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Computer-aided development of a high-performance liquid chromatographic method for the determination of hydroxyanthraquinone derivatives in Chinese herb medicine rhubarb

C.-L. Liu, P.-L. Zhu, M.-C. Liu*

Department of Chemistry, Lanzhou University, Lanzhou 730000, China

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Abstract

With computer simulation predicting separation in reversed-phase gradient elution, a method to separate and determine five hydroxyanthraquinone derivatives having a wide range of polarity in extract of Chinese herbal medicine rhubarb has been developed. The software DryLab was used to model the retention behavior of the compounds as a function of gradient conditions, based on data from two scouting gradient runs. Under the optimized conditions, i.e. column, Zorbax RX-C_{18}, 5 μm, 15×0.46 cm; solvent A, 36 m*M* triethylamine phosphate (TEAP), pH=2.5; solvent B, ACN; gradient, 36/36/80/80% B at 0/5.5/20.5/25.5 min; flow-rate, 1.00 ml/min; temperature, ambient, the method was successfully applied to monitor the quality of rhubarb from different sources. The effect of sample preparation procedures on extraction efficiency was also examined.

Keywords: Computer simulation; LC; Rhubarb; Optimization; Hydroxyanthraquinone derivatives

1. Introduction

Rhubarb is one of the important ingredients in Chinese traditional prescriptions. It contains hydroxyanthraquinone derivatives: rhein, emodin, aloe–emodin, chrysophanol, physcion and their glycosides having various pharmacological actions, such as purgation, antibacterial, antitumor [1]. Thus, the determination of the active components in rhubarb is required for the evaluation of its quality and the control of dosage during clinical studies. Several methods have been reported for the determination of the active components in rhubarb, including TLC [2], GC [3], CE [4], HPLC [5–8], and so on. Among HPLC methods, both normal and reversed-phase modes were set up. In a normal-phase method, the simultaneous separation of hydroxyanthraquinones, their glycosides and dimeric anthrone glycosides was carried out by tetrahydrofuran–15% acetic acid gradient on a dimethylamino bonded phase column. In this case, hydroxyanthraquinone peaks crowded at the head of the chromatogram and could not be separated from each other [6]. In the case of reversed-phase HPLC, using a C_{18} column and 0.1% $HClO_4$–methanol [7], or water–methanol [8] solvent combinations, five hydroxyanthraquinones were separated by isocratic elution. However, serious band broadening and tailing were observed. Recently, in a study about the constituents of senna, a HPLC method was developed to separate and determine 17

*Corresponding author.

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bianthranyls and anthraquinones [9]. HPLC was carried out by gradient elution, using a C_{18} column and 0.02 *M* phosphate buffer, pH 2–ACN solvent combination. So it is still necessary to develop a HPLC method which can be used for effective separation and quantitative determination of the five active components.

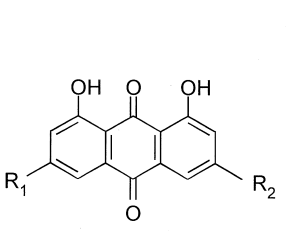
The DryLab software is a tool to develop HPLC methods in short time. In the case of gradient elution, the data from two scouting runs with a sample are input to simulate retention behavior of each component in the sample as a function of gradient conditions. Up to now the software has been applied to the HPLC method developments for various samples [10].

In this work, we developed a new HPLC method to analyze the hydroxyanthraquinone compounds in rhubarb, based on the prediction from DryLab simulation. In order to improve the extraction efficiency of hydroxyanthraquinone, several procedures for sample preparation were examined. The contents of the active components were compared for the rhubarb samples from different origins.

2. Experimental

2.1. Chemicals and reagents

Rhein, emodin, aloe–emodin, chrysophanol and physcion were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The structures of the five compounds are shown in Fig. 1. Rhubarb and other Chinese herbal samples were bought from a drug store. Two rhubarb samples from the different places of origin, Hebei and Gansu, were referred to as Sample H and Sample G. Acetonitrile (No.2 Chemical Reagent Factory, Tianjin, China) and triethylamine, TEA, (No.3 Chemical Reagent Factory, Shanghai, China), were of analytical grade and were distilled before use. Double distilled water was used for preparing solutions.



