Simultaneous determination of anthraquinones in rhubarb by high-performance liquid chromatography and capillary electrophoresis

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Abstract

High-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) were compared to simultaneously determine and separate 11 anthraquinones from rhubarb, including emodin, chrysophanol, rhein and their glucosides, aloe-emodin, sennoside A, and sennoside B. A UV-diode array detector (DAD) at 254 nm with a gradient elution of acetonitrile/water (method A: 0 min 6:94, 12 min 12:88, 15 min 20:80, 40 min 25:75, 53 min 55:45, 55 min 100:0; method B: 0 min 5:95, 2 min 15:85, 5 min 20:80, 12 min 25:75, 15 min 50:50, 19 min 98:2) at 28(±1) °C (method A) and 30–60 °C (method B) in HPLC or with 0.03 M borate buffer (pH 10.0) containing 25% (v/v) acetonitrile with 0.002 M 2,6-di-O-methyl-β-cyclodextrin (CD) and 0.005 M α-CD in CE effectively detected this separation in 25 min. The detection limits of anthraquinones from rhubarb were in the 0.02–0.2 μg/mL and 0.1–0.8 μg/mL ranges for HPLC and CE, respectively. The established HPLC and CE methods are suitable for quantitative determination of emodin, chrysophanol, aloe-emodin, emodin-1-β-d-glucoside, emodin-8-β-d-glucoside, chrysophanol-1-β-d-glucoside, chrysophanol-8-β-d-glucoside, and rhein-8-β-d-glucoside.

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1. Introduction

Rhubarb (Rheum sp.) is an important traditional crude drug in China and Japan, and is a laxative medicine in Europe. Various constituents have been isolated from rhubarb, which can be classified as anthraquinones, dianthrones, stilbens, anthocyanins, flavonoids, polyphenols, organic acids, and chromones [1]. However, the major constituents are anthraquinone derivatives, which include emodin, chrysophanol, rhein, aloe-emodin, physcion, and their glucosides, and are accepted as important active components with various pharmacological actions such as purgation, antibacterial, and antitumor activity [2].

As a continuation of our work on quinones, which are common in the plant kingdom and may have biological activity, we have developed a high-performance liquid chromatography (HPLC) method and a simple cyclodextrin-modified capillary zone electrophoresis (CD-CZE) method to separate and determine emodin, chrysophanol, aloe-emodin, rhein, emodin-1-β-d-glucoside, emodin-8-β-d-glucoside, chrysophanol-1-β-d-glucoside, chrysophanol-8-β-d-glucoside, rhein-8-β-d-glucoside, sennoside A, and sennoside B in rhubarb. Analyses of these compounds in pharmaceutical preparations or in complex matrices such as crude plant extracts are of special interest [3,4]. Rhubarb contains numerous components and to be able to simultaneously determine these different components, especially those with large polarity differences, is desirable for quality control purposes. Typical methods used to separate anthraquinones are HPLC [1,5–12], CE [13–22], and thin-layer chromatography (TLC) [23]. However, studies using these methods have been limited to a single component or to a few components. A few simultaneous analyses of different types of components have been conducted [1,7,11,21,22]. In general, HPLC analysis is based on the aglycons and take a long time. Studies related to the simultaneous determination by CE are especially rare. We have previously reported the separation and determination of emodin, chrysophanol, emodin-8-β-d-glucoside, and chrysophanol-8-β-d-glucoside by a CD-CZE method [21]. In this study, we have compared HPLC and CE methods, and developed a simple and rapid HPLC method and a CD-CZE method to simultaneously determine and separate...
2. Experimental

