A Quantitative Method for Simultaneous Determination of Four Anthraquinones with One Marker in *Rhei Radix* et *Rhizoma*

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Abstract: **Objective** To develop a quantitative method for simultaneously determining multi-components in *Rhei Radix* et *Rhizoma* using one chemical reference substance. **Methods** The contents of multi-components were calculated by the UV relative correction factors (RCFs) of chrysophanol, physcion, and rhein to emodin. **Results** The values of RCFs at 274 nm for rhein, chrysophanol, and physcion to emodin were 0.712, 0.674, and 1.051. The calibration curves were linear over the ranges of 0.02－4.08, 0.02－4.12, 0.07－12.92, and 0.02－3.68 μg/mL for rhein, emodin, chrysophanol, and physcion, respectively. The contents of emodin in 18 samples were determined by the external standard method, and the contents of the other three anthraquinone aglycones were calculated according to their RCFs. **Conclusion** No significant difference is found in comparison with the classical method, indicating that the RCFs have high reliability within their linear ranges and could be used in quality control of *Rhei Radix* et *Rhizoma*. The quantitative analysis of multi-component with a single marker is especially suitable for herbal medicines containing unstable or hard to be purified components as quality control markers.

Key words: anthraquinone; multi-component quantitative analysis; quality control; *Rhei Radix* et *Rhizoma*; UV relative correction factor

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Introduction

Herbal medicines have been widely used for thousands of years in many oriental countries and accepted by many occidental countries in recent years (Zhang *et al*, 2003; Li and Wang, 2004). However, a feasible quality control pattern for herbal medicines is urgently necessary to ensure their reliability for pharmacological and clinical utilization. During recent decades, several approaches have been developed for quality control of herbal medicines. In general, one or two markers, such as pharmacologically active components or specific compounds in herbs, are currently employed for evaluating quality (Wang, Gao, and Wang, 2007). But they failed to provide a comprehensive quality evaluation of herbal medicines (Yu *et al*, 2007). Moreover, the chemical constituents in herbal medicines varied depending on ecological environment, harvest season, cultivation conditions, plant origin, drying process, and other factors (Xie *et al*, 2006; Liu *et al*, 2007). In view of this situation, chromatographic fingerprinting is highly recommended for application to quality control of herbal medicines, because it could provide the whole profile (Liang, Xie, and Chan, 2004; Liu, Zhou, and Yan, 2007; Shao, 2009). Several chromatographic techniques, such as high-performance liquid chromatography (HPLC) (Singh *et al*, 2005), gas chromatography (GC) (Wei *et al*, 2006), thin-layer chromatography (TLC) (Liu *et al*, 2007), GC-MS (Huang *et al*, 2007), and HPLC-MS (Fan, Wang, and Cheng, 2006), are encouraged to establish fingerprint and quality assurance. But these techniques could be used only for qualitative evaluation but in the absence of the quantitative function (Chai, Li, and Li, 2005).

Another quality control approach for simultaneous determination of multiple components has been developed

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in recent years. Analytical techniques like HPLC (Chen *et al*, 2007), capillary electrophoresis (CE) (Li *et al*, 2000), GC-MS (Deng *et al*, 2006), and HPLC-MS (Tian *et al*, 2006; Zhang *et al*, 2006) are used, and the quality of many kinds of herbal medicines is controlled by determining the bioactive components as definitly as possible. However, the limitation of this method is that it is hard to be applied in practice, for it requires sufficient chemical purity standards or chemical reference substances, advanced analytical instruments, and expert analysts. In addition, high purity chemical standards or chemical reference substances of herbal medicines are expensive and insufficient, especially when the component is of low content level and hard to be purified from the plant. Moreover, some constituents of herbal medicines become unstable when they are purified from a complicated matrix. In considering of the above reasons, factories have many difficulties to apply this type of quality control in manufacture. It is urgently necessary to develop a convenient and low-cost approach for controlling the quality of herbal medicines.

