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Determination of Active Components in Rhubarb and Study of Their Hydrophobicity by Micellar Electrokinetic Chromatography

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Abstract—A micellar electrokinetic chromatographic (MEKC) method has been developed for the determination of five anthraquinones and one distyrene derivative in rhubarb. The separation conditions were optimized and two kinds of rhubarb plants and rhubarb-containing medicines were analyzed. The negatively charged solutes migrated toward the anode and were retarded by their interaction with the micelle. Hydrophobicity of the solutes was studied by both MEKC with SDS and SDS-free capillary zone electrophoresis in the buffer of 15 mmol/L NaH_2PO_4 + 20 mmol/L borax and 15% ethanol (v/v). Linear correlation between log k' and log P_{OW} was obtained for the five anthraquinones in SDS micelle system. The capacity factor, k', and free energy differences $\Delta(\Delta G)$ derived from this method provided fundamental information on the interaction between the solutes and the micelle.

Rhubarb, one of the ancient and best-known Chinese herbal medicines, has been used for thousands of years. It has the effect of purgation, purging heat, loosening the bowels, curing metal and renal disorders and removing bacterial dysentery, etc.[1] What is more, rhubarb has the action of antitumor and antimutagenicity.[2] Besides its pharmacological values, rhubarb can also be made as nourishing food. Most Rheum species are produced in China, and are exported under various commercial names according to their appearance, quality or place of production. Rhubarb contains anthraquinone derivatives, anthrones and tannins, etc., in which anthraquinone derivatives including emodin, aloe-emodin, rhein, physcion, chrysophanol and their glucosides are the accepted important active components. Rhaponticin, a distyrene derivative, only exists in non-quality (inferior-grade) rhubarb. In quality rhubarb and most exported rhubarbs, the content of rhaponticin are not allowed to be detected.

The methods commonly used for the determination of the anthraquinone compounds in Rhubarb are thin-layer chromatography (TLC)[3] and high performance liquid chromatography (HPLC).[4] But none of these methods are entirely adequate because of either low resolution or large consumption of organic solvent. High performance capillary electrophoresis (HPCE) has proved to be a highly efficient separation technique in the pharmaceutical industry. In 1995, Sheu et al.[5] used MEKC for the determination of three anthraquinones in rhubarb in SDS solution with acetonitrile. In the same year, Zong et al.[6] also adopted the MEKC method to separate five anthraquinones in rhubarb but determined only three of them. In recent years, Chai and Ji et al.[7,8] applied SDC micelle to the separation of five anthraquinones in rhubarb, and determined rhein, emodin and aloe-emodin three components. In the previous study, Shang et al.[9] reported the determination of six components in rhubarb by using a mixed micellar system. MEKC is a branch of CE, the principle of the separation is based on the distribution of the solute between an aqueous phase and a micellar phase. Therefore, MEKC is very effective for the separation of hydrophobic compounds. Hydrophobicity plays an important role in the biological and physicochemical behavior of numerous classes of organic compounds. It is well known that the hydrophobicity of drugs affects their absorbability and transportation properties. The estimation of hydrophobic parameters has been carried out by HPLC,[10] liposome capillary electrophoresis (LCE),[11] MEKC[12] and microemulsion electrokinetic chromatography (MEEKC).[13] The logarithm of *n*-octanol-water partition coefficient, log P_{OW}, has been widely used as a hydrophobic parameter. However, log P_{OW} of

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the five anthraquinones has not been determined yet. In this paper, we describe a MEKC method for the determination of rhaponticin, rhein, emodin, aloe-emodin, chrysophanol and physcion six compounds in rhubarb plant and its preparations. The log P_{OW} values of the five anthraquinones are determined and calculated. Solute-micelle interaction with SDS micelle system is investigated. Data of capacity factors and relative Gibbs free energy reflects the degree of hydrophobicity of the analytes.

Reagents

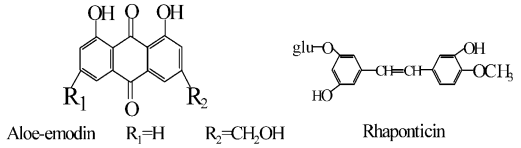
Emodin, aloe-emodin, rhein, rhaponticin, chrysophanol and physcion were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their structures are shown in Figure 1. SDS was purchased from the Academy of Military Medical Science (Beijing, China). *o*-Phthalic acid was bought from Beijing Chemical Factory (Beijing, China). Commercial rhubarb samples Hua-pe-da-huang and *R. palmatum* L. were bought from Tong Ren Tang drug store in Anguo (Hebei, China). Niu Huang Jie Du Pian and Jin Yi Niao Shi Granules were purchased from Tong Ren Tang drug store in Beijing (Beijing, China). *n*-Octanol was bought from Changsha Factory of Organic Reagent (Changsha, China). Sodium dihydrogenphosphate and borax were of analytical grade. Double distilled water was used for preparing solutions.