2.1. Materials and reagents

Emodin (1) and chrysophanol (2) were isolated from the leaves of *Cassia siamea* [24]. Emodin-1-β-D-glucoside (5) and emodin-8-β-D-glucoside (6) were isolated from the root of *Polygonum cuspidatum* Sieb. et Zucc. [25, 26]. Chrysophanol-1-β-D-glucoside (7) and chrysophanol-8-β-D-glucoside (8) were from cascara sagrada (the root of *Rhamnus purshiana* DC.) [27, 28]. Aloe-emodin (3), rhein (4), and rhein-8-β-D-glucoside (9) were isolated from *Rhei rhizoma* [29] (Fig. 1). Sodium tetraborate, sodium hydroxide, hydroxypropyl-β-CD (HP-β-CD), γ-CD, HP-γ-CD, and HPLC-grade acetonitrile were from Wako Pure Chemicals (Osaka, Japan). α-CD, 2,6-di-O-methyl-β-CD, and sennoside A (10) were from Nacalai Tesque (Kyoto, Japan). Sennoside B (11) was from Funakoshi (Tokyo, Japan), while boric acid was from Fluka (Buchs, Switzerland). Deionized water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). Rhubarb (*Rheum palmatum* L., *Rheum tanguticum* Maxim.) was purchased from The Iguchi Pharmacy (Kobe, Japan). The standards of 11 anthraquinones were purified and identified using 1H NMR, 13C NMR and MS.

2.2. HPLC conditions

HPLC separation was conducted on a Waters alliance 2690 system (Waters, MA, USA) equipped with an autosampler, a temperature-controlled column, and a photodiode array detector (PDA996: Waters, MA, USA) working at 254 nm. The anthraquinones were separated on a Cosmosil 5C18AR-II column, 4.6 mm I.D. × 150 mm, particle size 5 μm, Nacalai Tesque, Kyoto, Japan) (method A) and a fast LC column (Cosmosil 5C18 AR-II short column, 4.6 mm I.D. × 50 mm, particle size 5 μm, Nacalai Tesque, Kyoto, Japan) (method B). The mobile phase consisted of acetonitrile/water. The gradient profiles were as follows: method A: 0 min 6:94, 12 min 12:88, 15 min 20:80, 40 min 25:75, 53 min 55:45, 55 min 100:0; method B: 0 min 5:95, 2 min 15:85, 5 min 20:80, 12 min 25:75, 15 min 50:50, 19 min 98:2. The flow-rate was 1.0 mL/min. The column temperature was 28(±1) °C in method A and between 30–60 °C in method B. The injection volume was 20 μL. Among the chromatogram peaks, compounds 1–10 were identified by spiking with standards.

2.3. CE conditions

CE analyses were conducted using a Beckman MDQ and P/ACE System 5000 apparatus (Fullerton, CA, U.S.A.) equipped with a UV detector set at 254 nm, a diode array detector (DAD), and a Beckman untreated fused-silica capillary (75 μm I.D. × 570 mm; 500 mm effective length). The analytical conditions were as follows: sampling time 5 s (hydrodynamic mode; 0.5 p.s.i.); applied constant voltage 20 kV; column temperature 20 °C. The CD-CZE electrolyte was a buffer solution prepared by mixing 0.03 M sodium tetraborate with the appropriate volumes of 10% (w/v) sodium hydroxide (pH 10.0) and the subsequent additions of 0.002 M 2,6-di-O-methyl-β-CD, 0.005 M α-CD, and 25% (v/v) acetonitrile. Standard solutions were prepared by dissolving each compound in the electrolyte at a concentration of ~10 μg/mL. Among the peaks in the chromatogram, compounds 1–11 were identified by spiking with standards and comparing to a UV similarity index (the value was used to compare the UV spectrum of an unknown compound against the spectrum of a known sample).

2.4. Standard preparation and sample preparation of extracts

The standard stock solutions were prepared by dissolving 1 mg of each compound in 1 mL of methanol. These solutions were stored in dark glass bottles at 4 °C and were stable for at least 1 month. Working standards solutions were freshly prepared by dissolving suitable amount of the above solutions with methanol (HPLC) or borate buffer (pH 10.0) (CE) before injection. Powdered rhubarb (10 g) was extracted in a Soxhlet apparatus for 10 h with 100 mL of methanol. After concentrat-
the residue was diluted with methanol to 100 mL. This solution was passed through a 0.45 μm pore size filter. The methanolic extract solution (1 mL) was diluted to 10 mL with methanol for HPLC and with borate buffer (pH 10.0) for CE analysis, and then directly injected into the HPLC and CE systems.

