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Development and validation of a UPLC method for quality control of rhubarb-based medicine: Fast simultaneous determination of five anthraquinone derivatives

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ABSTRACT

A reverse phase ultra performance liquid chromatography (UPLC) method was developed for the rapid quantification of five anthraquinone derivatives (aloe-emodin, rhein, emodin, chrysophanol and physcion) in rhubarb using a Waters Acquity BEH C_{18}, 50 mm × 2.1 mm, 1.7 μm column. The runtime was as short as 3 min. The influence of flow rate and column temperature on resolution was investigated. The method was validated according to the regulatory guidelines with respect to precision, accuracy, linearity and robustness. Comparison of system performance with conventional HPLC was made with respect to analysis time, efficiency and sensitivity. The proposed method was found to be reproducible and convenient for quantitative analysis of five anthraquinone derivatives in three species of rhubarb and related preparations.

1. Introduction

With the objective of reducing analysis time and maintaining good efficiency, there has been substantial focus on high-speed chromatographic separations. Recently, the commercially available technique of ultra performance liquid chromatography (UPLC) has proven to be one of the most promising developments in the area of fast chromatographic separations [1]. It utilizes sub-2 μm particles as stationary phase. The use of these very small particles allows a drastic improvement of the resolution per time unit, because chromatographic efficiency and optimal mobile phase velocity are both inversely proportional to the particle size. Therefore, due to the high efficiency of sub-2 μm particles, the column length can be decreased to obtain equivalent resolution in a reduced analysis time [2–4]. Because of its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis [5,6]. It is also suitable for the routine analysis of botanical drugs consisting of multiple components. For example, it was successfully applied to simultaneous determination of 14 nucleosides and nucleobases in cultured *Cordyceps* [7].

Rhubarb (Dahuang in Chinese), a well-known Chinese herbal medicine, has long been used in oriental preparations and is officially listed in Chinese Pharmacopoeia, containing three species, *Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf. and *Rheum officinale* Bail. [8]. It is also officially listed in Japanese and European Pharmacopoeia [9,10]. This important herbal medicine has been recognized for centuries in traditional medicine for its multiple pharmacological actions include laxative, antibacterial, hemostatic and antispasmodic [11–13]. The major active components of the herb are five anthraquinones (AQs), namely aloe-emodin, rhein, emodin, chrysophanol and physcion. These are the basis for the quality control of rhubarb and other plant-derived drugs from Rheum, Cassia and Polygonum genera [14–17]. The chemical structures of these anthraquinone derivatives are shown in Fig. 1.

The methods commonly used for the separation and determination of all or some of the five major AQs in rhubarb are thin-layer chromatography (TLC) [17,18], high-speed counter current chromatography (HSCCC) [19,20], micellar electrokinetic chromatography (MEC) [21], capillary zone electrophoresis [22,23] and high performance liquid chromatography (HPLC) [24–26]. Among them, HPLC method is well established and robust, but it is time-consuming (25 min at least). Although a pressurized capillary electro-chromatographic (pCEC) method was developed to perform the separation of five AQs in rhubarb within 5 min, it

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Table 1
Comparison of system performance of HPLC and UPLC

Components	Elution time (min)		USP resolution		Tailing factor		USP plate count	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
Aloe-emodin	4.907	0.546	19.29	3.22	1.40	1.35	12658	3140
Rhein	6.252	0.788	6.70	5.20	1.44	1.46	12181	3382
Emodin	10.622	1.487	15.61	10.45	1.25	1.12	16344	5594
Chrysophanol	15.402	1.849	12.15	4.22	1.21	1.11	18480	6484
Physcion	22.861	2.751	13.21	9.27	1.18	1.07	18401	6874