Apparatus

The experiments were performed on a 1229 type HPCE Analyser system (Beijing Institute of New Technology and Application, Beijing, China) with an UV detector (254 nm). Separation was carried out in a bare fused-silica capillary (50 μm ID) whose effective length was 40 cm. The temperature was kept at 20±1 °C. The applied voltage was 15 kV. Sample solutions were introduced by electromigration at 10 kV for 5 s. Before doing the experiment, the capillary was cleaned consecutively by washing with 0.1 mol/L NaOH for 20 min, distilled water for 20 min and running buffer for 10 min, in that order.

Sample Preparation

Commercial rhubarb powder 0.5 g or 1 g sample of rhubarb-containing medicines was extracted in Soxhlet extractor with 15 mL of 2 mol/L H_2SO_4 and 70 mL anhydrous ethanol. The extraction was continued until the aqueous phase was almost colorless. Then the extract was evaporated to dryness on a water bath. The residue was dissolved by anhydrous ethanol. After addition of 50 μL internal standard (I.S.) solution (*o*-phthalic acid), the extract was diluted to 50 mL with NaH_2PO_4 solution. All solutions were filtered through a 0.45 μm membrane before injection.



Aloe-emodin	R_1=H	R_2=CH_2OH
Rhein	R_1=H	R_2=COOH
Emodin	R_1=OH	R_2=CH_3
Physcion	R_1=CH_3O	R_2=CH_3
Chrysophanol	R_1=H	R_2=CH_3

Figure 1. Structures of the six components in rhubarb.

Extraction Recovery Studies

Fixed amounts of authentic rhein, emodin, aloe-emodin, physcion, chrysophanol and rhaponticin were added to Hua-pe-da-huang of known contents, respectively. The mixtures were extracted and analyzed three times as the method of sample preparation.

Determination and Calculation of log P_{OW} for the Anthraquinones

The log P_{OW} values of the five anthraquinones were determined by traditional shake-flask method.[14] The compounds were dissolved by anhydrous ethanol (0.3 mg/mL). Absorbance of the sample was determined on a 721-type spectrophotometer (254 nm).

The log P_{OW} values of the five anthraquinones were calculated by 'fragmental' method of Leo/Hansch. The determination and calculation results were listed in Table 1.

Separation of Six Components by MEKC

SDS micelle was used as a pseudostationary phase in buffer solution of 15 mmol/L NaH_2PO_4 + 20 mmol/L borax (pH 9.7) and 15% ethanol (v/v). At pH 9.7, the six analytes were deprotonated to a different extent, so they had electrophoretic mobilities (μ) in the direction of the anode in the presence of an electric field. The anionic micelle also migrated towards the anode. Injections were made at the anode.

The effect of changes of SDS concentration on the separation was studied. A plot of capacity factor (k') of the solutes versus SDS concentration is plotted in Figure 2. k' values were calculated by eq 2 in the following section. The k' values of the solutes increased with increasing SDS concentration, especially chrysophanol

Table 1. Results of determination and calculation of log P_{OW} for anthraquinones

Compd	Log P_{OW}		
	Determination	Calculation	Deviation (%)
Rhein	2.42	2.37	2.06
Aloe-emodin	2.68	2.62	2.23
Emodin	2.91	2.87	1.37
Chrysophanol	3.63	3.39	6.61
Physcion	3.85	3.65	5.19

