A new microemulsion electrokinetic chromatographic method based on a butan-1-ol–ethyl acetate–water microemulsion for the separation and determination of five components in Rheum plant was developed. The migration behavior of these analytes was studied and the separation efficiency was optimized by the systematic examination of the microemulsion content and acetonitrile concentration in the run buffer. Resolution was enhanced for neutral compounds by adjusting the microemulsion and acetonitrile content. Extracts of a Rheum plant sample were analyzed and five active components were determined. An equation describing the migration behavior of compounds at different acetonitrile concentrations was also derived by simulation.

Keywords: Microemulsion electrokinetic chromatography; capillary electrophoresis; active component; Rheum plant

Capillary electrophoresis (CE) is one of the most attractive analytical separation techniques to be developed in the past 20 years. Since it shows high efficiency in the separation of organic and inorganic substances, there has been a substantial increase in the use of CE for the separation of pharmaceutical compounds and biomolecules in recent decades.1–4 With the increasing number of analyses of neutral molecules by CE, modifications of the buffer and capillary have been developed. Ionic micelles have been widely accepted for the modification of buffer solutions in CE5,6 and the technique using micelles as carriers was named micellar electrokinetic chromatography (MEKC). It has been proved to be effective in selectivity manipulation in CE. Many substances, including amino acids, phenols and pharmaceuticals, have been analyzed by this technique.7–9

In 1991, Watarai10 reported the modification of the buffer with an oil–water (o/w) microemulsion. Compared with micellar media methods, a wider migration time window and a higher resolution could be obtained with this new modification, and this technique of modification of CE was named microemulsion electrokinetic chromatography (MEEKC). The fundamental characteristics of MEEKC have also been studied from the viewpoint of selectivity and efficiency.11,12

In this paper, we propose a new MEEKC method, which was applied to the separation and determination of rein, emodin, aloe-emodin, chrysophanol and physcion (for structures, see Fig. 1) in natural products of commercial Rheum plant. This

Fig. 1 Structures of analytes.
stable, transparent emulsion was prepared by mixing ethyl acetate, sodium dodecyl sulfate (SDS), butan-1-ol and water (o/w), and sodium phosphate was used as the buffer system (pH 7.2). Acetonitrile was used as another organic additive to enhance the selectivity of method, as it reduces the interaction of the very hydrophobic compounds chrysophanol and physcion with the microemulsion, and improves the separation efficiency and selectivity. A systematic investigation of the influence of acetonitrile concentration on the migration behavior of these pharmaceuticals was also made. It was found that the introduction of acetonitrile affects not only the separation efficiency but also the migration order of these five components.

Rein, emodin, aloe-emodin, chrysophanol and physcion are bioactive components of a Chinese traditional pharmaceutical, Chinese rhubarb, a commercial Rheum plant. Chinese rhubarb is used as a bacteriostatic agent in Chinese traditional clinical treatment of many diseases, and the analysis of its active component is of pharmacological importance.

Some HPLC and TLC methods have been reported for the separation of these five compounds, and a MEKC method has also been reported for the separation and quantitative identification of three components. However, no MEEKC method for the separation and determination of these five substances has been reported. Compared with other methods, MEEKC is an easier and more stable technique for the separation and determination of all five compounds, with higher resolution and higher efficiency.

Experimental

Instrumentation

MEEKC was carried out by using a Waters Quanta-4000 system (Waters Chromatography Division, Milford, MA, USA) with a positive power supply at a detection wavelength 254 nm and a temperature of 25 ± 1 °C. Separation was performed in uncoated fused silica capillaries manufactured by Waters (Accusep). Capillaries of 75 μm id, 42.4 cm effective length (50 cm total length) were used, and a detection window was created by removing the polyamide coating at 7.6 cm from the cathodic end of the capillary. Direct UV detection was performed with an Hg lamp and 254 nm optical filter. Samples were introduced from the anodic end of the capillary by hydrodynamic injection where the sample vial was raised by 10.0 cm for 2 s. Data acquisition was carried out with a Maxima 820 chromatography workstation. A 5 min wash cycle with 0.5 m NaOH was followed by 3 min with distilled water and a 5 min wash with separation buffer was necessary to condition the capillary.