	R_1	R_2
Aloe-emodin	H	CH_2OH
Emodin	OH	CH_3
Rhein	H	COOH
Physcion	OCH_3	CH_3
Chrysophanol	H	CH_3

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

36 m*M* TEAP buffer, pH=2.5. Add 5 ml of TEA to ≈950 ml of water in a beaker, with stirring. Adjust to pH 2.5 with 85% phosphoric acid. Transfer the solution to a 1 l volumetric flask and QS with water. Pass the solution through a 0.45 μm membrane filter and degas by vacuum.

2.2. Equipment

High-performance liquid chromatograph system consists of automated gradient controller, 501 and 510 HPLC pumps (Waters, Milford, MA, USA), Rheodyne 7125 injection valve with 6μl loop (Cotati, CA, USA), Perkin-Elmer LC-95 UV/visible spectrophotometer detector (Norwalk, CT, USA), and 3066 recorder (the No.4 Instrument and Meter Plant, Sichuan, China). The dwell volume of the system is 1.20 ml. The chromatographic conditions were set up as follows: analytical column, 15×0.46 cm Zorbax RX-C_{18}, 5 μm (Rockland Technologies, Newport, DE, USA); gradient solvent A, 36 m*M* TEAP, pH 2.5; solvent B, acetonitrile; flow-rate, 1.00 ml/min; detection, UV 254 nm; temperature, ambient.

In order to cleanup the aqueous solvent A in gradient elution, a valve-switching technique [11] was adopted. A precolumn packed with Supelclean™ LC-18 SPE packing (Supelco, Bellefonte, PA, USA) was placed between the A-solvent pump and the high-pressure mixer. A six-port valve (Japan Spectroscopic Co., Kyoto, Japan) was used for valve switching. The procedure of cleanup is given in [11].

The software DryLab used for computer simulation was provided by LC Resources (Walnut Creek, CA, USA). The optimized gradient profile (see Section 3.2) was 36/36/80/80% B at 0/10/25/30 min. The precolumn for cleaning up solvent A was turned to cleanup and flushing positions by valve-switching at 0 and 30 min, respectively. Then the valve kept on the flushing position with 80% B for 5 min. The sample injection was carried out at 4.5 min.

So the actual gradient profile for the separations was 36/36/80/80% B at 0/5.5/20.5/25.5 min.

2.3. Sample preparation

The procedures of sample preparation are the following:

Method 1. Extract 0.05 g of rhubarb powder with 30 ml of 95% ethanol for 30 min by refluxing three times. Then combine and evaporate the ethanol extracts on a water bath to dryness. Add 15 ml of 2 *N* H_2SO_4 to dissolve the residue and keep the solution on the water bath for 30 min. Extract the hydrolyzed solution with 10 ml of chloroform five times and evaporate the combined extracts on a water bath to dryness. At last dissolve and transfer the residue into a 25 ml volumetric flask by 36 m*M* TEAP (pH=2.5)–ethanol (v/v 65:35). The solution was filtered with a 0.45 μm membrane before injection.

Method 2. Extract 0.05 g of rhubarb powder with 15 ml of 2 *N* H_2SO_4 and 20 ml of chloroform for 30 min by refluxing three times. Take out the chloroform layer only after each refluxing. Then combine and evaporate the chloroform extracts on a water bath to dryness. Dissolve and transfer the residue into a 25 ml volumetric flask by 36 m*M* TEAP (pH=2.5)–ethanol (v/v 65:35). The solution was filtered with a 0.45 μm membrane before injection.