Table 2
Calibration curves, LOD and LOQ of the investigated components

Components	Regression equation ($y = ax + b$)[a]	R^2	Linear range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
Aloe-emodin	$y = 10224x + 320.5$	0.9999	0.40–40.0	0.04	0.12
Rhein	$y = 9112x - 78.2$	0.9999	0.40–40.0	0.06	0.20
Emodin	$y = 8043x + 416$	0.9998	0.40–40.0	0.06	0.20
Chrysophanol	$y = 12892x + 238.5$	0.9999	0.40–40.0	0.06	0.20
Physcion	$y = 6674x + 48.1$	0.9999	0.40–40.0	0.12	0.40

[a] y is the peak area, x the corresponding injection concentration, a is the slope and b is the intercept of the regression line, respectively.

depends on a not commercially available and specially prepared polymeric monolithic column [27]. It is, therefore, felt necessary to develop a rapid and reliable quality control method for simultaneous determination of five AQs in rhubarb and its related preparations using ultra-performance liquid chromatography (UPLC).

2. Experimental

2.1. Materials and reagents

Rhubarb samples were collected in Gansu province (*Rheum palmatum* L.), Qinghai province (*Rheum tanguticum* Maxim. ex Balf.) and Sichuan province (*Rheum officinale* Bail.), China. Sanhuang tablet (Handan Pharmaceutical Co. Ltd., Lot No. 1100) and Paidu Yangyan capsule (Panlong Yunhai Pharmaceutical Co. Ltd., Lot No. 070311) were bought from the pharmacy of 302 Hospital of PLA, Beijing, China. Sanhuang tablet is composed of Radix Et Rhizoma Rhei (rhubarb), Radix Scutellariae and Berberine Hydrochloride. Paidu Yangyan capsule is composed of Radix Et Rhizoma Rhei (rhubarb), Fructus Aurantii Immaturus, Natrii Sulfas and six other herbs. Aloe-emodin, rhein, emodin, chrysophanol and physcion were supplied by National Institute for the Control of Pharmaceutical and Biological Products. HPLC grade methanol and phosphoric acid were purchased from Beijing Chemical Regents Company. High purity water was obtained by Millipore Milli Q water purification system.

2.2. High performance liquid chromatography

The HPLC system used for initial chromatographic development was Waters Alliance separation module with a photo diode array detector. A Kromasil C_{18}, 250 mm × 4.6 mm, 5 μm column was used for separation. Mobile phase consisting of a mixture of 0.1% aqueous phosphoric acid and methanol in the ratio of 15:85 (v/v) with the flow rate of 1 ml/min was employed. The injection volume was 10 μl while detector was set at 254 nm. The column was maintained at 25 °C.

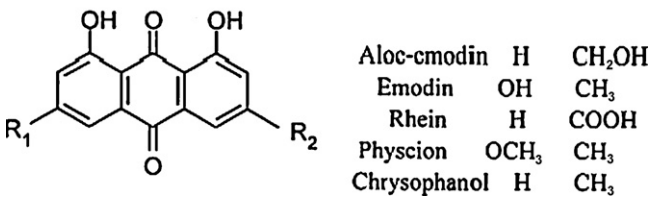

Fig. 1. Structures of rhein, emodin, aloe-emodin, chrysophanol and physcion.

2.3. Ultra performance liquid chromatography

UPLC was performed using a Waters Acquity system equipped with binary solvent delivery pump, an auto sampler and photo diode array detector. The chromatographic separation was performed using a Waters Acquity BEH 50 mm × 2.1 mm, 1.7 μm, C_{18} column. The mobile phase consisting of a mixture of 0.1% aqueous phosphoric acid and methanol in the ratio of 31:69 (v/v) with the flow rate of 750 μl/min was used. The detector wavelength was set at 254 nm. The injection volume was 1 μl while the column was maintained at 35 °C.

