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Optimization of the capillary electrophoretic separation of bisbenzylisoquinoline alkaloids by an overlapping resolution mapping scheme

Shao-Wen Sun*, An-Cheng Wu

School of Pharmacy, National Taiwan University, 1 Jen-Ai Road Sec. 1, Taipei, Taiwan

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Abstract

The micellar electrokinetic chromatographic (MEKC) separation of eight bisbenzylisoquinoline (BBI) alkaloids, aromoline, berbamine, colorflammine, homoaromoline, isotetrandrine, obamegine, tetrandrine and thalrugosine, was optimized by using the overlapping resolution mapping (ORM) scheme. Three critical parameters of the electrophoretic media, i.e. surfactant (sodium cholate), organic modifier (acetonitrile) and pH, were chosen for optimization, with their working ranges being appropriately defined. Seven experiments were conducted to obtain the overlapped resolution diagram from which the area of maximum separations could be located. Using the conditions of a point in this area, the eight BBI compounds were baseline separated within 20 min. The elution order of the compounds was found to be related to their lipophilicity. The resolution, run time, efficiency and the limits of detection of the MEKC method were compared with those of the high-performance liquid chromatography method developed previously.

Keywords: Overlapping resolution mapping; Optimization; Buffer composition; Bisbenzylisoquinolines; Alkaloids

1. Introduction

Bisbenzylisoquinoline (BBI) alkaloids, occurring primarily in menispermaceae, berberidaceae, monimiaceae and ranunculaceae [1], were reported to have many cardiovascular activities, for example, calcium antagonistic, antiarrhythmic and platelet-aggregating actions have been shown in tetrandrine [2–4], calcium channel blocking, isoproterenol and histamine antagonizing actions were found with berbamine [5,6], and α-adrenoreceptor blocking and hypotensive activities were found in obamegine [7].

Recent research on the alkaloidal constituents from the leaves of *Dehassia triandra* Merr., a Lauraceous plant indigenous to Taiwan, revealed that the plant contained eleven BBI alkaloids [8]. This shows that the Lauraceous plants, especially the *Dehassia* genus, might also be a source of these pharmacologically interesting molecules.

To facilitate the study of the BBI constituents of Lauraceous plants (as well as the other above-mentioned plants), a reversed-phase ion pair chromatographic method was developed in our laboratory [9]. With this method, nine BBI alkaloids were separated using a mobile phase composed of acetonitrile and acidic phosphate buffer, to which diethylamine and

*Corresponding author.

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sodium heptanesulfonate were added. Capillary electrophoresis (CE), because of its merits, such as high resolution, high mass sensitivity, small sample volumes, extraordinarily small mobile phase consumption and rapid separation, has become an alternative technique to high-performance liquid chromatography (HPLC) in natural product research. Micellar electrokinetic chromatography (MEKC) extends the application range of capillary electrokinetic techniques to neutral molecules and, due to such versatility, MEKC counts among the more important modes of CE.

In developing a CE analytical method, the univariate approach to an optimal separation is generally employed. This step-by-step approach varies one factor at a time while keeping all other factors constant to see the effect of the varied factor on separation. The factors, however, are not always independent and interactions between them may occur. On following this approach, the optimal separation is either not found or it is not a true one, if found. Based on this consideration, chemometric approaches are used to undertake the optimization of separation. These approaches are multivariate in which several factors are varied simultaneously to carry out the experiments. On applying these strategies, a large number of experiments can be saved and a true optimum can be obtained. In recent years, many chemometric approaches to the systematic optimization used in HPLC separations have been employed in CE, among which, Placket-Burman design [10,11], the overlapping resolution mapping (ORM) scheme [12–16], central composite design [17,18] and Box-Behnken design [19] are examples. Placket-Burman design is a fractional factorial design. Its main use is to observe the effects of a large number of factors on separations. If the interactions between the factors are to be investigated, one should resort to the full factorial design. The aim of the ORM, central composite and Box-Behnken designs lies in the establishment of a response surface from the least number of experiments for the prediction of the conditions of a separation optimum.