Method 3. Extract 0.05 g of rhubarb powder with 30 ml of chloroform for 30 min by refluxing three times. Combine and transfer the extracts into a 100 ml volumetric flask by chloroform. Evaporate a 50 ml aliquot on a water bath to dryness. Dissolve and transfer the residue into a 10 ml volumetric flask by 36 m*M* TEAP (pH=2.5)–ethanol (v/v 65:35). The solution was filtered with a 0.45 μm membrane before injection.

3. Result and discussion

3.1. The selection of mobile phase

Generally ionic or ionizable species are retained weakly on non-polar silica gel based stationary phases and give poorly defined peaks with severe tailing. If ionization is suppressed by adjusting pH of mobile phase or forming associates with ion-pair reagents, the retention of the solutes would be increased and the peak shape improved. Two phenolic groups locating in 1,8-hydroxyanthraquinone display weak acidity ($pK_{a,1}$=8.30 and $pK_{a,2}$=12.46). Among the five hydroxyanthraquinone derivatives, only rhein has a carboxylic group (Fig. 1). Although the pK_a value of the group is unknown, it can be estimated to be around 4. Thus, the TEAP, pH 2.5–ACN solvent combination was tried first to be used as the mobile phase in the chromatographic separation. The low pH is necessary for ion suppression and the TEA cation might eliminate the deleterious effect of silanol groups existing on the surface of packing. And the phosphate buffer with pH 2.5 has buffering capacity high enough to keep pH constant. The results obtained in this work show that the TEAP, pH 2.5–acetonitrile is an appropriate choice for the separation of hydroxyanthraquinone compounds. (See below)

3.2. Simulation of chromatography with DryLab

The theory and procedure to optimize chromatographic conditions by DryLab are given in [12]. In

Table 1
Retention data from two scouting runs

Compounds	Retention time (min)	
	Run 1 (30–80% B in 30 min)	Run 2 (30–80% B in 40 min)
Aloe–emodin	11.20	12.45
Rhein	12.10	13.65
Emodin	18.20	21.15
Chrysophanol	26.00	31.65
Physcion	28.80	35.40