2.4. Standard solution preparation

The standard stock solutions of emodin, rhein, aloe-emodin, chrysophanol and physcion (40 μg/ml of each) were prepared in

Table 3
Accuracy of the investigated components

Components	Quantity added (μg/ml)	Quantity found (μg/ml)	Recovery (%)	R.S.D. (%)
Aloe-emodin	4.52	4.40	97.32	3.98
	9.03	8.92	98.81	3.63
	13.55	13.29	98.09	3.89
Rhein	3.65	3.58	97.96	4.29
	7.30	7.10	97.21	4.18
	10.95	10.78	98.47	3.99
Emodin	5.73	5.65	98.56	4.38
	11.45	11.25	98.27	4.59
	17.18	16.98	98.86	3.18
Chrysophanol	6.34	6.16	97.12	3.12
	12.67	12.49	98.57	3.14
	19.01	18.76	98.68	4.36
Physcion	1.74	1.69	97.08	4.79
	3.48	3.41	97.94	3.19
	5.22	5.08	97.28	4.47

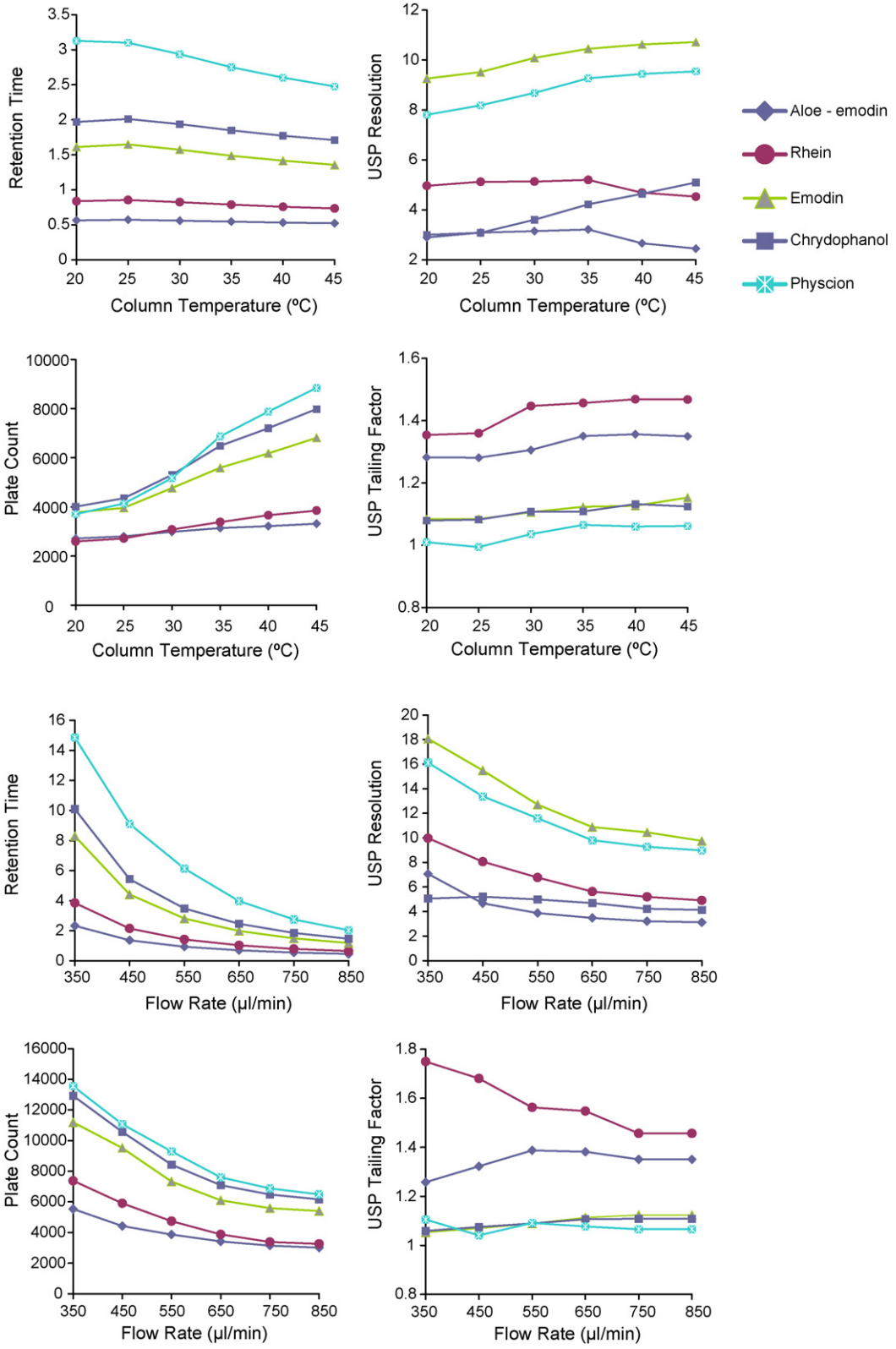

Fig. 2. Influence of flow rate and column temperature on UPLC chromatographic performance.

methanol. These solutions were stored in dark glass bottles at 4 °C and were stable for at least 1 month. Working standard solutions containing 4 μg/ml each of five standards were freshly prepared by diluting suitable amounts of the above solutions with methanol before injection.

2.5. Sample solution preparation

Powdered rhubarb or its preparations (0.15 g) was extracted with 25 ml methanol by refluxing for 60 min. The extracted solution was prepared by the method of weight relief, by which the weight

lost in the extraction procedure was compensated. After filtering, 5 ml filtrate was transferred into a flask and was then evaporated to dryness. Subsequently, 10 ml of 2 M HCl and 20 ml of chloroform were added to dissolve the residue and keep the solution on a water bath for 1 h. The hydrolyzed solution was extracted with 10 ml of chloroform four times and the combined extract was then evaporated to dryness. The residue was dissolved and transferred into a 50 ml volumetric flask by methanol. The solution was filtered through a 0.22 μm filter before injection.

2.6. Validation of UPLC method

The UPLC method was validated in terms of precision, accuracy and linearity according to ICH guidelines [28]. Assay method precision was determined using six independent test solutions. The intermediate precision of the assay method was also evaluated using different analyst on 3 different days. The accuracy of the assay method was evaluated with the recovery of the standards from samples. Three different quantities (low, medium and high) of the authentic standards