Herein, we propose a method involving the use of a single standard to assay simultaneously multi-components in herbal medicines. Among them, one component is determined with external standard method, while the amounts of the other components are calculated by their UV relative correction factors (RCFs) at specific wavelength.

There are some reports about the application of the RCFs. In *United States Pharmacopoeia*, conversion factors were used for quantification analysis of different diterpene lactones in the plants of *Andrographis* Wall. It was calculated that conversion factors for andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin were 1.00, 3.90, 1.45, and 2.65, respectively. And the approximate relative retention time of the different diterpene lactones was as follows: 1.00 min for andrographolide, 1.16 min for neoandrographolide, 1.31 min for 14-deoxy-11,12- didehydroandrographolide, and 1.50 min for andrograpanin (The United States Pharmacopeial Convention, 2011). Relative response factor (RRF) was used in food chemical analysis. (+)-Catechin was selected as the reference compound for calculating the RRFs of the catechins. The RRFs for catechins were quite similar at 210 nm of detection under different analytical conditions (columns, elution systems, and HPLC instruments). It was confirmed that by using these RRFs, the quantification of catechins in tea and related products could be carried out with only catechin as a reference substance (Wang, Gordon, and Keith, 2003). A case study was also reported to determine the RRFs of paclitaxel-related impurities by HPLC equipped with a UV detector and charged aerosol detector (CAD) in tandem. And by these RRFs, the percent of all the impurities in paclitaxel drug substance could be evaluated (Sun *et al*, 2008). And the content of the total lignoside in extract of arctium fruit was determined by correction factors by HPLC (Huang, Zheng, and Zeng, 2005).

In this paper, a Chinese herbal medicine, *Rhei Radix* et *Rhizoma*, roots and rhizomes of *Rheum officinale* Baill., *R. palmatum* L., and *R. tanguticum* Maxim ex Balf. (Pharmacopoeia Committee of P. R. China, 2010), has been selected as a model to develop and validate our method for evaluating the quality of herbal medicines by guarantee levels of these four effective components. *Rhei Radix* et *Rhizoma* is one of the most well-known herbal medicines for the treatment of constipation and inflammation (Xiao, He, and Wang, 1984). Chemical and pharmacological investigations indicated that derivatives of anthraquinones, including physcion, emodin, rein, and chrysophanol, are the effective constituents, with strong laxative, antibacterial, anti-oxidative, and antipyretic actions (Tang and Eisenbrand, 1992; Newall, Anderson, and Phillipson, 1996; Lv *et al*, 2010).

Materials and methods

Materials and reagents

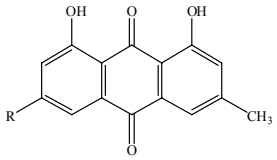
A total of 18 samples of *Rhei Radix* et *Rhizoma* were collected from 10 provinces of China (Table 1). All samples were authenticated by Prof. WANG Zhi-min and Dr. ZHU Jing-jing. The voucher specimens (CGSM-0404, CGSM-0405, and CGSM-0406) were deposited in Herbarium of Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences (Beijing, China).

HPLC-grade methanol was provided by Tedia Company, Inc. (USA). Phosphoric acid was of chromatographic grade. Water was purified with an Milli-Q