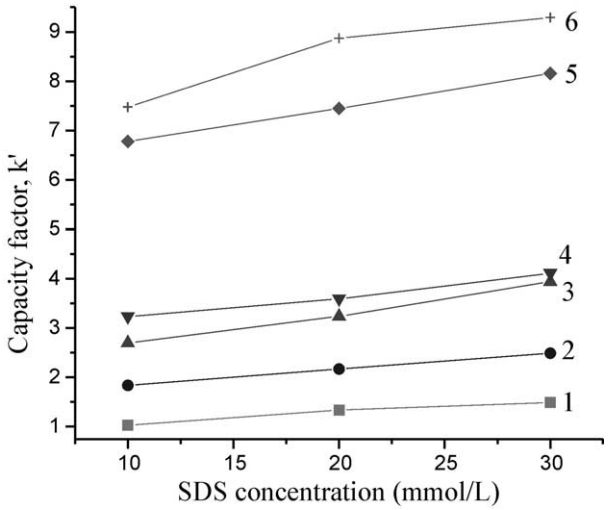


Figure 2. Effect of SDS concentration on capacity factor of solutes. (1) Rhaponticin, (2) rhein, (3) aloe-emodin, (4) emodin, (5) chrysophanol, (6) physcion. Conditions: 15 mmol/L NaH_2PO_4 + 20 mmol/L borax + 15% ethanol (v/v), pH 9.7, 18 kV.

and physcion had obviously larger capacity factors than other solutes. With increase of SDS concentration, differences of k' of the solutes became large, which was conducive to improve the resolution. The solutes were retarded by their partition and interaction with the micelle, accordingly, the ionic mobility and migration times of the solutes changed. The more the migration time is, the more is the interaction with micelle, and the solutes migrate with the micelle more slowly. At SDS concentration of 20 mmol/L, good resolution was achieved, and the smallest resolution between chrysophanol and physcion was 2.09.

The effect of buffer pH on the retention and resolution was investigated in the range of 8.5–10.3. Figure 3 shows the variation of pH on electrophoretic mobilities of the

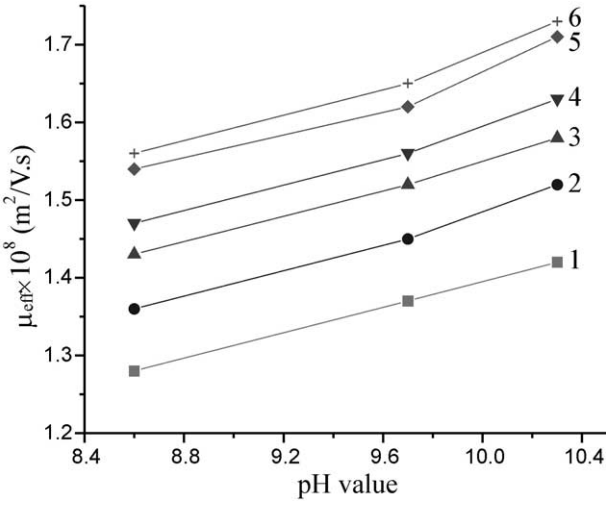


Figure 3. Effect of pH on electrophoretic mobilities of solutes. (1) Rhaponticin, (2) rhein, (3) aloe-emodin, (4) emodin, (5) chrysophanol, (6) physcion. Conditions: 15 mmol/L NaH_2PO_4 + 20 mmol/L borax + 15% ethanol (v/v) and 20 mmol/L SDS, 18 kV.

solutes with pH values. The magnitude of μ for the solutes were smaller than that for SDS, and therefore they had greater net velocities in the direction of the cathode since the velocity of the electroosmotic flow (EOF) was much greater than the electrophoretic velocities in the opposite direction. Variation of pH changed the electroosmotic velocity of the aqueous phase and the solutes. As the pH value increased, the net velocities of the solutes increased, and their migration times decreased. Seen from the figure, at pH 10.3, the solutes had a good peak resolution with the shortest migration times. Electrolyte pH values greater than 10.3, had a much higher current level, which produced excessive Joule heating, resulting in band broadening.

Influence of an organic modifier on the separation was investigated. Different contents of ethanol ranging from 0 to 20% in solution (15 mmol/L NaH_2PO_4 + 20 mmol/L borax and 20 mmol/L SDS) at pH 10.3 were prepared. Migration times of rhaponticin, rhein, emodin and aloe-emodin increased as more ethanol was added, which was due to the decrease of the EOF. As to chrysophanol and physcion, because ethanol had a great effect on their solubilization in micelle and aqueous phases than on the others, the migration times of them decreased firstly and then gradually increased. Although the addition of ethanol increased migration times of the solutes, it enhanced solubilization of the solutes in aqueous phase and improved their peak shapes. At ethanol content of 15% (v/v), good peak profiles and resolution were obtained.

Method Evaluation

The linearity response of the six components in standard solution was studied at five concentration levels. For each ingredient, calibration graph was constructed by concentration (y, μg/mL) versus ratio of corrected peak area to I.S. (x). The corrected peak area is defined as the ratio of the measured area to migration time of the peak. The slopes and intercepts of regression equations, linearity ranges and correlation coefficients are listed in Table 2.