2.5. Calibration curves

Working standard solutions, which spanned a concentration range from 5 μg/mL to 200 μg/mL for HPLC analysis and from 5 μg/mL to 100 μg/mL for CE analysis, were prepared.

3. Results and discussion

The study aimed to develop a simple, sensitive, and rapid method with a high degree of specificity, to simultaneously determine the anthraquinones of rhubarb. Fig. 1 shows the structures of 11 anthraquinones and bianthaquinones from rhubarb. Among these compounds, emodin, chrysophanol, aloe-emodin, and rhein are free anthraquinones (aglycon); emodin-1-β-D-glucoside, emodin-8-β-D-glucoside, chrysophanol-1-β-D-glucoside, chrysophanol-8-β-D-glucoside, and rhein-8-β-D-glucoside are glycosides; sennoside A and sennoside B are bianthrone glycosides. These 11 compounds encompass a wide range of lipophilicity and molecular mass. Two methods have been achieved to provide optimum separations; HPLC with an acetonitrile/water solvent gradient and CE with 0.03 M borate buffer (pH 10.0) that contains 25% acetonitrile, 0.002 M 2,6-di-O-methyl-β-CD, and 0.005 M α-CD.

3.1. HPLC analysis

Conventional HPLC analysis of the anthraquinones in rhubarb was conducted on a reversed-phase (RP) column using organic solvents such as acetonitrile or methanol, and aqueous acetic acid or aqueous phosphoric acid. Preparative HPLC, which involves the addition of non-volatile or acidic material to the mobile phase, complicates the isolation of the product after separation. Therefore, we developed a new HPLC method to characterize anthraquinone glycosides and aglycons using a gradient elution system with a simple acetonitrile/water mobile phase. Most optimization procedures for HPLC methods neglect the influence of temperature. Even in the late 1970’s, temperature was acknowledged primarily for its influence on retention, with increased temperatures producing shorter retention times. The observation that in general shorter retention times give poorer resolution [30] argued against the use of elevated temperature as a routine practice. But column temperature is now recognized as an important variable in controlling selectivity and retention in RP-HPLC separations [31]. The selectivity effects observed for temperature changes in RP-HPLC generally are complementary to those observed for mobile phase strength changes, so it is often possible to improve resolution by simultaneous optimization of temperature and mobile phase percent organic or gradient steepness. Simply programmed in many HPLC systems, changes in temperature during method development are more convenient than solvent type or pH changes. In this work, the separations using shorter column has demonstrated the usefulness of temperature improve the separation speed and the resolution of the peaks.

The individual anthraquinones were well separated on a Cosmosil 5C18 AR-II column (4.6 mm I.D. × 150 mm, reversed-phase column). Fig. 2 shows a HPLC chromatogram of a standard mixture of the 11 compounds using method A (acetonitrile/water gradient in 65 min). This is the first report to simultaneously separate and determine emodin-1-β-D-glucoside, emodin-8-β-D-glucoside, chrysophanol-1-β-D-glucoside, chrysophanol-8-β-D-glucoside, and rhein-8-β-D-glucoside. However, this method did not separate rhein-8-β-D-glucoside, sennoside A, and sennoside B very well. Fig. 3 shows a HPLC chromatogram of a methanolic extract of rhubarb. The peaks for compounds 1–9 could be assigned by the retention time, comparing to the UV spectrum of known compounds, and spiking with standards.