Table 1 Eighteen Samples of *Rhei Radix* et *Rhizoma*

No.	Species	Geographical origin	Collecting time
1	*R. palmatum*	Taiyuan, Shanxi	July, 2006
2	*R. palmatum*	Shihezi, Xinjiang	September, 2006
3	*R. palmatum*	Wulumuqi, Xinjiang	August, 2006
4	*R. tanguticum*	Lanzhou, Gansu	October, 2006
5	*R. palmatum*	Tianshui, Gansu	September, 2006
6	*R. palmatum*	Longxi, Gansu	April, 2005
7	*R. palmatum*	Chengdu, Sichuan	October, 2006
8	*R. tanguticum*	Anguo, Hebei	September, 2006
9	*R. palmatum*	Xinxiang, Henan	February, 2007
10	*R. palmatum*	Baoji, Shaanxi	February, 2007
11	*R. palmatum*	Kunming, Yunnan	March, 2007
12	*R. palmatum*	Xining, Qinghai 1	August, 2006
13	*R. palmatum*	Xining, Qinghai 2	August, 2006
14	*R. tanguticum*	Xining, Qinghai 3	August, 2006
15	*R. palmatum*	Xining, Qinghai 4	August, 2006
16	*R. tanguticum*	Xining, Qinghai 5	August, 2006
17	*R. palmatum*	Dingxi, Gansu	August, 2006
18	*R.officinale*	Enshi, Hubei	February, 2007

system (Millipore, USA) and subsequently filtered through a 0.45 μm membrane (Millipore, USA). The other solvents, purchased from Beijing Chemical Factory (Beijing, China), were of analytical grade. Four reference compounds (rhein, emodin, chrysophanol, and physcion, Fig. 1) were purchased from National Institute for Food and Drug Control (Beijing, China).



R=H chrysophanol
R=OCH_3 physcion
R=OH emodin
R=COOH rhein

Fig. 1 Structures of four anthraquinone aglycones

Instruments and chromatographic conditions

Analysis was performed on two HPLC systems with a Waters 2695-2996 series, including a quaternary pump, a phodiode array detector, a vacuum degasser, a thermostated autosampler, a column compartment, and Empower work station, and an Agilent 1100 series, including a quaternary pump, a diode array detector, a vacuum degasser, a thermostated autosampler, a column compartment, and a data system (Agilent Chem Station). The chromatographic separation was performed on an Agilent Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm) and an Alltima C_{18} column (250 mm × 4.6 mm, 5 μm).

The eluent consisted of methanol-water (85:15) containing 0.4% phosphoric acid. The flow rate was kept at 1 mL/min. Column temperature was kept constant at 25 ℃, and the injection volume was 10 μL. The detection wavelength of the PDA detector was 274 nm with 360 nm as a reference wavelength, both at 4 nm bandwidth with full spectral scanning 200－300 nm and 0.5 nm resolution. Fig. 2 shows the HPLC-PDA chromatograms of the samples of *Rhei Radix* et *Rhizoma* (Fig. 2A) and mixed anthraquinone standards (Fig. 2B).

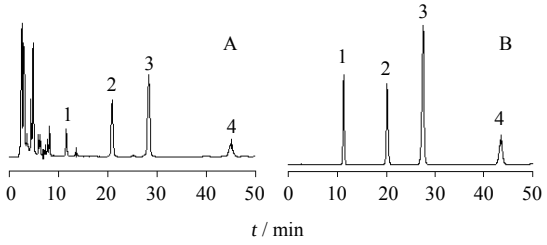


Fig. 2 Representative HPLC profiles of *Rhei Radix* et *Rhizoma* (Longxi, Gansu, A) and mixed anthraquinone reference substances (B)

1: rhein 2: emodin 3: chrysophanol 4: physcion

Sample preparation

Rhei Radix et *Rhizoma* was powdered to a homogeneous size in a mill, passed through a 60-mesh sieve and dried at 50 ℃ until constant weight was achieved. Approximately 0.30 g of the pulverized sample was weighed accurately and macerated in 50 mL of acetone. After keeping at room temperature for 2 h, the sample was extracted for 40 min in a ultrasonic bath, cooled, and the loss of weight due to evaporation of solvent was replenished with acetone. The extract was filtered and 25 mL of the filtrate was concentrated *in vacuo*. A gum residue was obtained and dissolved in 10 mL methanol and filtered through a 0.45 μm filter membrane. Then the filtrate (10 μL) was injected into the HPLC system for each analysis.

