopoeia Committee of China, 2020), Japanese Pharmacopoeia (17^{th} edn) (Pharmaceutical and Food Safety Bureau, 2016) and European Pharmacopoeia (8.0^{th} edn) (European Pharmacopeia Convention, 2013), is a well-known herbal medicinal plant and has been used for thousands years in China. It has been recognized for many centuries in traditional medicine for its multiple pharmacological actions include cardioprotective actions (Evans, 2020), protecting diabetic nephropathy (Li *et al.*, 2017; Zeng *et al.*, 2021), anti-inflammatory (Hu *et al.*, 2021), anti-cancer (Semwal *et al.*, 2020; Trinh *et al.*, 2019), antimicrobial activity and cytotoxic properties (Keser *et al.*, 2020).

According to the original herbs materials, Rhubarb

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formula granules included Rhei Radix et Rhizoma formula granule (RRFG, processed directly from original herbs materials), Winy Rhei Radix et Rhizoma formula granule (WRFG, processed from herbs, which was cooked with Yellow rice wine) and Rhubarb charcoal formula granule (RCFG, processed from herbs, which was fried into charcoal). However, in practical applications, the three drugs are easily confused and doctors often inaccurately prescribe. Therefore, in this paper, we investigated these three rhubarb formula granules and the application of QAMS was used to determine the components of several rhubarb anthraquinone in different processed products.

For the good analytical performance and wide accessibility, high performance liquid chromatography (HPLC) was usually used in quality control of herbal products and chemical drugs (Satyajit and Lutfun, 2015; Manirul and Elaref, 2017). QAMS is a new analysis method in multi-component measurement and it has been reported in many literatures now (Zhang *et al.*, 2017; Cui *et al.*, 2019; Li *et al.*, 2019).

It is well known that these components and their glycosides are the main bioactive ingredients in rhubarb (National Pharmacopoeia Committee of China, 2020). In this paper, a simple HPLC method was established for determination of anthraquinone components from RRFG, WRFG and RCFG. This method was successfully applied for quantitation and identification of 5 natural anthraquinones, including emodin, aloe-emodin, rhein, chrysophanol and physcion (fig. 1). In addition, emodin was chosen as the internal reference standard to develop the QAMS method, because it is inexpensive and easily achieved.

MATERIALS AND METHODS

Instrumentation and Reagents

Chromatographic separation was performed on the Waters Alliance HPLC-2695 equipped with Diode Array Detector (DAD-2998) and Empower Software system (Waters, USA). Other apparatus were used as following: Ultrasonic cleaner (USC-502, Shanghai Bolong Electronic Equipment Co., Ltd.); Electric-heated thermostatic water bath (DKS-14, Jiaxing Zhongxin Medical Instruments Co., Ltd.); Electronic balance (Ohaus DV215CD, Sartorius BSA224S, Mettler Toledo XS205DU).

Aloe-emodin (lot number: 110795-201609, content 99.5%), rhein (lot number: 110757-200206, content 99.7%), emodin (lot number: 110756-200110, content 99.5%), chrysophanol (lot number: 110796-201319, content 99.6%), physcion (lot number: 110758-201415, content 99.1%), all of them were purchased from National Institutes for Food and Drug Control. HPLC grade methanol (TEDIA Chemical Reagent Co., Ltd), ultrapure water (Wahaha Co., Ltd) and the rest of reagents were analytical grade.

Materials

RRFG (lot number: 6013323, 6013333), WRFG (lot number: 5104653, 5104663) and RCFG (lot number: 502149, 509031), All the samples were provided by Guangdong Yifang Pharmaceutical Co., Ltd, who was the first fixed manufacture for single TCM concentrated granules designated by China SFDA.

Preparation of the Sample Solution

The powder was screened through 50-mesh sieve and precisely weighed (0.1g), immersed with 25mL of 70% ethanol in beaker flask. Additional ethanol was added to apply the loss after ultrasonic extraction for 30min, the extraction was cooled to room temperature and filtered. Prior to HPLC analysis, all the sample solution to be determined were filtered through 0.45μm filter membranes.