For the quantitative analysis, method A was used to correlate the peak area and sample concentrations for 1–11. The curves (peak-area, Y, versus concentration, x (μg/mL)) were constructed in the range of 5–200 μg/mL for all compounds. The regression equations of these curves and their correlation coeffi-

![Fig. 2](image_url)

Fig. 2. Chromatograms of the standard compounds by HPLC using Cosmosil 5C18AR-II. Eluent: method A; acetonitrile/water: 0 min 6:94, 12 min 12:88, 15 min 20:80, 40 min 25:75, 53 min 55:45, and 55 min 100:0; flow-rate, 1.0 mL/min; column temperature 29 °C. Peaks: 1, emodin; 2, chrysophanol; 3, aloe-emodin; 4, rhein; 5, emodin-1-β-D-glucoside; 6, emodin-8-β-D-glucoside; 7, chrysophanol-1-β-D-glucoside; 8, chrysophanol-8-β-D-glucoside; 9, rhein-8-β-D-glucoside; 10, sennoside A; 11, sennoside B.

![Fig. 3](image_url)

Fig. 3. Simultaneous determinations for anthraquinones in the extract of rhubarb by HPLC using Cosmosil 5C18AR-II. Eluent: method A, column temperature: 29 °C. Peaks: 1, emodin; 2, chrysophanol; 3, aloe-emodin; 4, rhein; 5, emodin-1-β-D-glucoside; 6, emodin-8-β-D-glucoside; 7, chrysophanol-1-β-D-glucoside; 8, chrysophanol-8-β-D-glucoside; 9, rhein-8-β-D-glucoside.
coefficients were calculated as follows: 1, \( Y = 49160x + 98050 \) \((r^2 = 0.998)\); 2, \( Y = 75650x - 137890 \) \((r^2 = 1.000)\); 3, \( Y = 76720x - 8980 \) \((r^2 = 1.000)\); 4, \( Y = 10870x - 17270 \) \((r^2 = 0.994)\); 5, \( Y = 35790x - 34430 \) \((r^2 = 0.999)\); 6, \( Y = 13590x - 33140 \) \((r^2 = 0.995)\); 7, \( Y = 23310x - 400 \) \((r^2 = 1.000)\); 8, \( Y = 29010x - 44650 \) \((r^2 = 0.998)\); 9, \( Y = 12270x + 13700 \) \((r^2 = 0.999)\); 10, \( Y = 9650x - 20200 \) \((r^2 = 1.000)\); 11, \( Y = 10210x + 57800 \) \((r^2 = 0.998)\). At a signal-to-noise ratio of 3, the detection limits of the analytes were: 1, 0.04 \( \mu g/mL \); 2, 0.02 \( \mu g/mL \); 3, 0.03 \( \mu g/mL \); 4, 0.1 \( \mu g/mL \); 5 and 6, 0.08 \( \mu g/mL \); 7 and 8, 0.05 \( \mu g/mL \); 9, 0.2 \( \mu g/mL \); 10 and 11, 0.1 \( \mu g/mL \). The extraction recovery was tested by adding known amounts of 1 and 2. The recoveries were 103.5% for emodin and 98.6% for cryptophanol \((n = 3)\). Table 1 shows the concentrations of compounds 1–9 determined in these sample extracts.

The individual anthraquinones were well separated on a Cosmolsil 5C18 AR-II column. However, these HPLC conditions required more time of method A. Thus, the attempt was made to shorten the run time by modifying the chromatographic conditions. A shorter column (5C18 AR-II column: 4.6 mm I.D. \( \times \) 50 mm), which enabled a fast separation of the anthraquinones, was tested in the method B. It was found that the column temperature significantly influenced the separation of compounds 6–8. An increased temperature usually improved the loadability and solubility, but decreased the viscosity of the mobile phase.