were added to the known real sample. The mixtures were extracted as described in Section 2.5, and were analyzed using the developed UPLC method. Linearity test solutions were prepared by diluting the mixed standards stock solution. The LOD and LOQ for five AQs were estimated by injecting a series of dilute solutions with known concentration. Precision study was also carried at the LOQ level. To determine the robustness of the method, the final experimental conditions were purposely altered and the resolution was examined. The flow rate was varied by (±) 5%. The percentage of organic modifier was varied by (±) 1%. Column temperature was varied by (±) 1 °C.

3. Results and discussions

3.1. HPLC method development and transfer to UPLC

Using the HPLC conditions described in Section 2.2 there was no complete separation between rhein and aloe-emodin and the peak purity of these two AQs in real samples was not satisfactory.

The basic chromatographic conditions like stationary phase, solvents and UV detection, employed in HPLC were taken into account when developing the new UPLC method. The detection wavelength, type of stationary phase, buffer and solvent used in HPLC were kept constant. At the beginning the same ratio of buffer to methanol used in HPLC mode was chosen. Using these conditions the runtime was around 1 min, but the separation between rhein and aloe-emodin was not satisfactory. Attempts to improve the chromatographic separation were performed with varying the composition of mobile phase. The separation between rhein and aloe-emodin was definitely improved when the ratio of buffer to methanol was increased and the total runtime was prolonged. Acetonitrile was also tried to replace methanol in order to get lower backpressure and higher flow rate. However, acetonitrile was found to be less selective to the separation of rhein and aloe-emodin than methanol while the analysis time was not decreased either. To achieve total resolution of the five analytes and shortest possible runtime, the final mobile phase was chosen as described in Section 2.3. To improve detector sensitivity and to achieve equivalent system performance compared with conventional HPLC at same sample concentration UPLC instrument manufacturer recommends to use low injection volume (see Acquity UPLC Columns Calculator software (Version 1.1.1) of Waters Corporation).