Preparation of Standard Solution

The mixed standard solution was prepared by dissolving each weighed compound in a 100mL volumetric flask and obtained the mix standard solution with concentration of aloe-emodin 0.1604g•L^{-1}, rhein 0.0793g•L^{-1}, emodin 0.1684g•L^{-1}, chrysophanol 0.1551g•L^{-1} and physcion 0.0807g•L^{-1}, respectively. At least six different concentrations were further diluted with methanol for establishing a calibration curve. In addition, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated.

Chromatographic conditions

Analysis was carried out on the Columns: Agilent ZORBAX SB-C_{18} (4.6x250mm, 5μm), InertSustam® C_{18} (4.6x250mm, 5μm), xBridge® C_{18} (4.6x250mm, 5μm), Agilent Eclipse XDB-C_{18} (4.6x250mm, 5μm) and under the following chromatographic conditions: the operating temperature of the column was maintained at 25℃; the injection volume was 10μL; the wavelength was set at 254n; the mobile phase was composed of Methanol/0.1% phosphoric acid solution at a flow rate of 1.0mL•min^{-1}. According to the peak area of modin peak, the number of theoretical plates should not be less than 3000. Each sample was measured twice and took the average. HPLC of sample solution and mixed standard solution are shown in fig. 2.

Principle of QAMS

Within the linear concentration range, the concentration of component is proportional to the sensitivity of the detector with the equation W=fA (W is the concentration of the analyte, A is a peak area). In the case of TCM multicomponent quality evaluation, the characteristic component of the economic reference standard is used as internal reference standard. The RCFs of other analytes of the internal reference standard are then finally established and their contents are calculated by RCFs. The RCFs can be calculated as following:

$$fc/x = (Ax \times Wc)/(Ac \times Wx)$$

Where A*x* is the peak area of the internal reference standard, W*x* is its concentration, A*c* is the response peak area of the analyte C, W*c* is the concentration of analyte C in sample.

STATISTICAL ANALYSIS

All original data were obtained from the Empower®3 Software system (Waters, USA), and the calculation of values including RSD was carried out in Microsoft Excel 2019.

RESULTS

Linearity

According to the chromatographic conditions and operation methods as described in "Preparation of Standard Solutions", all of the above different concentrations of mixed standard solutions were injected (10μL) into the HPLC system. The calibration curves for each analyte was produced by plotting the peak areas (Y) versus the concentration of each component (X, mg•L^{-1}) and the linear regression analysis was calculated with least square method. As shown in table 1, good calibration curves of 5 components were obtained, high coefficient values of correlation (r>0.9997) were shown with good linearity at a certain wide range of concentrations. LOD and LOQ were showed a high sensitivity under the established chromatographic condition.

Precision

The intro-day precision was tested by the mixed standard solution repeatedly injected six times in one day. The results showed that the RSDs of aloe-emodin, rhein, emodin, chrysophanol and physcion were 0.50%, 0.32%, 0.32%, 0.35% and 0.33%. In addition, the inter-day precision was tested by the solution injected three times for five consecutive days and their RSDs were 0.55%, 1.20%, 1.08%, 1.82% and 2.24%, respectively. These results indicated that the developed method had a good precision.

Stability

The stability of the sample solution was analyzed at 0, 6, 12, 24, 36 and 48h at room temperature (25±2°C), their RSDs were also calculated. The RSDs of aloe-emodin, rhein, emodin, chrysophanol and physcion were 0.47%, 0.82%, 0.87%, 0.51%, 0.66% and 0.80%, respectively, indicated that the sample solution was stable within 48h.

Repeatability

To confirm the repeatability of this method, six independently prepared solutions from the same sample (6013323) were analyzed. RSDs of the peak areas of 5 Anthraquinone components were calculated. The average contents of these five components were 0.872, 1.963, 1.606, 3.308 and 1.298mg•g^{-1}, respectively. RSDs were 1.65%, 1.38%, 1.84%, 0.93% and 1.92%, respectively. These results indicated that this method had good reproducibility.

Recovery

Recoveries studies were carried out to evaluated the accuracy of the method. Pre-analyzed samples were spiked with the standard solution at three different concentration levels (50, 100 and 150%). The mixtures were extracted and analyzed by the method mentioned in "Preparation of the Sample Solution" and three replicates were performed for each test level. The recoveries of the 5 Anthraquinone components were 97.55%, 97.21%, 101.07%, 100.02% and 98.93%, RSDs were 1.21%, 0.62%, 0.89%, 0.82% and 1.19%, respectively, which indicating that the recovery of this method was of good accuracy.