Reagents

Authentic Rein, emodin, aloe-emodin, physcion and chrysophanol (Fig. 1) were obtained from the National Institute for the Control of Pharmaceutic and Biological Products, China. A sample of Chinese rhubarb was obtained from the Lanzhou
where N is the theoretical plate number and \( t_{wh} \) is the peak width at half-height.

### Results and discussion

#### Influence of microemulsion on separation

The differences in structures between the investigated substances (Fig. 1) are due to variations in the R₁ and R₂ group. Of these five compounds, emodin, aloe-emodin, physcion and chrysophanol are hydrophobic, especially physcion and chrysophanol, which are very hydrophobic substances.

It is known ¹⁰,¹¹ that the MEEKC method, where butan-1-ol, heptane and water are used in the run buffer, provides better resolution for neutral substances than micellar media. In this work, a microemulsion system of ethyl acetate (3.2% v/v)–SDS (3.5%, m/v)–butan-1-ol (0.8% v/v), which was effective in the separation of these analytes, was adopted. This MEEKC method provides even better resolution of analytes than that of micellar media, as illustrated in Fig. 2. Fig. 2(A) shows an electropherogram obtained by the MEEKC method and Fig. 2(B) that of micellar media; the peaks are numbered according to the compound numbers in Fig. 1. The resolution of the aloe-emodin peak from the emodin, chrysophanol and physcion peaks in the MEEKC system is satisfactory, although the larger size of the microemulsion droplets broadens the peaks, owing to the difference in hydrophobic interactions between the analytes and microemulsion droplets. There was hardly any influence on the peak shape and migration behavior of rein whether the microemulsion or micelle was used as the medium because rein is an anionic molecule here, and it was difficult to enter the core of a surface anionic microemulsion or micelle, and its migration behavior was only influenced by the EOF and pH of the buffer.

The content of microemulsion is important for dissolving all analytes and obtaining good peak shapes. With a low microemulsion content, very hydrophobic compounds could not be found in the electropherogram (Fig. 3), because they are insoluble in the buffer system under these conditions and are substantially adsorbed by the capillary inner wall.

#### Influence of acetonitrile concentration

It was found in the experiments that there was a considerable influence of acetonitrile concentration on the effective mobilities of these five analytes, and the content of the acetonitrile was the main parameter utilized for method optimization. The system without acetonitrile as a modifier gave low resolution for emodin, chrysophanol and physcion [Fig. 2(A)]. At an acetonitrile concentration of 15% v/v, sufficient resolution could be obtained for these three analytes, but the peak shape was not satisfactory [Fig. 4(A)]. This was due to the adsorption of analytes on the capillary inner wall. A better peak shape could be obtained with a system containing a 30% v/v acetonitrile concentration. With this system, all five components were separated without serious band broadening [Fig. 4(B)]. A further increase in the acetonitrile concentration (40%...
destroyed, and there was still a distribution between the acetonitrile and the microemulsion system could not be stable and transparent, because the SDS was insoluble in extracts.

In order to obtain good results for relative migration time reproducibility and calibration curve

Table 4 Calibration data (detection at 254 nm)

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Slope</th>
<th>Intercept</th>
<th>Correlation coefficient</th>
<th>Linearity range/ µg ml⁻¹</th>
<th>Detection limit/ (S/N = 3)/ µg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rein</td>
<td>0.44</td>
<td>-0.11</td>
<td>0.9982</td>
<td>5–40</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>Aloe-emodin</td>
<td>0.56</td>
<td>-0.20</td>
<td>0.9995</td>
<td>10–90</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>Emodin</td>
<td>0.57</td>
<td>-0.22</td>
<td>0.9900</td>
<td>20–150</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>Chrysophanol</td>
<td>0.59</td>
<td>0.85</td>
<td>0.9998</td>
<td>10–120</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>Physcion</td>
<td>0.27</td>
<td>0.32</td>
<td>0.9991</td>
<td>10–150</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The migration order of the compounds was apparently affected by changes in acetonitrile concentration. Under the conditions of Fig. 4(A), the migration order is 1, 2, 3, 4 and 5, and with increase in acetonitrile concentration the polarity of buffer was decreased and the solubilities of hydrophobic compounds in mobile phase were increased, and their effective mobilities were also changed, with the migration order changing to 2, 4, 5, 3 and 1 [Fig. 4(B) and (C)]. The best separation was obtained with the conditions in Fig. 4(B).