Fig. 4 shows the temperature-dependent chromatograms of a mixture of the 11 compounds over a temperature span of 30 to 60°C. A baseline separation of anthraquinones (1–8) was achieved in 21 min at 60°C. As the temperature increased from 30 to 60°C, the elution sequence for compounds 6–8 was reversed. Emodin-8-\( \beta \)-d-glucoside (6) was co-eluted with 7 and 8 at 30°C, and was well separated at 60°C.
with chrysophanol-1-β-d-glucoside (7) and chrysophanol-8-β-d-glucoside (8) at a column temperature of 30 °C. At approximately 40 °C, 6 and 7 were co-eluted, but the elution order of 6 and 7 was reversed at 50 °C. Compounds 6–8 were satisfactorily separated at 60 °C. Consequently, these changes in the retention time aided in peak separation. It is apparent that adjusting the column temperature resulted in selectivity differences. Fig. 5 shows a HPLC chromatogram of a methanolic extract of rhubarb. Thus, a satisfactory separation was achieved for compounds 1–8 at an increased temperature (60 °C). However, the resolution of compounds 9–11 is unacceptable and must be improved.

The precision of the chromatographic determination for the proposed method, which is expressed as a relative standard deviation (RSD), was calculated for five replicate injections of each sample in methods A and B. The RSDs for the 11 anthraquinones were between 0.17%–5.10% for method A and 0.05%–3.96% for method B at 30 °C (Table 2).

3.2. CE analysis

Recently, we published the simultaneous determination of 9 anthraquinones by CD-CZE [21]; the running electrolyte used in this method was 0.005 M α-CD in 0.03 M borate buffer (pH 10.0) containing 20% acetonitrile. However, chrysophanol-1-β-d-glucoside (7) and chrysophanol-8-β-d-glucoside (8) were eluted together and could not be separated using the above conditions. Because compounds 1–11 all possess phenolic hydroxy or carboxy groups, CE using a buffer solution of borate with different pH values as the carrier was employed in our preliminary trials. The pH-dependence of the migration time of emodin (1) was examined with 0.03 M borate buffer in a pH range 8–11. The migration time became longer as the pH value increased. We tried to separate all the compounds in the mixture using CZE by changing the buffer solutions. It is well known that when the concentration of the buffer increases, the electro-osmotic flow decreases, which increases the migration time. After examining a series of buffer solutions with differing pHs (8–11), concentrations (0.005–0.1 M), and modifiers (α-CD, β-CD, γ-CD, HP-β-CD, HP-γ-CD, 2,6-di-O-methyl-β-CD), and with varying concentrations of CDs (0.002–0.03 M), it was found that 0.03 M borate buffer (pH 10.0) with 0.002 M 2,6-di-O-methyl-β-CD and 25% acetonitrile could resolve a standard mixture of the 11 compounds. However, further addition of α-CD to the above electrolyte was required to obtain a satisfactory resolution of the compounds in rhubarb extract. Thus, CDs were successfully applied to separate several hydrophobic compounds. In the inclusion-complexation mechanism, either the entire molecule or its hydrophobic part fits into the CD cavity. In this work, the α-CD may have an important role in separating the extracts from a large number of co-existent interferences. In CZE, the main factor affecting the migration velocity of a sample molecule is generally determined by the molecular size/charge ratio. The