Compared with other separation strategies, the triangular ORM scheme is simple, rapid and effective. It was employed in this work to undertake the optimization of an MEKC separation of eight BBI alkaloids (Fig. 1) in which seven were isolated from

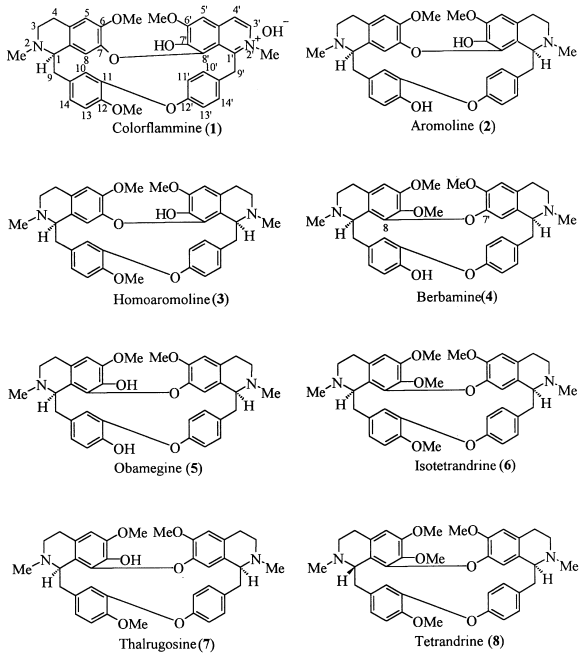

Fig. 1. Bisbenzylisoquinoline alkaloids studied in the optimization procedure.

a Lauraceous plant *Dehassia triandra* Merr. In this scheme, three factors affecting the separations to a great extent were selected to run the process. Only seven experiments had to be conducted for the prediction of resolution at any point within the triangle.

2. Experimental

2.1. Apparatus

All experiments were performed on a CE system consisting of a Lauer Labs. Prince programmable injector, a 30-kV high-voltage supplier (Emmen, The Netherlands) and a Dynamax UV-C absorbance detector (Rainin, Emeryville, CA, USA) for UV detection. The electropherograms were recorded with a EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA) on a 486 DX2 66 PC with an appropriate ADC card and interface. A fused-silica capillary of 50 μm I.D. and 375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) with a total length of 66 cm and a detection length of

51 cm was used. A Mettler delta 320 pH meter with an InLab 410 combination electrode (Essex, UK) was employed for pH measurement.

2.2. Chemicals and reagents

Tetrandrine was obtained from First Medical University of Shanghai (China) and the other seven alkaloids, aromoline, berbamine, colorflammine, homoaromoline, isotetrandrine, obamegine and thalrugosine, were isolated from the leaves or twigs of the Lauraceous plant *Dehaasia triandra* Merr. [8]. The identities of the substances were verified by UV, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. Sodium dodecylsulfate (SDS), sodium cholate, sodium deoxycholate, sodium taurocholate and sodium taurodeoxycholate from Sigma (St. Louis, MO, USA), sodium tetraborate and sodium dihydrogen phosphate from Merck (Darmstadt, Germany), cetyltrimethylammonium bromide (CTAB) from Acros (Geel, Belgium), and methanol and acetonitrile of chromatographic grade from Mallinckrodt (Paris, KY, USA) were used. Water was purified on a Barnstead water purification system (Dubuque, IO, USA).

2.3. Working solutions

Stock solutions for each of the above eight BBI compounds were prepared at 4 mg ml^{-1} in methanol. Two hundred and fifty μl of each stock solution were mixed and the methanol of the mixed solution was evaporated off. The addition of 1 ml of methanol to the dried residue gave a working solution containing each of the compounds at 1 mg ml^{-1}.

2.4. Electrophoretic conditions

The experiments were conducted at 18 kV and 25°C. The total length and the detection lengths of the capillary were 66 and 51 cm, respectively. Samples were injected hydrodynamically at 40 mbar for 1.2 s, such that the injected volume was about 1 nl. The detection wavelength was set at 214 nm.