Calculation of RCFs

In the experiment, Emodin was chosen as the internal reference standard, therefore, the quantitative analyses of aloe-emodin (A), rhein (B), chrysophanol (D) and physcion (E) can be achieved by detecting the concentration of the internal reference standard (C) and then calculated by their RCFs (table 2).

Factors influencing RCFs and reproducibility evaluation

In the application of QAMS, the key is to establish the RCFs between internal standards and the analytes. The flow rate, column temperature, column, etc. are the main factors affecting RCFs (Wang *et al.*, 2016; Cui *et al.*, 2016). In order to obtain a good reproducibility of RCFs, we investigated the influence of the above factors.

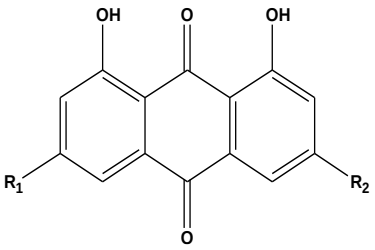
Flow Rate Effects of different flow rates (0.8, 1.0 and 1.2mL•min^{-1}) on the RCFs were investigated using a Waters Alliance HPLC system (Waters 2695-2998) and InertSustam® C_{18} column. The RCFs were well reproducible with RSD<2% (table 3).

Columns Temperature Influence of Different Columns Temperature on RCFs was performed on Waters Alliance HPLC system (Waters 2695-2998) and InertSustam®C_{18} with four different temperature. The RCFs were well reproducible (table 4).

Columns In the study, analysis test was performed on Waters Alliance HPLC system under the conditions mentioned in 2.1 terms with four different columns (Agilent ZORBAX SB-C_{18}, InertSustam®C_{18}, xBridge®C_{18} and Agilent Eclipse XDB-C_{18}). The RCFs were well also reproducible (table 5).

Establishment of RCFs

Finally, we achieved the average RCFs in consideration of the above affect factors as follows: aloe-emodin(fc/a) 1.276, rhein (fc/b) 1.066, chrysophanol (fc/d) 1.466 and physcion (fc/e) 0.671, the RSDs of these 4 Anthraquinone components were 1.01%, 0.24%, 1.09% and 0.58%, respectively.



	R_1	R_2
emodin	OH	CH_3
aloe-emodin	H	CH_2OH
rhein	H	COOH
chrysophanol	H	CH_3
physcion	OCH_3	CH_3

Fig. 1: Chemical structures of 5 anthraquinone components.