The standard addition recoveries of the six components from an inferior-grade rhubarb sample Hua-peda-huang were assessed by the ratio of the amount produced and the amount added. When added concentrations of rhaponticin, rhein, emodin, aloe-emodin, chrysophanol and physcion were at 45.2, 27.6, 28.5, 27.1, 30.3 and 33.2 μg/mL, respectively, the recoveries of them were 93.1, 100.8, 103.4, 101.7, 93.9 and 97.6%, respectively.

The intra- and inter-day reproducibility of the method was determined at three concentration levels by measuring ten and nine replicate injections, respectively. For each compound, the intra- and inter-day relative standard deviation (RSD) values were 1.32 and 1.93% for rhaponticin, 1.15 and 1.81% for rhein, 0.78 and 1.19% for emodin, 0.97 and 1.49% for aloe-emodin,

1.62 and 2.03% for chrysophanol and 1.64 and 2.08% for physcion.

Detection limit was estimated as a peak with a signal-to-noise ratio of 3. Quantitation limit was calculated as a peak with a signal-to-noise ratio of 10. Data of detection limits and quantitation limits are listed in Table 1. Six replicate injections at the quantitation limit level gave an average RSD value of 5.6% for the ratio of corrected peak area (relative to I.S.).

Quantitative Analysis of Six Components in Rhubarb Plants and its Preparations

Under the optimized conditions, two kinds of Rhubarb plants, of which *R. palmatum* L. is quality rhubarb and Hua-pe-da-huang is non-quality rhubarb, and two kinds of Rhubarb-containing medicines Niu Huang Jie Du Pian and Jin Yi Niao Shi granules were analyzed. Typical electropherograms are shown in Figure 4. No interference was found in the electropherograms. The contents of the six components in real samples were determined in Table 3. Just as our previous study, rhaponticin does not appear in *R. palmatum* L., while it exists in the non-quality rhubarb sample Hua-pe-da-huang. In the two kinds of medicines, active ingredients and their contents are different.

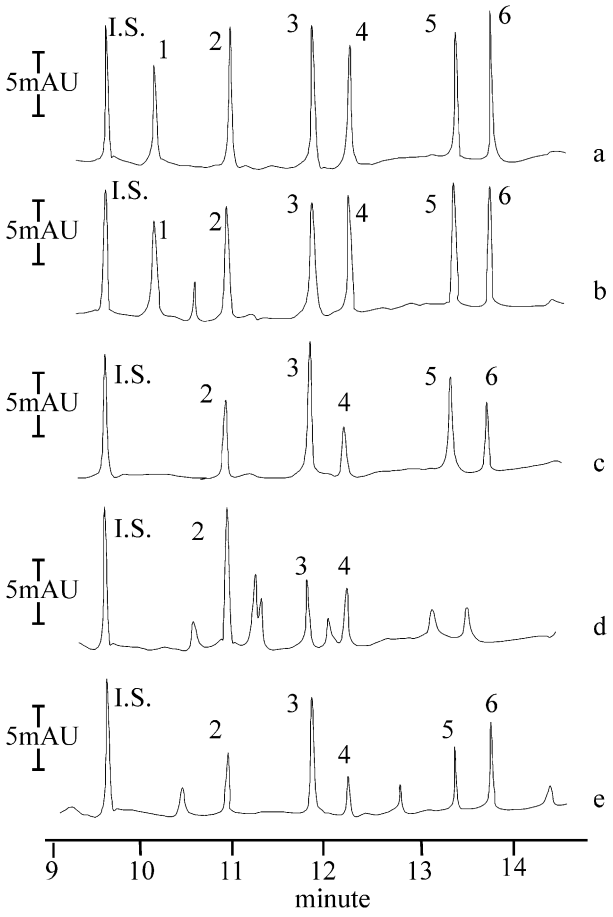


Figure 4. Typical electropherograms of real samples. (1) Rhaponticin, (2) rhein, (3) aloe-emodin, (4) emodin (5) chrysophanol, (6) physcion. Conditions: 15 mmol/L NaH_2PO_4 + 20 mmol/L borax + 15% ethanol (v/v) and 20 mmol/L SDS, pH 10.3; (a) Standard samples, (b) Hua-pe-da-huang, (c) *R. palmatum* L. (d) Niu Huang Jie Du Pian (e) Jin Yi Niao Shi granules.