Stock solutions of sodium borate and sodium dihydrogen phosphate at 200 m*M* were prepared. The electrophoretic media were prepared by adding accurately weighed sodium cholate to the mixtures of the above two stock solutions, the proportions of which were determined by the desired pH values. The required volume of acetonitrile was added. Water was finally added to make up the volume. The solutions were filtered through a 0.45-μm filter (Millipore, Bedford, MA, USA) before use.

When a new capillary was used, it was flushed with 1.0 *M* sodium hydroxide for 10 min and then with 0.2 *M* sodium hydroxide for 10 min. Between runs, the capillary was flushed with 0.2 *M* sodium hydroxide for 2 min, followed by run buffer for 3 min.

The relative standard deviation of migration times for six replicated injections, using the above instrumental settings, rinsing procedure and the optimum conditions obtained from the following ORM scheme, were within 0.6% for each of the BBI compounds.

3. Results and discussion

Simple capillary zone electrophoresis using borate/phosphate buffers at pH values of 4.6–9.2 gave electropherograms with serious overlapping of peaks. At pH values below 7.0, all of the BBI compounds were positively charged and the peaks migrated before the electroosmotic flow (EOF). At pH values between 7.0 and 8.5, only a large peak overlapped with the EOF marker, indicating that all of the BBIs were practically electroneutral. At pH values greater than 8.8, some peaks appeared after the EOF because the BBIs were gradually becoming negatively charged.

MEKC was therefore employed. SDS (10–100 m*M*) was tested, but either a singe broad peak or a multi-split peak was obtained, even when organic modifiers were added. The change to 5–25 m*M* CTAB, a cationic surfactant often used in MEKC, did not improve the separation either. These phenomena suggested that the straight-chain hydrocarbon surfactant could not provide good selectivity for the separation. Compared with such surfactants, bile salts have steroid structures and higher polarity, and the partition coefficients can be lowered for molecules of higher lipophilicity. Four bile salts, sodium cholate, sodium taurocholate, sodium deoxycholate and sodium taurodeoxycholate, were selected for

tests and the former two were found to show very striking improvements both in peak shape and in separation, compared with the use of SDS and CTAB. Sodium cholate was chosen for subsequent use for economic reasons.

Physical factors also affect the separation. The applied voltage, capillary temperature, capillary length, injection pressure and injection time were all tested. Appropriate values of these are listed in Section 2.4.

3.1. Electrophoretic conditions

3.1.1. Choice of parameters and setting of their working ranges

Chemical factors, i.e., the factors involved in the composition of the electrophoretic media, are of pivotal importance to CE separations. Quite often, they provide more of a chance of manipulating the selectivity than physical factors do. In this work, the buffer media included four chemical factors, namely, the concentration of sodium cholate, the volume percentage of acetonitrile added as buffer modifier, the pH of the buffer and the concentration of the buffer electrolyte. Based on their relative importance to the separation, the former three were chosen to undertake the optimization by the three-parameter ORM scheme. The working ranges of the parameters are of such importance to the separation that they should be deliberately set before carrying out the optimization process. Preliminary studies on the chemical factors and for setting their working ranges are shown below.

3.1.1.1. Sodium cholate concentration

As generally found in MEKC, increasing the concentration of sodium cholate increased the migration times of the BBI molecules. At 18 kV and 25°C, the effect of sodium cholate was investigated in the concentrations range 10–200 m*M*. At concentrations below 100 m*M*, resolution between peaks decreased. At concentrations higher than 180 m*M*, the resolution increased little, there was an uneven baseline, the noise level was larger and peaks were shaped irregularly. A range of 120–160 m*M* was therefore decided upon for the optimization work.