It was found that there was a functional relationship between the effective mobility of the compounds and the concentration of acetonitrile (%). This relationship was simulated by the following equation:

\[ \mu_{\text{eff}} = \frac{(\mu_L - \mu_h) e^{dc}}{1 + e^{-dc}} + \mu_h \]

where \( \mu_L \) is the effective mobility of analyte at low acetonitrile concentration, \( \mu_h \) is the effective mobility at high acetonitrile concentration, \( c \) is the acetonitrile concentration, \( c_{eq} \) is a constant and \( dc \) is a concentration unit. The results obtained by experiment are shown in Fig. 5 as a scatter graph and the simulated curve is shown in Fig. 5 with a line graph. The parameters of this equation obtained by simulation are given in Table 1. The mechanism of the influence of acetonitrile concentration on the effective mobility could be explained satisfactorily with this simulated equation, that is, there are maximum effective mobilities of compounds at low acetonitrile concentration and minimum mobilities at high acetonitrile concentration. The effective mobility of every compound varied between their maximum and minimum effective mobility values with the change of \( \exp(c-c_{eq})/(dc) \). This was due to the change in solubility of the analytes in acetonitrile–water phase with change in acetonitrile concentration.

The concentration of acetonitrile was also an important factor that influences the separation efficiency of compounds, and this is illustrated in Table 2. A higher concentration of acetonitrile provided a higher plate number for the analyte compounds, and a 30% acetonitrile concentration was satisfactory.

**Applied voltage**

At the higher voltages necessary for rapid analysis, resistance to mass transfer in the mobile phase appears to be the primary source of band broadening. When there was a lower voltage, because the migration of compound becomes slow, the band will also be broadened due to diffusion effects. At an applied voltage of 15–18 kV, the highest separation efficiency could be obtained for each analyte.

**Relative migration time reproducibility and calibration curve**

In order to obtain good results for relative migration time reproducibility, the buffer should be changed in each run and a seal is necessary for the buffer vial because evaporation of organic solvents will occur, and this will affect the method performance and reproducibility. The method was validated for

\( v/v \) provided a relatively low resolution between chrysophanol and physcion [Fig. 4(C)].

At a high concentration of acetonitrile, the run buffer was still stable and transparent, because the SDS was insoluble in acetonitrile and the microemulsion system could not be destroyed, and there was still a distribution between the water–acetonitrile mobile phase and oil pseudo-stationary phase. With increase in acetonitrile concentration, the solubility of the analytes in the mobile phase was increased and their distribution behavior was also changed.
reproducibility of the relative migration time of the analytes. The migration stability of these five components is given in Table 3. RSDs ranging from 0.14 to 2.52% were obtained.

The calibration curve data are given in Table 4. The correlation coefficients, linearity ranges and detection limits (S/N = 3) are also given.

Applications
Ethanolic solutions of sample extracts were injected directly and separated under the conditions of Fig. 5(B). An electropherogram for a real sample is shown in Fig. 6 and the results are given in Table 5. Peaks were identified by addition of standard substances of rein, emodin, aloe-emodin, chrysophanol and physcion. The results for the determination of these five compounds is satisfactory.

Conclusion
Microemulsion electrokinetic chromatography has been shown to provide selectivity and high efficiency in the separation and determination of bioactive components with similar structures in Rheum plants. Modification of the capillary with microemulsion provides a wide range of organic modifiers, including many hydrophobic organic solvents. Acetonitrile as another organic modifier performs an important role in adjusting the selectivity and efficiency for the five components studied. The manipulation of the pseudo-stationary phase in MEEKC is easy, requiring only that the capillary be rinsed and filled with the modified microemulsion system. This technique has potential in the analysis of complicated samples of natural products.

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