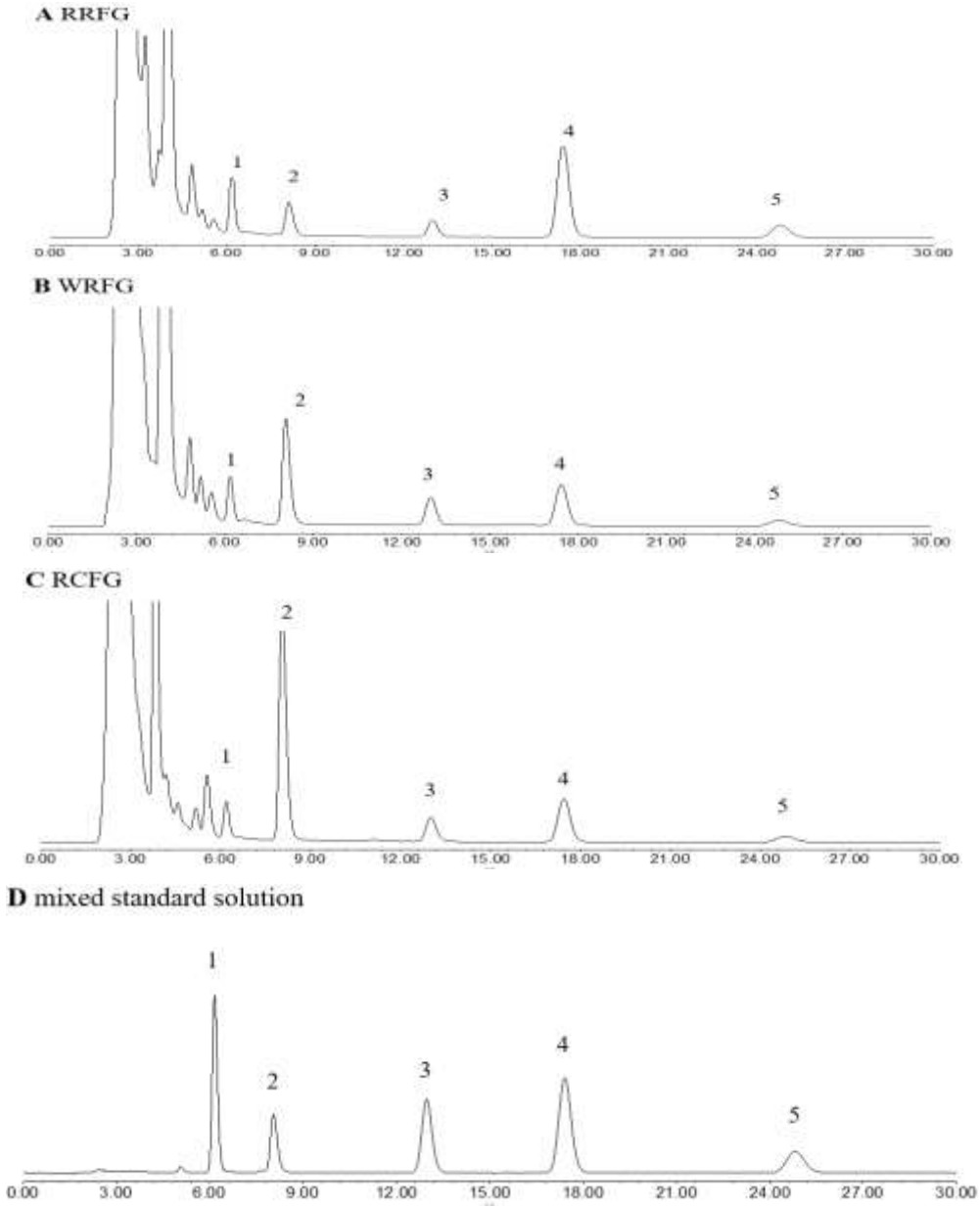


Fig. 2: HPLC of Sample Solution: RRFG (A), WRFG (B), RCFG (C) and mixed standard solution (D); 1: aloe-emodin, 2: rhein, 3: emodin, 4: chrysophanol and 5: physcion.

Table 1: Calibration curves of the 5 anthraquinone components determined

Analyte	Calibration curve	R	Linear range (mg•L-1)	LOD (mg•L-1)	LOQ (mg•L-1)
aloe-emodin	y = 46717x + 2679.3	1.0000	0.3208～160.40	0.1005	0.3208
rhein	y = 40573x + 3646.8	1.0000	0.1586～ 79.32	0.0442	0.1586
emodin	y = 37187x - 23127	0.9998	0.3368～168.40	0.0821	0.3368
chrysophanol	y = 52492x + 1558.9	1.0000	0.3101～155.06	0.0885	0.3101
physcion	y = 19723x + 13604	0.9997	0.1613～ 80.67	0.0423	0.1613

Table 2: RCFs of Analytes in RRFG

NO.	RCFs			
	Aloe-emodin fc/a	Rhein fc/b	Chrysophanol fc/d	Physcion fc/e
1	1.262	1.069	1.435	0.677
2	1.275	1.059	1.423	0.668
3	1.288	1.073	1.445	0.682
4	1.276	1.086	1.473	0.679
5	1.291	1.081	1.522	0.657
6	1.258	1.048	1.461	0.660
Average	1.275	1.069	1.460	0.670
SD	1.33	1.42	3.53	1.08
RSD/%	1.04	1.33	2.42	1.61

Table 3: RCFs by different flow rate

Flow rate mL·min-¹	RCFs			
	Aloe-emodin fc/a	Rhein fc/b	Chrysophanol fc/d	Physcion fc/e
0.8	1.315	1.062	1.461	0.661
1.0	1.288	1.073	1.445	0.682
1.2	1.269	1.053	1.474	0.682
Average	1.290	1.063	1.460	0.675
RSD/%	1.79	0.98	0.99	1.81