Attempts to improve the chromatographic performance were made by altering the flow rate and column temperature. The results are shown in Fig. 2. The influence of flow rate was investigated with column temperature of 35 °C, and the flow rate was set at 750 μl/min when column temperature was altered.

The resolution and theoretical plates of aloe-emodin and rhein, which were relatively more difficult to separate than other components, showed unremarkable decline trend with the flow rate increased, compared to a dramatical decline of the retention time of physcion. Considering system backpressure and running time, flow rate of 750 μl/min was preferred.

The resolution, theoretical plates and tailing factor obtained for emodin, chrysophanol and physcion showed some improvement with the increase of column temperature. The resolution of aloe-emodin and rhein showed a declining trend above 35 °C. Hence, the column temperature of 35 °C was preferred. At this column temperature and flow rate of 750 μl/min, a satisfactory and rapid separation was achieved in 3 min with a backpressure of 11,000 psi.

3.2. Comparison study of chromatographic performance

Comparative data on chromatographic performance of HPLC and UPLC have been obtained by injecting a solution of mixed standards (4 μg/ml each). The performance parameters of both the systems are shown in Table 1. The runtime of UPLC was reduced by 8-fold to that of HPLC. The UPLC method showed comparatively better analysis efficiency than HPLC, though the resolution and theoretical plates obtained for five AQs in UPLC were somewhat poorer. Typical chromatograms HPLC and UPLC chromatograms are depicted in Fig. 3.

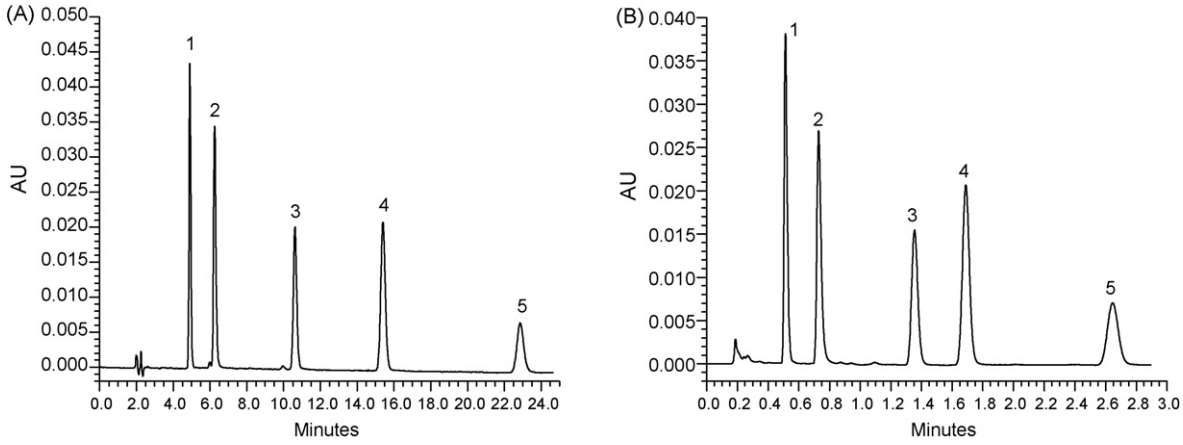

Fig. 3. Comparison of chromatograms of mixed standard obtained from (A) HPLC and (B) UPLC. *Key*: (1) aloe-emodin; (2) rhein; (3) emodin; (4) chrysophanol; and (5) physcion.