Standard solution preparation

A mixed stock solution containing reference standards of four anthraquinones was prepared by dissolving weighed accurately samples of each

compound in methanol at concentration of 36.8－129.2 μg/mL and diluting to appropriate concentration for the establishment of calibration curves and RCFs. A single standard stock solution of emodin was prepared by dissolving accurately weighed emodin in methanol at concentration of 40.8 μg/mL as an external standard in the proposed analytical method.

Statistical method

According to the principle that within a concentration range, the absorption of analyte was linearly proportional to sample concentration and their relations could be shown with the formula $W = fA$ (Yu, 1992), where W is the sample concentration, A is the response value, and f is the correction factors (CF). The value of CF is a constant related with the detected substance and the sensitivity of the detector. Supposed several components coexisting in Chinese materia medica sample, every component could be shown as formula (1).

$$\frac{W_i}{A_i} = f_i (i = 1,2,...,k,...,m) \quad (1)$$

If component s was used as an internal standard, the RCFs between components s and m (f_{sm}) is established through formula (2):

$$f_{sm} = \frac{f_s}{f_m} = \frac{W_s \times A_m}{W_m \times A_s} \quad (2)$$

Then quantitative formula (3) could be deduced, where A_s and W_s are the peak area and concentration of inter standard substance, while A_m and W_m are the peak area and concentration of target component.

$$W_m = \frac{W_s \times A_m}{f_{sm} \times A_s} \quad (3)$$

If the content of component s was authentically determined, the content of component m could be calculated through their RCFs.

Results and discussion

Optimization of sample pre-treatment

Several experiments were carried out in order to optimize sample preparation and chromatographic conditions. The peak areas of anthraquinone aglycons were investigated in each test condition. Methanol, acetone, and acetone-water were used when extracted. As a result, 50 mL acetone was chosen as the extract solvent for sample powder (0.3 g).

Extract methods such as ultrasonic extraction, reflux, and Soxhlet's extraction were compared. The proper time and extract temperature of each step were also optimized. The optimal extract method was obtained by macerating in acetone for 2 h and then extracting for 40 min with ultrasonic bath assistance.

Calibration curves and linear ranges

According to the concentration of samples, the mixed stock solutions of reference substances were diluted to the appropriate concentration for establishing calibration curves. Each calibration curve was created at six different concentration in triplicate, and then the calibration curves were produced by plotting the integrated peak areas (Y) versus the concentration of each component (X, mg/mL) and by the linear regression analysis. The regression equations and linear ranges for the four markers are shown in Table 2. The limits of detection (LOD) and quantification (LOQ) were calculated as the amount of the injected sample which resulted in a signal-to-noise ratio of 3 and 10, respectively. The calibration curves were linear over the ranges of 0.02－4.08, 0.02－4.12, 0.07－12.92, and 0.02－3.68 μg/mL for rhein, emodin, chrysophanol, and physcion, respectively, with correlation coefficients for the linear regression analyses falling into the range of 0.9992－0.9999. The UV RCFs at 274 nm of rhein, chrysophanol, and physcion to emodin were 0.712, 0.674, and 1.051, respectively, and with good repeatability (RSD = 0.44%－0.95%) (Table 3).

Method validation

Validation studies proved that this method had good precision and accuracy. The overall intra- and inter-day variability in retention time and peak areas of the four target constituents were less than 2.2% and 3.3%, respectively. The accuracy of this analytical method was determined by adding different amounts of reference substances to the sample powder with known contents of target analytes. Then the samples were treated following the same procedure. The results were shown in Table 4. The average recoveries were in the range of 94.5%－104.3% with good repeatability (RSD = 0.30%－4.2%), which indicated that the proposed method had an adequate degree of accuracy for the simultaneous determination of the four target constituents in the samples. Reproducibility was determined in Tables 5 and 6. The results showed that the method had good reproducibility (RSD = 0.29%－0.89%). All the above method-validation results