Table 4: RCFs by different column temperature

Columns Temperature ºC	RCFs			
	Aloe-emodin fc/a	Rhein fc/b	Chrysophanol fc/d	Physcion fc/e
20ºC	1.302	1.067	1.522	0.698
25ºC	1.288	1.073	1.445	0.682
30ºC	1.268	1.061	1.425	0.662
35ºC	1.259	1.058	1.419	0.623
Average	1.279	1.065	1.453	0.666
RSD/%	1.52	0.65	3.27	4.87

Table 5: RCFs dertermined by different columns

Columns	RCFs			
	Aloe-emodin fc/a	Rhein fc/b	Chrysophanol fc/d	Physcion fc/e
Agilent ZORBAX SB-C18	1.223	1.067	1.485	0.663
InertSustam® C18	1.288	1.073	1.445	0.682
xBridge® C18	1.265	1.053	1.498	0.668
Agilent Eclipse XDB-C18	1.260	1.066	1.530	0.677
Average	1.259	1.065	1.489	0.673
RSD/%	2.14	0.80	2.36	1.30

Table 6: Contents of each component in different formulation granules of rhubarb by external standards method and QAMS

	Sample	Method	Emodin	Aloe-emodin	Rhein	Chrysophanol	Physcion
RRFG	6013323	ESM	1.657	0.893	2.443	3.053	1.219
		QAMS		0.873	2.379	3.152	1.243
		RSD%		1.58	1.87	2.26	1.35
	6913333	ESM	1.549	0.875	2.413	2.663	1.185
		QAMS		0.884	2.431	2.625	1.206
		RSD%		0.74	0.52	1.03	1.25
WRFG	5104653	ESM	0.519	0.451	1.733	0.896	0.338
		QAMS		0.451	1.726	0.874	0.340
		RSD%		0.02	0.29	1.80	0.46
	5104663	ESM	0.519	0.457	1.744	0.909	0.341
		QAMS		0.455	1.724	0.882	0.342
		RSD%		0.36	0.82	2.12	0.16
RCFG	501249	ESM	0.130	0.060	1.485	0.157	0.061
		QAMS		0.060	1.482	0.152	0.062
		RSD%		0.04	0.12	2.17	0.62
	509031	ESM	0.121	0.057	1.490	0.145	0.057
		QAMS		0.057	1.473	0.143	0.057
		RSD%		0.57	0.79	0.74	0.20

DISCUSSION

In this paper, external standard method (ESM) was used to determine the content of anthraquinones in six batches of rhubarb and different formulations and calculated the contents of each anthraquinone component in different formulation granules of rhubarb by QAMS method. Then, the results of these two methods were compared. It showed that there was no significant difference in contents measured by the two methods and all the RSD$\leqslant$ 2.26% (table 6), the method of QAMS established has good credibility. This result further showed that it was feasible to use QAMS for rapid and simple quality control of traditional Chinese medicine formula granules in the absence of some reference substances.

Emodin was chosen as the internal reference standard, in addition to the price, easy to obtain, moderately polar and relatively high content, were the main reasons for its selection. In addition, the linearity, precision, stability, repeatability and recovery of the determination method were verified, all of the investigations ensured the applicability and feasibility of the QAMS method. In this proces, flow Rate, columns temperature and chromatographic columns are likely to have an influence on RCFs. Therefore, we have also investigated the influence of the above factors on RCFs and found that different flow rates, column temperatures and column types have little influence on RCF calculation, which illustrated that the calculation method of RCFs was stable and credible.

According to the experimental results, RRFG was rich in free and binding anthraquinone components, which had intense purgative activity (Cirillo and Capasso, 2015; Feng et al., 2013). These components in RRFG were more obvious than those in other two formula granules, and RCFG had the lowest content. This result indicated that anthraquinone components will be significantly reduced after be cooked with Yellow rice wine or fried into charcoal. Therefore, the effect of these three drugs may be different obviously because of their varied pharmacodynamic basis. Thus, in order to use these drugs safely, the results deserved more attention for doctors and it was necessary to rigorously prescribed according to the degree of patient's illness.

CONCLUSION

In this paper, three types of rhubarb formula granule were investigated, the QAMS method was successfully applied to the analysis of RRFG, WRFG and RCFG. The results demonstrate that the QAMS method was a simple and feasible quantitative method for simultaneously determining the other four components (aloe-emodin, rhein, chrysophanol and physcion) with emodin as the internal reference standard, and this method can provide reference for the quality control of rhubarb and other traditional Chinese medicines containing anthraquinone components.

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