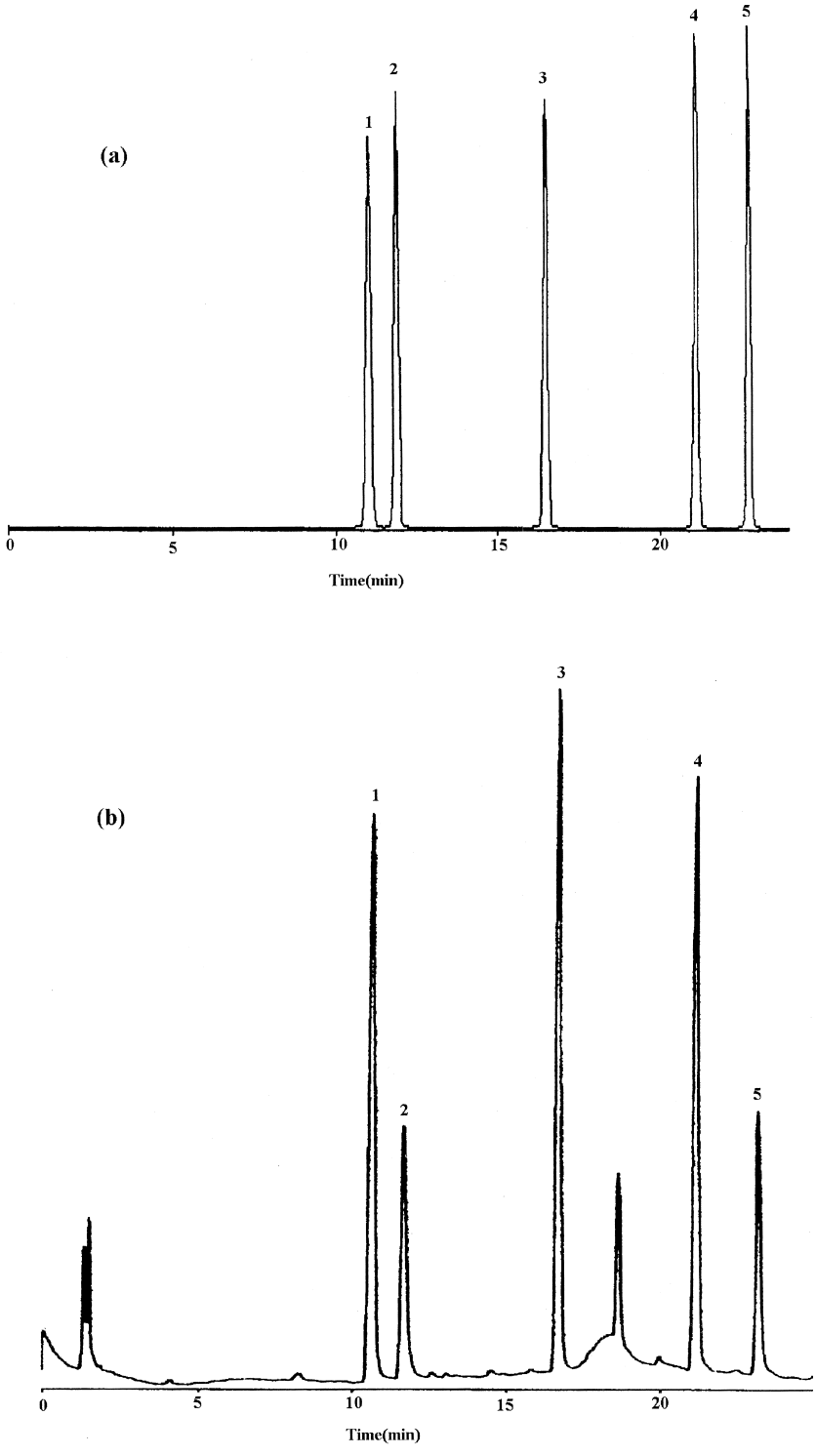


Fig. 2. (a) Predicted and (b) actual separations. Compounds: (1) aloe–emodin; (2) rhein; (3) emodin; (4) chrysophanol; and (5) physcion. Chromatographic conditions: see Section 2.2.

Table 2
Calibration curve data

Compounds	Correlation coefficient of regression equation	Range (μg/ml)
Aloe–emodin	0.9997	1.2–24
Rhein	0.9996	0.6–12
Emodin	0.9994	1.3–26
Chrysophanol	0.9999	0.95–38
Physcion	0.9994	0.48–9.6

the case of gradient elution, two scouting runs are carried out prior to computer simulation to predict separation as a function of gradient and column conditions. The composition of natural products is so complex that it is impossible to prepare synthetic samples containing most of components in realistic ones. Therefore, two steps are involved in the optimization of chromatographic conditions for natural products. Firstly, an optimized method for a synthetic sample containing all of the determined components is established. Then, the method is used to analyze realistic samples. If a satisfactory separation is obtained, the method will be accepted without any modification. Otherwise, it will be adjusted further by DryLab to obtain the final procedure.

In this work, five hydroxyanthraquinones have a wide range of capacity factor k'. For instance, the isocratic elution with 50% B has a 1.5–45.9 k' range. According to the first initial run with 5–90% B in 15 min, the gradient profile conditions for two scouting runs, 30–80% B in 30 and 40 min, were selected to obtain the retention data listed in Table 1. Predicted by DryLab, the critical pair in the separation is aloe–emodin/rhein. The resolution between the two components increases with the decreasing strength of the mobile phase while the isocratic elution is favorable to their separation. Thus, 36% B is kept at the beginning of the elution for 5.5 min, followed by a linear gradient. In this case, both retention time and resolution are given consideration. The optimized gradient profile for the separation of hydroxyanthraquinones is 36/36/80/80% B at 0/5.5/20.5/25.5 min. The comparison between the predicted and experimental separations is shown in Fig. 2. It can be seen that the chromatographic behavior predicted by DryLab is consistent with the experimental result.