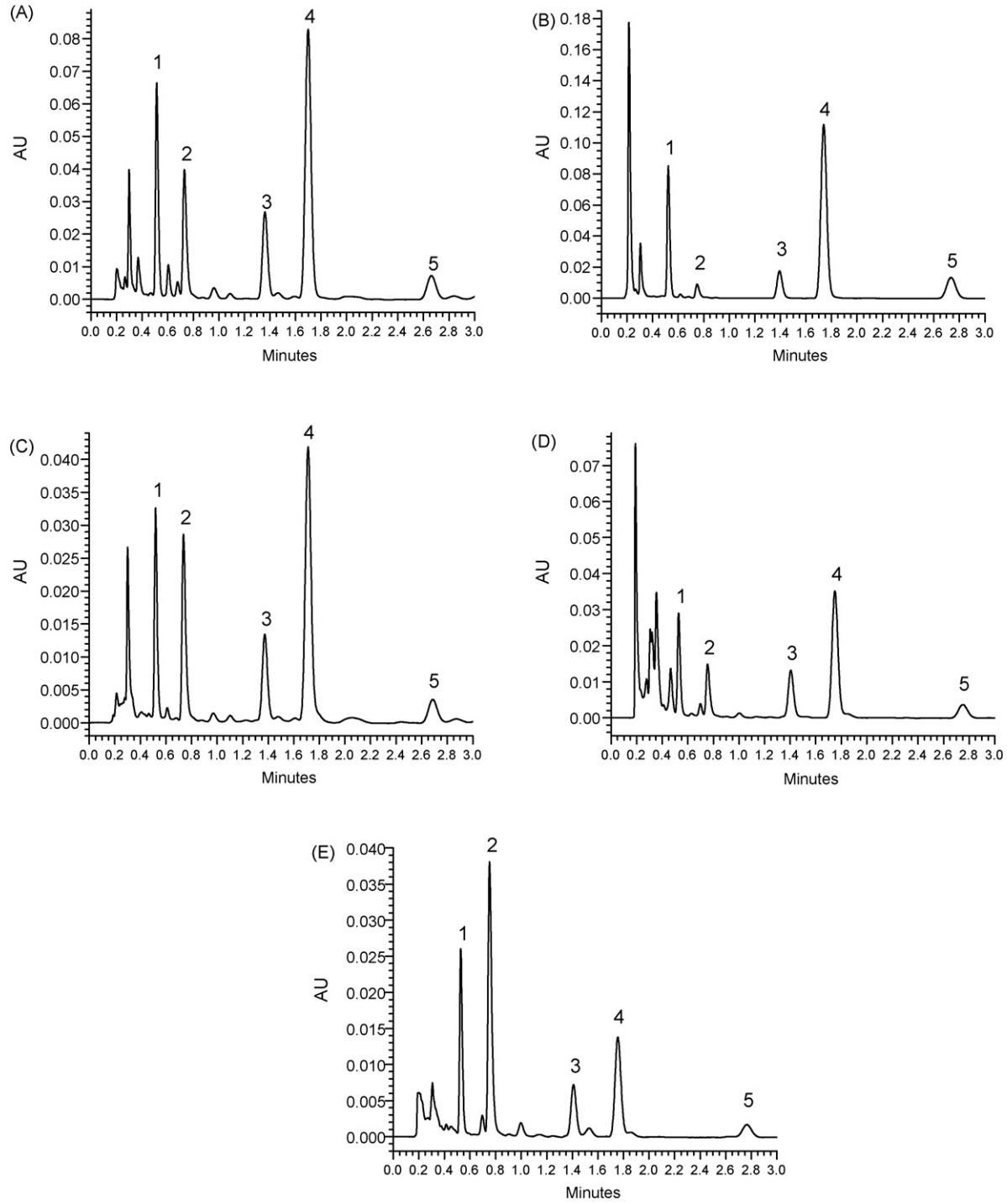

Fig. 4. UPLC-UV chromatograms of (A) R. *palmatum* L.; (B) R. *Tanguticum* Maxim. Ex Reg.; (C) R. *officinale* Baill.; (D) Sanhuang tablet; and (E) Paidu Yangyan capsule. *Key*: see Fig. 3. Chromatographic conditions: see Section 2.3.

3.3. UPLC method validation

3.3.1. Precision

The precision was evaluated by carrying out six independent assays. The %RSD was within the acceptable limit of 2%. The intermediate precision was also within 2%.