Correlation of Log k′ with log P_{OW} for the Five Anthraquinones

If the distribution mechanism of the analytes in MEKC follows the same free energy relationship as the distribution in the octanol–water system, log k' will be linearly related to log P_{OW}. The retention factor relationship to the log P_{OW} takes the form of a Collander equation,

$$\log k' = a\log P_{OW} + b$$

Figure 5 shows the correlation of log k' with log P_{OW} for the five anthraquinones. A good linearity was found between log k' and log P_{OW} in this SDS micelle system. The regression line for log k' versus log P_{OW} is

$$\log k' = 0.579\log P_{OW} - 1.024 \quad (r = 0.9816)$$

Table 2. Calibration data, detection limits and quantitation limits

Compd	Slope	Intercept	Correlation coefficient	Linearity range (μg mL^{-1})	Detection limit (S/N = 3) (μg mL^{-1})	Quantitation limit (S/N = 10) (μg mL^{-1})
Rhaponticin	10.39	0.27	0.9991	6–90	0.92	2.78
Rhein	11.15	0.16	0.9993	4–40	0.90	2.75
Aloe-emodin	12.07	−0.21	0.9992	4–40	0.71	2.26
Emodin	15.36	0.05	0.9996	4–55	0.89	2.72
Chrysophanol	11.33	0.18	0.9981	5–76	1.04	3.14
Physcion	13.22	−0.19	0.9985	5–85	1.08	3.39

Table 3. Contents of the six components in rhubarb plants and rhubarb-containing medicines

Sample	Content (mg g^{-1})					
	Rhaponticin	Rhein	Aloe-emodin	Emodin	Chrysophanol	Physcion
Hua-pe-da-huang	0.217	0.832	1.152	1.168	0.773	0.655
R. palmatum L.	—	2.813	1.786	2.571	5.829	2.246
Niu Huang Jie Du Pian	—	0.437	0.785	0.117	—	—
Jin Yi Niao Shi Granules	—	0.661	0.389	0.580	0.858	0.216

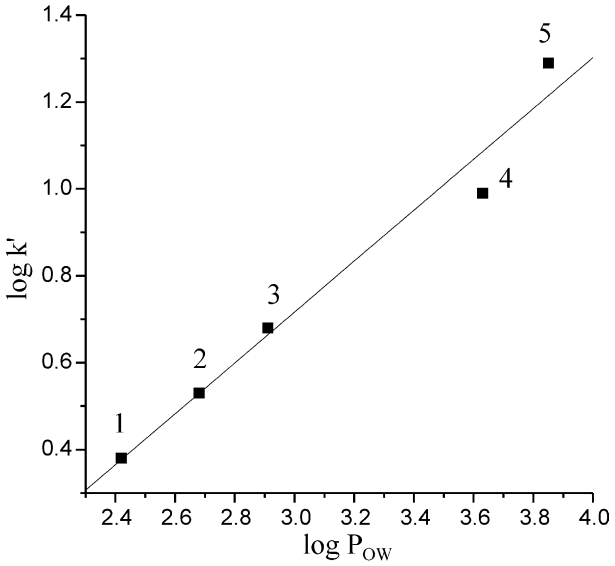


Figure 5. Correlation of log k'-log P_{OW} for the anthraquinones. (1) Rhein, (2) aloe-emodin, (3) emodin, (4) chrysphenol, (5) physcion. Conditions: 15 mmol/L NaH_2PO_4 + 20 mmol/L borax + 15% ethanol (v/v) and 20 mmol/L SDS (pH 10.3).

Thermodynamic Analysis

The movement from injection to detection is given a positive sign. Methanol was used as the EOF marker and Sudan III was used as micelle marker. The effective mobility (μ_{eff}) of the solutes were determined at pH 10.3 using the following equations:

$$\mu_{eff} = \left(\frac{1}{t_0} - \frac{1}{t_R}\right)\frac{lL}{V} \tag{1}$$

where t_0 and t_R are the migration times of the EOF marker and the solute respectively, l is the effective capillary length to the detector, L is the total length of the capillary, V is the applied voltage. The velocity of any solute is given by its electrophoretic mobility, μ, times the field strength, E, or $v = \mu E$.