3.3. Calibration curves

Under the specified chromatographic conditions, the peak heights were regressed against the concentrations of the five hydroxyanthraquinone derivatives to give the correlation coefficients of calibration curves shown in Table 2. We were interested especially in the region around 0.5–50 μg/ml of active components for the samples we wanted to evaluate. The data in Table 2 shows us good linearity in the concentration range.

3.4. Sample preparation

In the literature, various solvents were used for extracting the active components from rhubarb, including methanol, ethanol, chloroform and so on. However, no discussion about the effect of solvents on the extraction efficiency was given. In this work, we selected ethanol and chloroform as extracting solvents and compared their extraction efficiencies with each other.

The hydroxyanthraquinone derivatives in rhubarb exist in the free forms and their glucosides. In

Table 3
Analytical results of Sample H prepared by Method 1 and 2

Compounds	Content (%)			
	Sample H prepared by Method 1		Sample H prepared by Method 2	
	Average (n=3)	Std. Dev. (%)	Average (n=3)	Std. Dev. (%)
Aloe–emodin	0.15	15.1	0.33	3.0
Rhein	0.03	0.0	0.07	0.0
Emodin	0.09	6.2	0.20	9.9
Chrysophanol	0.52	9.4	1.02	14.1
Physcion	0.05	28.6	0.18	11.5