3.3.2. Sensitivity

The LOD and LOQ of five AQs for UPLC are summarized in Table 2. The better sensitivity of UPLC over HPLC can be characterized by comparison of LOQ values of physcion in both systems. The LOQ for UPLC was found to be 0.40 μg/ml, with RSD 2.8% at injection volume of 1 μl. The LOQ concentration for HPLC was found to be 1 μg/ml, with RSD 5.4% at injection volume 10 μl.

3.3.3. Linearity

Linear calibration plots of five AQs were obtained at seven concentration levels in triplicate (Table 2). The results showed excellent correlation between the peak area and concentration of five AQs.

Table 4
Determination of the five components in three species of rhubarb and related preparations

Compounds	Content ($n = 3$, mean ± S.D., mg/g)				
	R. palmatum L.	R. *Tanguticum* Maxim. Ex Reg.	*R. officinale* Baill.	Sanhuang tablet	Paidu Yangyan capsule
Aloe-emodin	11.42 ± 0.27	14.65 ± 0.3	5.56 ± 0.14	5.26 ± 0.11	4.37 ± 0.09
Rhein	10.72 ± 0.27	2.51 ± 0.07	7.63 ± 0.14	4.06 ± 0.11	10.16 ± 0.18
Emodin	11.5 ± 0.22	7.64 ± 0.12	5.52 ± 0.13	5.73 ± 0.1	3.25 ± 0.08
Chrysophanol	24.53 ± 0.52	33.85 ± 0.79	12.81 ± 0.2	10.95 ± 0.3	4.03 ± 0.11
Physcion	6.54 ± 0.12	12.49 ± 0.25	3.07 ± 0.08	3.4 ± 0.07	1.49 ± 0.04
Total	64.71 ± 0.7	71.14 ± 0.765	34.59 ± 0.345	29.4 ± 0.345	23.3 ± 0.25

3.3.4. Accuracy

The accuracy of the method was determined by spiking known amount of mixed standards in known rhubarb samples (R. *Tanguticum* Maxim. Ex Reg.) in triplicate at levels 50%, 100% and 150% of the specified limit. The recoveries of five AQs were calculated and given in Table 3. The recovery of the investigated components ranged from 97.0% to 99.0%, and their RSD values were all less than 5.0% characterizing good reliability and accuracy of the method.

3.3.5. Robustness

In all the deliberately varied chromatographic conditions, the chromatogram of sample solution showed satisfactory resolution.

3.4. Application

The developed UPLC method was applied to the simultaneous determination of five AQs in three species of rhubarb and two preparations. The target components were identified by comparing their retention times and UV spectra with those presented in the chromatogram of the mixture standard solution. The peak purity of target components in these samples was verified using photo diode array (PDA) detector. The peak purity of five AQs in three rhubarb species and Paidu Yangyan capsule was found to be satisfactory. Although the peak purity of aloe-emodin in Sanhuang tablet was lower than that of others, it is still acceptable. The chromatograms are depicted in Fig. 4 and the results are presented in Table 4. Although the total content of five AQs in three tested rhubarb samples was above the current quality control criterion of the Chinese Pharmacopoeia (15 mg/g), the content of five AQs in different rhubarb species was quite variable, which influences the stability of the quality. Therefore, it is recommendable to control the contents of the individual active components rather than their total content. The current Chinese Pharmacopoeia [29] does not contain simultaneous determination of the five AQs in Sanhuang tablets: it should contain no less than 3.88 mg/g of emodin and physcion totally. There is no quality criterion in Chinese Pharmacopoeia for Paidu Yangyan capsule. The developed UPLC method seems to be a promising tool to improve the quality control of these two preparations.

4. Conclusion

The UPLC method developed for the quantification of emodin, rhein, aloe-emodin, chrysophanol and physcion was found to be capable of giving faster analysis with good resolution than that achieved with conventional HPLC. The method was completely validated showing satisfactory data for all the parameters tested. This method is also eco-friendly for its low consumption of organic solvents as compared to other analytical techniques. Overall, it is suitable for rapid and accurate quality control of rhubarb and its related preparations.

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