3.1.1.2. Acetonitrile volume percentage

In MEKC, organic solvents are often used to improve the separation. The interactions between solutes and micelles are weakened and this helps to enhance the separation of lipophilic substances. In this work, acetonitrile provided better selectivities than the other solvents tested, such as methanol, isopropanol and dimethylformamide, therefore, it was chosen as the buffer modifier. Volume percentages of 5–30% acetonitrile were tested to determine the working range. At quantities lower than 23%, the overlapping of peaks occurred. At quantities higher than 28%, the time window (i.e. the migration time between the first and the last peaks) was reduced greatly and the resolution decreased. Thus, an acetonitrile range of 23–28% (v/v) was set for the ORM work.

3.1.1.3. pH of the buffer solution

The eight BBI alkaloids to be separated were either phenolic or non-phenolic. The pK_a values of their amine functions and phenolic groups (if any) were about 7.0 and 10.0, respectively. For the separation of mixtures of acidic (or basic) compounds, the optimum pH is often found to be near the mean value of their pK_a values. By analogy, the optimum pH for the separation of amphoteric substances can be sought from near the mean value of their isoelectric points (p*I* values). Buffer solutions of pH 6.0–9.0 were therefore tested to determine the working pH range. At pH values below 6.9, the peaks of homoaromoline and berbamine completely overlapped, as did the peaks of isotetrandrine, thalrugosine and norobaberine. At pH values below 7.5 and above 8.0, the peaks of homoaromoline and berbamine again overlapped, accompanied by the minor overlapping of other peaks. Therefore, the pH range of 7.5–8.0 was chosen for the optimization work. This narrow range is due to the close similarity in structures of the tested BBI compounds.

3.1.1.4. Buffer electrolyte concentration

The borate–phosphate combination buffers cover a wide buffer range and have low absorbances at shorter wavelengths. The current generated is also minimal with the borate, which has low mobility. Generally speaking, increasing the ionic strength of the buffer is helpful for separations as a result of the

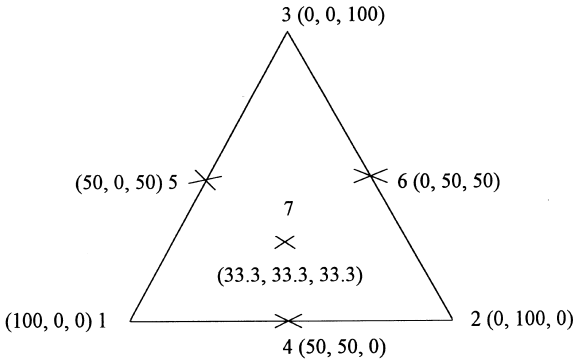

Fig. 2. Experimental design of the seven ORM experiments.

decrease in both the zeta potential and the wall-adsorption of solutes. After setting the working ranges of the three parameters, the ionic strength of the electrophoretic media was adjusted with the buffer electrolyte. Total concentrations of sodium borate–sodium dihydrogen phosphate in the range 50–120 m*M* were tested. It was found that, at concentrations below 70 m*M* and above 110 m*M*, both the efficiency (peak sharpness) and resolution were decreased. The current was too high at concentrations above 110 m*M*. Therefore, 90 m*M* was chosen as a compromise and this concentration was used throughout the optimization process.

3.2. Overlapping resolution scheme

The triangular ORM scheme requires seven experiments to be conducted at selected points in a triangle. The positions of the seven points are shown in Fig. 2, with the figures in parentheses indicating the percentages for the three parameters. The working range of each parameter has been specified in the above section. From this, the conditions of the seven preplanned experiments were set up, as depicted in Table 1 (relation between Fig. 2 and Table 1 was as follows: for sodium cholate, concentrations of 120 and 160 m*M* in Table 1 were taken as 0 and 100% compositions in Fig. 2, thus a concentration of 140 m*M* corresponded to 50% composition and a concentration of 133 m*M* corresponded to 33.3% composition. The percentage of acetonitrile and the pH followed the same relationship). From the seven electropherograms obtained from these experiments, the resolution, R_s, between adjacent peaks was calculated using the equation