Table 2
Reproducibility of retention and migration times of anthraquinones

<table>
<thead>
<tr>
<th>Compound</th>
<th>HPLC</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5C18ARII (150 mm)</td>
<td>5C18ARII (50 mm)</td>
</tr>
<tr>
<td></td>
<td>Retention time (min)</td>
<td>RSD of Retention time (%) (n = 5)</td>
</tr>
<tr>
<td></td>
<td>30 °C</td>
<td>60 °C</td>
</tr>
<tr>
<td>Emodin</td>
<td>58.83</td>
<td>20.33</td>
</tr>
<tr>
<td>Chrysophanol</td>
<td>60.41</td>
<td>21.38</td>
</tr>
<tr>
<td>Aloe-emodin</td>
<td>53.48</td>
<td>18.62</td>
</tr>
<tr>
<td>Rhein</td>
<td>23.20</td>
<td>8.84</td>
</tr>
<tr>
<td>Emodin-1-β-d-glucoside</td>
<td>28.35</td>
<td>10.72</td>
</tr>
<tr>
<td>Emodin-8-β-d-glucoside</td>
<td>40.27</td>
<td>14.79</td>
</tr>
<tr>
<td>Chrysophanol-1-β-d-glucoside</td>
<td>38.99</td>
<td>14.32</td>
</tr>
<tr>
<td>Chrysophanol-8-β-d-glucoside</td>
<td>41.49</td>
<td>15.09</td>
</tr>
<tr>
<td>Rhein-8-β-d-glucoside</td>
<td>12.04</td>
<td>5.27</td>
</tr>
<tr>
<td>Sennoside A</td>
<td>14.90</td>
<td>5.94</td>
</tr>
<tr>
<td>Sennoside B</td>
<td>12.91</td>
<td>5.48</td>
</tr>
</tbody>
</table>
long migration time of rhein-8-β-d-glucoside, sennoside A, and sennoside B can be explained by the fact that alkaline conditions are required to induce ionization of the carboxy group. Although the migration time of rhein was too long (over 50 min), attempts to shorten this time by changing the conditions were unsuccessful. Fig. 6 is an electropherogram, showing the separation of ten authentic substances, and Fig. 7 is an electropherogram of the methanolic extract of rhubarb (except rhein).

For quantitative analyses, correlations between the peak area and the sample concentrations (1–3, 5–9) were determined. The calibration curves (peak-area; Y, versus concentration; x (µg/mL)) were constructed in the range of 5–100 µg/mL for all compounds. The regression equations of these curves and their correlation coefficients were calculated as follows: 5, \( Y = 352x + 799 \) \((r^2 = 0.999)\); 7, \( Y = 930x - 8770 \) \((r^2 = 0.993)\). Except for 5 and 7, the regression equations are nearly identical to the preceding paper [21]. At a signal-to-noise ratio of 3, the detection limit for 5 and 7 was 0.8 µg/mL. The extraction recovery was tested by adding known amounts of 1 and 2. The level of recovery was 105.8% for emodin and 101.1% for chrysophanol \((n=5)\). Table 1 shows the concentrations of these compounds, except for 4, found in the sample extracts.

The reproducibility, expressed as the RSD, was calculated on the basis of the migration time over five replicate injections and is shown in Table 2. The reproducibility (RSD) of the migration time for an individual compound was between 0.74% and 1.99%.

4. Conclusion

Two efficient methods, HPLC and CD-CZE, have been developed to simultaneously analyze 11 anthraquinones, which consist of anthraquinones and their glucosides that show a large difference in polarity. It should be emphasized that emodin (1), chrysophanol (2), emodin-1-β-d-glucoside (5), emodin-8-β-d-glucoside (6), chrysophanol-1-β-d-glucoside (7), and chrysophanol-8-β-d-glucoside (8) were simultaneously separated and determined in 25 min in the untreated rhubarb extract using either the HPLC or CE method. However, the CE method more selectively separates sennosides and rhein-8-β-d-glucoside (9), but the HPLC method may be improved in the future to encompass the separation of sennosides and rhein-8-β-d-glucoside. Though both HPLC and CE provide acceptable analysis times (except rhein in CE), the detection limits of CE are lower than HPLC (CE: 0.1–0.8 µg/mL; HPLC: 0.02–0.2 µg/mL). If attention is focused on the simultaneous determination and separation of emodin (1), chrysophanol (2), emodin-1-β-d-glucoside (5), emodin-8-β-d-glucoside (6), chrysophanol-1-β-d-glucoside (7), and chrysophanol-8-β-d-glucoside (8), then a significant difference between these two methods was not demonstrated. On the basis of the present study, having many-sided experience with the possibilities of the most common, two techniques, it is hard to declare the unambiguous advantage of either one.

References