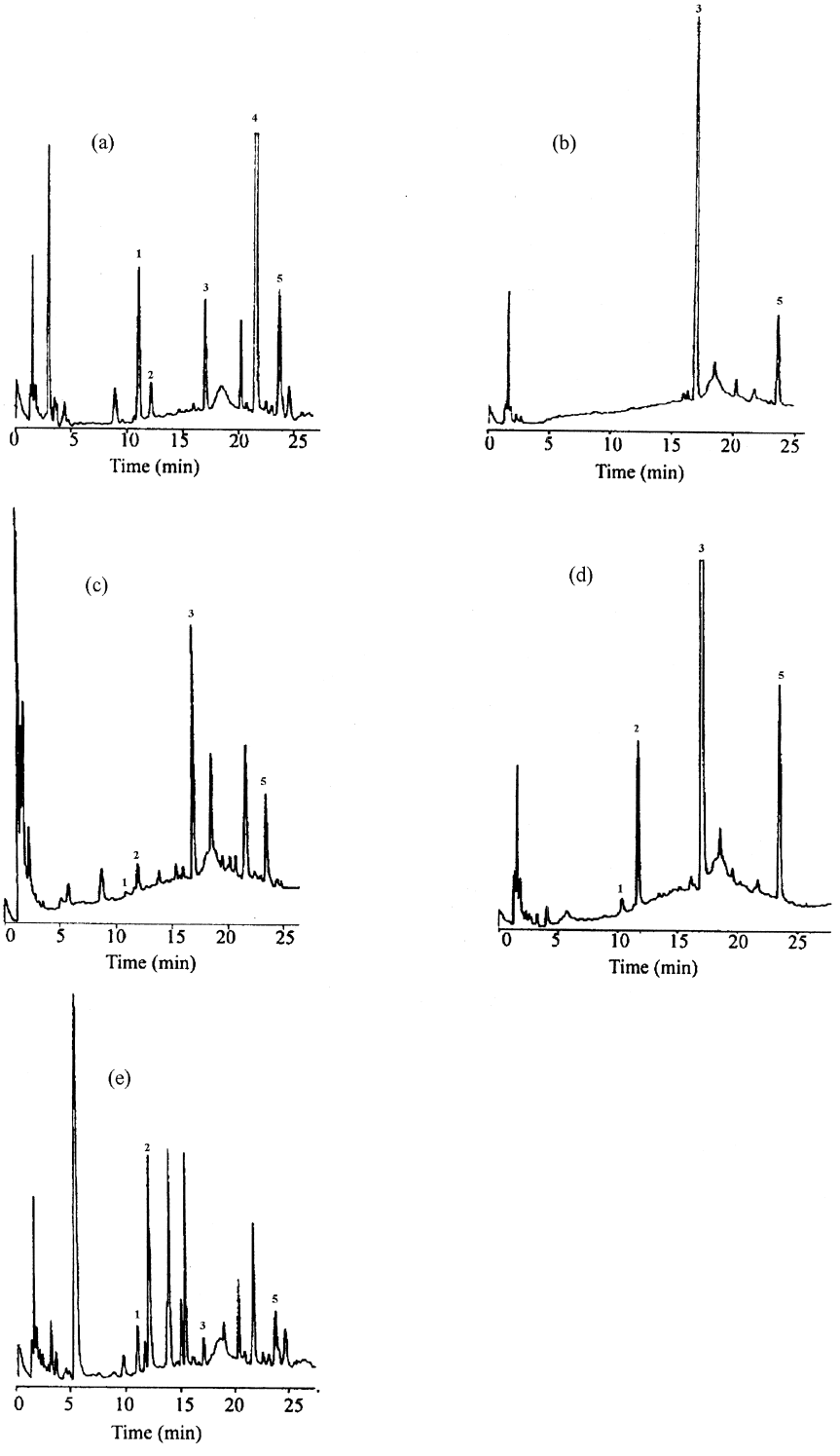


Fig. 3. Chromatograms of (a) rhubarb; (b) fleeceflower tuber; (c) fleeceflower stem; (d) giant knotweed; and (e) senna leaf. Compounds: (1) aloe–emodin; (2) rhein; (3) emodin; (4) chrysophanol; and (5) physcion. Chromatographic conditions: see Section 2.2.

Table 4
Analytical results of five active compounds in Sample H and Sample G

Compounds	Content (%) of Sample H		Content (%) of Sample G	
	Average (n=3)	Std. Dev. (%)	Average (n=3)	Std. Dev. (%)
Aloe–emodin	0.33	3.0	0.03	0.0
Rhein	0.07	0.0	0.01	0.0
Emodin	0.20	9.9	0.31	1.9
Chrysophanol	1.02	14.1	0.66	0.9
Physcion	0.18	11.5	0.09	11.1
Total	1.80		1.10	

Method 1 of sample preparation, the glycosides are transformed to their free forms by hydrolysis so that the total amount of anthraquinones can be determined from the chromatographic analysis. In Method 2, the extraction by chloroform and hydrolysis are carried out simultaneously. In the case, the total amount of anthraquinones is determined also. Two procedures were followed for sample preparation as described in Section 2.3. The difference between Method 1 and 2 is that extraction is carried out prior to hydrolysis step in Method 1, but extraction and hydrolysis are completed in one step in Method 2. Starting with the samples before extraction and hydrolysis, three analyses were carried out for each method. Comparing the analytical results in Table 3 obtained by both methods, it can be seen that Method 2 gives higher contents than Method 1 does. That implies that some of anthraquinone glycosides could not be fully extracted by ethanol. To prove that hydroxyathraquinones could be extracted completely by Method 2, the remains of rhubarb was treated once again by 20 ml of chloroform. The content of the compounds in the extract was less than 2% total amount. Thus, chloroform was selected as the solvent for sample preparation. The sample used for the determination of the free anthraquinones is prepared by Method 3 (see Section 2.3) where hydrolysis step is omitted. The total amount obtained from the sample that is prepared by Method 2 minus the amount of free forms is equal to one of glycosides.