$$R_s = \frac{1.18(t_2 - t_1)}{W_{1/2_1} + W_{1/2_2}} \quad (1)$$

where t_1 and t_2 are the migration times and $W_{1/2_1}$ and $W_{1/2_2}$ are the half-height peak widths of two adjacent peaks, respectively. The calculated R_s values for all of the peak pairs are shown in Table 2. These resolution values were then fitted to a polynomial equation:

$$R_s = a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{123}X_1X_2X_3 \quad (2)$$

where a_i are the coefficients and X_i are the percentages of each parameter as defined in Fig. 2. The values of a_i for each adjacent pair of peaks were determined (Table 3) using the BASIC program developed by Berridge [20]. From Eq. (2), the resolutions for each adjacent pair of peaks could be calculated for any composition of the three parameters in the triangle. Venn diagrams of each adjacent pair of compounds were then generated in which the various symbols represented the specified resolution

Table 1
Experimental conditions for the seven experiments

Experiment[a]	pH	Acetonitrile (%, v/v)	Sodium cholate (m*M*)
1	8.00	23.0	120
2	7.50	28.0	120
3	7.50	23.0	160
4	7.75	25.5	120
5	7.75	23.0	140
6	7.50	25.5	140
7	7.67	24.7	133

[a] All experiments were performed using 90 m*M* sodium borate–sodium dihydrogen phosphate.

Table 2
Resolution between adjacent peaks obtained from the seven experiments in Table 1

	Resolution, R_s, for peak pairs						
Experiment	1–2	2–3	3–4	4–5	5–6	6–7	7–8
1	16.47	5.48	0.00	2.74	2.85	2.68	2.36
2	7.08	4.03	2.95	2.07	2.44	2.36	0.66
3	16.57	6.47	0.91	1.71	6.79	2.12	2.85
4	14.75	6.11	1.89	3.03	2.28	3.26	1.18
5	17.11	6.99	1.10	2.56	4.25	2.85	2.29
6	9.95	7.56	0.98	1.22	5.65	1.67	2.21
7	14.16	7.35	1.48	2.11	4.08	2.29	1.75

levels [20]. By overlapping all seven Venn diagrams and then plotting the symbols representing the lowest resolution among all of the individual diagrams, areas defining the composition of buffer that would give the desired resolution among all of the peaks in the BBI alkaloid mixture were established. The resulting diagram for the eight BBIs is shown in Fig. 3. The regions marked by the symbol * should give a minimum resolution of 1.6 between all of the adjacent peak pairs. Point 1 was considered to give an optimum separation. The experiment was conducted with the conditions corresponding to this point (sodium cholate, 132 m*M*; acetonitrile, 25.8% (v/v); pH, 7.58). To test the validity of this optimization procedure, the condition of point 2, representing the predicted resolution level between 1.0 and 1.3, was used to conduct the experiment. The two electropherograms are shown in Figs. 4 and 5, respectively. The electropherogram obtained with the conditions predicted at point 1 shows that all eight peaks were baseline resolved in 20 min.

In view of the small area in the overlapping resolution diagram, which allows baseline separations for all adjacent peaks, the method does not seem to be rugged. This means that the resolution is very sensitive to small variations in the relevant parameters. To obtain a satisfactory separation, the operating conditions should be carefully followed.