Table 2 Regression data of four anthraquinone aglycones in *Rhei Radix* et *Rhizoma*

Compounds	Linearity ranges / (μg·mL^{-1})	Calibration equations[a]	r^2
rhein	0.02－4.08	$Y = 2.68 \times 10^6X - 2.63 \times 10^4$	0.9999
emodin	0.02－4.12	$Y = 3.80 \times 10^6X - 4.07 \times 10^4$	0.9997
chrysophanol	0.07－12.9	$Y = 2.55 \times 10^6X - 9.74 \times 10^4$	0.9992
physcion	0.02－3.68	$Y = 2.51 \times 10^6X - 3.52 \times 10^4$	0.9995

[a] Regression curves were obtained by plotting concentration (mg·mL^{-1}) (X) and peak area (Y). r^2 is the coefficient of determination

Table 3 UV RCFs of anthraquinone aglycones in *Rhei Radix* et *Rhizoma* at 274 nm

Injection volumes / μL	RCF_{UV}[a]		
	$f_{r,e}$[b]	$f_{c,e}$	$f_{p,e}$
0.5	0.721	0.671	1.044
1	0.701	0.680	1.050
2	0.713	0.664	1.053
5	0.712	0.673	1.045
25	0.710	0.672	1.054
100	0.712	0.681	1.055
mean	0.712	0.674	1.051
RSD / %	0.89	0.95	0.44

[a] RCF_{UV} is UV RCFs to emodin at 274 nm

[b] Letters r, e, c, p represent rhein, emodin, chrysophanol, and physcion, respectively, Tables 5 and 6 are same

Table 4 Recoveries of anthraquinone aglycones (n = 3)

Analytes	Reference substances added into samples / μg	Average recovery rate / %	RSD / %
rhein	27.2	102.3	2.40
	35.2	97.7	0.48
	43.1	94.5	3.30
emodin	36.9	104.3	1.80
	46.8	101.7	0.45
	54.3	96.3	4.20
chrysophanol	107.7	103.5	1.20
	136.6	98.2	0.30
	165.7	102.3	0.75
physcion	25.1	97.3	2.40
	34.6	100.7	1.20
	41.8	103.2	2.30

demonstrated that the RCFs had high reliability, and that the method could be used for HPLC quantitative determination.

A total of 18 samples were injected for HPLC analysis. In this paper, to validate this method, two routes have been arranged for quantifying the four target components. The first is to determine the content of emodin by the external standard method, then to calculate the other three contents according to their RCFs. The second is to determine the four target components by the use of the external standard method. These two group results are compared in Table 7. No significant difference was found between the two methods, indicating that this proposed method has potential for developing a pattern for quality control of herbal medicines.

Table 5 RCFs by different instruments and columns (n = 6)

Instruments	Chromatograph columns	RCFs		
		$f_{r,e}$[b]	$f_{c,e}$[b]	$f_{p,e}$[b]
Agilent 1100	Alltima	0.711	0.669	1.058
	Agilent Zorbax	0.706	0.672	1.051
Waters 2695-2996	Alltima	0.712	0.669	1.050
	Agilent Zorbax	0.707	0.673	1.053
mean		0.709	0.671	1.053
RSD / %		0.42	0.31	0.29

Table 6 RCFs values validated in different laboratories (n = 3)

Injection volumes / μL	$f_{r,e}$[b]		$f_{c,e}$		$f_{p,e}$	
	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2
0.5	0.733	0.713	0.671	0.671	1.042	1.055
1	0.740	0.710	0.670	0.671	1.042	1.053
2	0.730	0.710	0.670	0.672	1.056	1.055
5	0.731	0.712	0.672	0.671	1.069	1.056
25	0.731	0.711	0.682	0.674	1.069	1.069
100	0.733	0.702	0.681	0.672	1.058	1.056
mean	0.733	0.710	0.674	0.672	1.042	1.055
RSD / %	0.80	0.88	0.89			