The influence of acidity on hydrolysis of glycoside was examined by increasing the concentration of

Table 5
Recovery data by standard addition

Compounds	Concentration (μg/ml)	Added (μg/ml)	Obtained (μg/ml)	Recovery (%)
Aloe–emodin	2.69	1.92	4.70	101.9
		2.88	5.47	98.4
		3.36	5.87	97.2
Rhein	0.58	0.96	1.52	98.7
		1.44	2.04	101.0
		1.68	2.16	95.6
Emodin	1.51	2.08	3.64	101.4
		3.12	4.52	97.8
		3.64	4.94	96.0
Chrysophanol	7.75	3.04	11.32	104.9
		4.56	12.16	99.4
		5.32	12.81	98.0
Physcion	1.32	0.77	2.17	103.6
		1.16	2.50	100.8
		1.35	2.62	98.1

Table 6
Analytical results of five active compounds in fleeceflower tuber, fleeceflower stem, giant knotweed and senna leaf

Compounds	Content (%)[a]			
	Fleeceflower tuber	Fleeceflower stem	Giant knotweed	Senna leaf
Aloe–emodin	–[b]	0.002	0.02	0.08
Rhein	–	0.002	0.13	0.20
Emodin	0.38	0.013	1.98	0.02
Chrysophanol	–	–	–	–
Physcion	0.06	0.003	0.10	0.02
Total	0.44	0.020	2.23	0.32

[a] Average of duplicate determinations.
[b] –: Not detected.

hydrochloric acid from 2 *N* to 9 *N*. No difference was found. It was shown that 2 *N* HCl is enough to break the band of O-glycoside.

3.5. Applications

The developed method in this work was applied to evaluate the quality of the rhubarbs from two places of origin, Sample H and Sample G (see Section 2.1). A chromatogram of Sample H is shown in Fig. 3 and the analytical results are listed in Table 4. The recovery of the method was examined by adding standards into Sample H as shown in Table 5. It is seen from the chromatogram that the established gradient profile for synthetic sample (see Section 3.2) can be used to analyze the realistic samples without further adjustment.

The hydroxyanthraquinone derivatives in some Chinese herbal medicines such as fleeceflower tuber, fleeceflower stem, giant knotweed and senna leaf could be determined by the same procedures also. The chromatograms and analytical results are given in Fig. 3 and Table 6, respectively. The peaks of hydroxyathraquinones were identified by known standards.

4. Conclusions

The analytical method and sample preparation procedures developed in this work can be applied to the determinations of hydroxyanthraquinone derivatives in rhubarb and some herbal medicines. For sample preparation, chloroform is preferred to ethanol to obtain high extraction efficiency. With the gradient profile varied, the method might be developed further to determine more anthraglycosides.

References

[1] Dictionary of Traditional Chinese Medicine, 1st ed, New Medical College of Jiangsu, People's Publisher of Shanghai, 1997, pp. 102.
[2] P.P. Rai, T.D. Turner, S.L. Greensmith, J. Pharm. Pharmacol. 26 (1974) 722.
[3] T. Furuya, S. Shibata, H. Iizuka, J. Chromatogr. 21 (1966) 116.
[4] Y.-Y. Zong, M.-T. Jin, Yaoxue Xuebao 30 (1995) 594.
[5] P.P. Rai, T.D. Turner, J. Chromatogr. 110 (1975) 401.
[6] Y. Ohshima, Y. Ohno, K. Kajiyama, K. Takahashi, J. Chromatogr. 360 (1986) 303.
[7] W.-L. Luo, J. Ping, Yaowu Fenxi Zazhi 9 (1989) 259.
[8] S.-L. Lin, L.-H. Zhao, Z.-N. Wu, Zhongcaoyao 20 (1989) 250.
[9] W. Methger, K. Reif, J. Chromatogr. A 740 (1996) 133.
[10] L.R. Snyder, J.L. Glajch, J.J. Kirkland, in: Practical HPLC Method Development, 2nd ed, Wiley-Interscience, New York, 1997, Chap. 10.
[11] P.-L. Zhu, L.R. Snyder, J.W. Dolan, J. Chromatogr. A 718 (1995) 429.
[12] J.W. Dolan, D.C. Lommen, L.R. Snyder, J. Chromatogr. 485 (1989) 91.