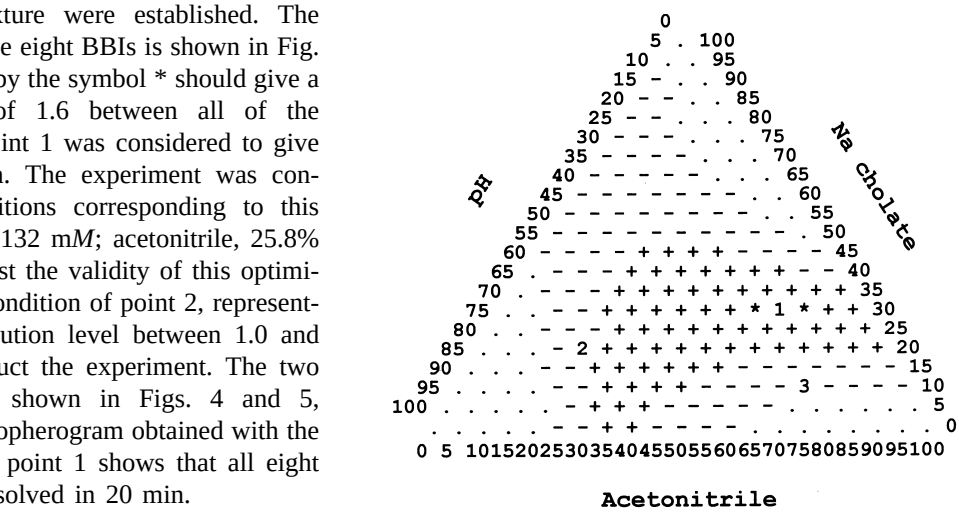

Fig. 3. Overlapped Venn diagram for the seven pairs of peaks. Notation: (●) $R_s<1.0$; (−) $1.0\leq R_s<1.3$; (+) $1.3\leq R_s<1.6$; (*) $R_s\geq 1.6$.

Table 3
Coefficients of Eq. (2) for adjacent peak pairs from the seven experiments

Peak pair	Coefficients						
	a_1	a_2	a_3	a_{12}	a_{13}	a_{23}	a_{123}
1–2	16.47	7.08	16.57	11.90	2.36	−7.50	0.96
2–3	5.48	4.03	6.47	5.42	4.06	9.24	−1.53
3–4	0.00	2.95	0.91	1.66	2.58	−3.80	3.90
4–5	2.74	2.07	1.71	2.50	1.34	−2.68	−5.19
5–6	2.85	2.44	6.79	−1.46	−2.28	4.14	0.24
6–7	1.68	2.36	2.12	4.96	3.80	−2.28	−13.05
7–8	2.36	0.66	2.85	−1.32	−1.26	1.82	−3.30

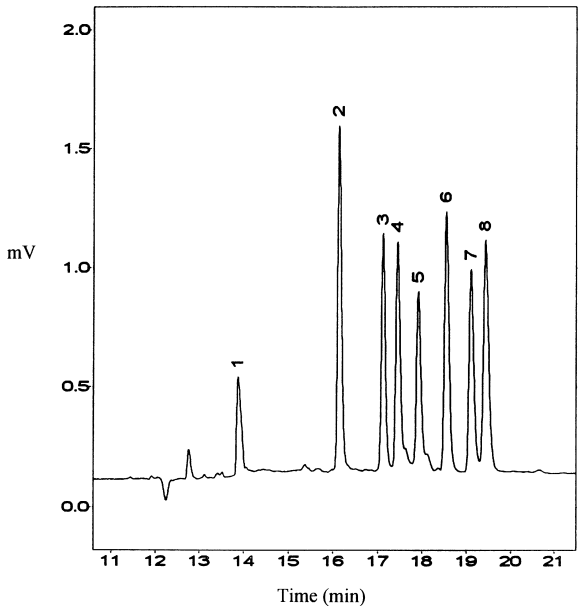

Fig. 4. Electropherogram of the BBI alkaloids obtained using the optimum electrophoretic conditions corresponding to point 1 in Fig. 3. Conditions: 90 m*M* sodium borate–NaH_2PO_4, 132 m*M* sodium cholate, pH 7.58–acetonitrile (74.2:25.8, v/v); fused-silica capillary, 50 μm I.D., total length of 66 cm, detection length of 51 cm; injection, 40 mbar, 1.2 s; voltage, 18 kV; temperature, 25°C; UV detection, 207 nm. The compounds' numbers are shown in Fig. 1.