The capacity factor, k', of an ionic solute in MEKC is defined as:

$$k' = \frac{\mu_{aq} - \mu_{eff}}{\mu_{eff} - \mu_{me}} \tag{2}$$

where μ_{aq} and μ_{me} are the electrophoretic mobilities of the solute in the aqueous phase and micelle phase, respectively, and μ_{eff} is the effective mobility in the micellar solution. The μ_{eff} was calculated by eq 1. The μ_{me} was obtained by measuring the migration times of Sudan III, and the μ_{aq} was obtained by capillary zone electrophoresis in free solution.

When the volume of the micellar phase, V_m, is constant, k' is proportional to the partition coefficient between the two phases, P_{mw}, as follows:

$$k' = P_{mw}(V_m/V_{aq}) = P_{mw}\phi \tag{3}$$

where V_{aq} is the volume of the aqueous phase, and ϕ is the phase ratio.

The free energy (ΔG) of the interaction between solutes and surfactant was derive by:[15]

$$\Delta G = -R_g T \ln\left(\frac{\nu_{aq} - \nu_s}{\phi\left(\frac{l}{t_{obs}} - \nu_E - \nu_s\right)} - \frac{1}{\phi}\right) \tag{4}$$

where R_g is the gas constant, and T is the absolute internal temperature of the capillary, v_{aq} is the electrophoretic velocity of the solute in the absence of the surfactants, v_s is the electrophoretic velocity of the surfactant, v_E is the electro-osmotic velocity, and t_{obs} is the observed migration time of the solute in buffer solution with micelle.

The absolute ΔG value can be determined by the method in reference,[16] which needs to operate the capillary system at different temperatures. However, relative free energy differences ($\Delta(\Delta G)$) between solutes can be obtained by eq 5 just by canceling the phase ratio term. Because rhein had the smallest k' value, the $\Delta(\Delta G)$ value of rhein is noted as 0, and the $\Delta(\Delta G)$ values of the other substances are calculated by the differences between them and rhein, respectively.

$$\begin{aligned}\Delta(\Delta G) &= (\Delta G_2 - \Delta G_1) \\ &= -R_g T \ln \frac{\left[\left(\nu_{aq2} - \nu_s\right)/\left(\frac{l}{t_{obs2}} - \nu_E - \nu_s\right)\right] - 1}{\left[\left(\nu_{aq1} - \nu_s\right)/\left(\frac{l}{t_{obs1}} - \nu_E - \nu_s\right)\right] - 1}\end{aligned} \tag{5}$$

The subscripts 1 and 2 correspond to rhein and other solutes, respectively.

With the gas constant $R_g = 8.314$ J/mol/K, the internal temperature of capillary $T = 293$ K, and the effective length of capillary $l = 40$ cm, the $\Delta(\Delta G)$ values of the solutes in SDS solution were calculated in Table 4. With forced air-cooling, the measurement of internal temperature of capillary was not so accurate. In spite of that, the $\Delta(\Delta G)$ values enabled us to gain some infor-

Table 4. The free energy differences $\Delta(\Delta G)$ values of the five compounds

Compd	$\Delta\Delta G$ (KJ/mol)
Rhein	0
Aloe-emodin	−0.041
Emodin	−0.065
Chrysophanol	−0.104
Physcion	−0.113

mation about the interaction of the solutes with micelle. The more negative ΔG is, the more is the equilibrium moved to the micelle side. Chrysophanol and physcion had large negative values compared with other analytes, which indicates they had strong interaction with micelle. The affinity strength of the solutes to SDS micelle was found to be as follows:

physcion > chrysophanol≫emodin > aloe-emodin > rhein

Conclusions

In pharmaceutics it is important to study the hydrophobicity/hydrophilicity of drugs between aqueous and micelle phases. The proposed MEKC method had determined six components in rhubarb within 15 min in buffer solution of 15 mmol/L NaH_2PO_4 + 20 mmol/L borax + 15% ethanol (v/v) and 20 mmol/L SDS. The hydrophobic interactions between components and micelle were studied as well. The buffer solution employed in this method is comparatively simple, friendly to the operator and the environment. Compared with our previous study, in this method, the retention time is shorter and the detection limits are improved. This MEKC method offers an advantage over some of the other chromatographic methods such as HPLC, MEEKC and LCE in its simplicity. The $\log k' - \log P_{OW}$ of the five anthraquinones correlates well. Thermodynamic analysis gives a quantitative assay for hydrophobicity of the anthraquinone components. The equation derived for calculation of the differences in free energy, $\Delta(\Delta G)$, provides a possibility to study or predict the solute-micelle interactions. Although the methodology lies in limitations, it is simple and feasible to characterize competitive interaction of analyte with micelle.

Acknowledgements

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