Identification of target chromatographic peaks

When this method is applied, it is essential to find a convenient means to identify correctly the four target

Table 7 Comparison of anthraquinones content determined by two methods (mg·g^{-1}, n = 3)

No.	rhein			emodin	chrysophanol			physcion		
	a	b	SD / %	a	a	b	SD / %	a	b	SD / %
1	0.173	0.176	1.73	0.491	1.31	1.34	2.45	0.564	0.561	0.47
2	0.315	0.321	1.91	0.452	1.26	1.31	3.24	0.789	0.798	1.25
3	0.185	0.189	2.16	0.300	1.17	1.21	2.90	0.646	0.653	1.13
4	0.367	0.375	2.18	0.903	2.26	2.34	3.41	1.267	1.284	1.31
5	0.203	0.209	2.96	0.446	1.45	1.46	0.414	0.757	0.768	1.37
6	0.414	0.419	1.21	0.532	1.49	1.54	3.22	0.908	0.913	0.52
7	0.118	0.114	3.39	0.311	2.44	2.53	3.31	1.564	1.589	1.58
8	0.132	0.136	3.03	0.432	3.53	3.65	3.26	1.803	1.832	1.61
9	0.252	0.258	2.38	0.387	1.37	1.42	3.28	0.761	0.768	0.93
10	0.103	0.105	1.94	0.272	2.28	2.35	2.99	0.907	0.911	0.52
11	0.113	0.112	0.885	0.383	3.07	3.17	3.26	1.366	1.382	1.22
12	0.162	0.168	3.70	0.211	0.35	0.355	2.31	0.202	0.200	0.81
13	0.154	0.159	3.25	0.394	0.85	0.875	2.46	0.444	0.448	0.93
14	0.223	0.228	2.24	0.301	0.343	0.349	1.75	0.298	0.300	0.55
15	0.214	0.217	1.40	0.292	0.242	0.248	2.48	0.316	0.316	0.08
16	0.144	0.148	2.78	0.184	0.308	0.309	0.325	0.169	0.168	0.66
17	0.124	0.127	2.42	0.322	2.26	2.34	3.27	1.416	1.429	0.89
18	0.296	0.305	3.041	0.461	1.29	1.33	2.63	0.756	0.765	1.16

a: contents were determined by the traditional external standard method

b: contents were calculated by the proposed method

components from the sample. In our paper, the four target peaks in the HPLC profile of *Rhei Radix* et *Rhizoma* (Fig. 2A) were clear and easy to be identified; The parameter of relative retention was used to locate the target peaks. Using emodin as the external standard, the relative retentions between the other target marker and emodin were obtained in different columns and various HPLC systems (Table 8). The value of relative retention was insensitivity to different HPLC systems and columns (RSD = 0.72% — 2.7%). Combining relative retentions, peak shapes, and their UV absorption characteristics, three target chromatographic peaks could be correctly identified.

Table 8 Relative retention of four target anthraquinones by different instruments and columns

Instrument	Column	$r_{r,e}$[a]	$r_{e,e}$	$r_{c,e}$	$r_{p,e}$
Agilent	Alltima	0.562	1.00	1.36	3.82
1100	Agilent zorbax	0.574	1.00	1.36	3.63
Waters	Alltima	0.564	1.00	1.35	3.70
2695-2996	Agilent zorbax	0.590	1.00	1.38	3.60
mean		0.573	1.00	1.36	3.69
RSD / %		2.3	—	0.72	2.7

Conclusion

Using the UV RCFs, our proposed method by which we used a single standard substance in the sample to quantify other co-existing components, succeeded in the simultaneously quantitative and qualitative analysis of four anthraquinones in 18 samples of *Rhei Radix* et *Rhizoma*. The proposed method is a simple and low-cost quality control pattern for herbal medicines which is especially suitable for determination of the unstable constituents.

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