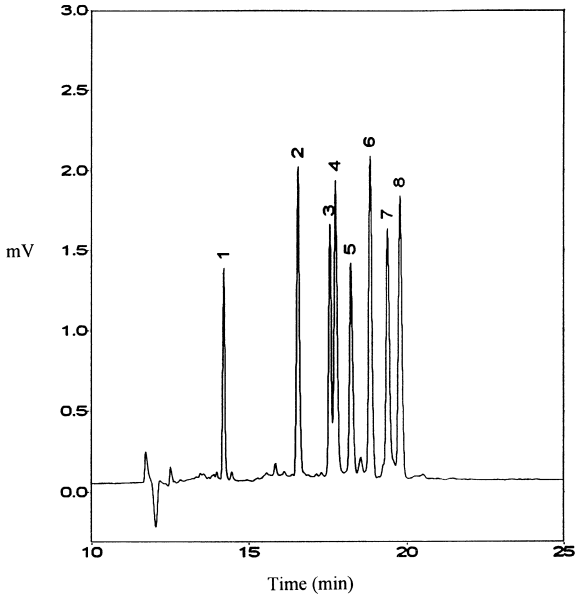

Fig. 5. Electropherogram of the BBI alkaloids obtained using the electrophoretic conditions corresponding to point 2 in Fig. 3. Conditions: 90 m*M* sodium borate–NaH_2PO_4, 128 m*M* sodium cholate, pH 7.80–acetonitrile (76:24, v/v); other conditions as shown in Fig. 4. The compounds' numbers are shown in Fig. 1.

3.3. Elution order of the BBI compounds

At pH 7.58 under the optimum separation conditions, the negatively charged cholate micelles (pK_a of cholic acid, 6.4) migrated the slowest with respect to the BBI molecules to the cathode. Basically, the more lipophilic the BBI molecule was, the stronger the interaction between the molecule and the micelles would be, and the more time it would take to elute the molecule. The elution of homoaromoline, isotetrandrine and thalrugosine after aromoline, berbamine and obamegine, respectively, illustrated this.

The eight analytes belong to two types of BBI alkaloids. Aromoline, homoaromoline and colorflammine are type VI BBI alkaloids, while the others are type VIII BBI alkaloids. Both types have two diphenyl-ether linkages, with one linkage (C_{11}–O–$C_{12'}$) being identical, whereas the other was of the type C_7–O–$C_{8'}$ or C_8–O–$C_{7'}$. Previous HPLC results suggested that type VIII BBIs were more lipophilic than type VI isomers [9]. In this work, the same elution order was observed: aromoline eluted before obamegine and homoaromoline eluted before thalrugosine. Isotetrandrine and tetrandrine are both non-phenolic and belong to the type VIII BBI alkaloids. They are more lipophilic and eluted more slowly than the others.

3.4. Comparison with the HPLC separation

To compare this MEKC method with the previous HPLC method [9] for the detection of these BBI alkaloids, the run time, resolution, efficiency and the limits of detection of the compounds are listed in the Tables 4 and 5. It can be seen that there is not much difference in resolution and run time between the two methods, however, the efficiency of the MEKC method is considerably greater than that of the HPLC method. Due to the very small volume injected, the detection limits in amount injected of the compounds

Table 4
Comparison of run time, minimum resolution and efficiency between the MEKC and HPLC methods

	Run time (min)	Minimum R_s [a]	Efficiency [b] (plate no.)
MEKC	19.4	1.62	126 383
HPLC	16.7	2.24	9163

[a] Calculated using the equation $R_s = 2(t_2 - t_1)/(w_1 + w_2)$.
[b] Average of the peaks, calculated using the equation $N = 5.54(t/w_{1/2})^2$.

achieved with the MEKC method are about one tenth of that with the HPLC method.

4. Conclusions

The ORM scheme was successfully used in this work for the separation of eight BBI alkaloids that only had very minor structural differences. With this scheme, the alkaloids were baseline separated within a moderate analysis time. The scheme itself is simple, straightforward and does not require a theoretical model to describe the migration behavior of the solutes. However, to use this scheme effectively, attention should be paid to two things. First, the three parameters for the scheme should be chosen suitably. They are preferably the ones that affect the migration of solutes with greater discrimination than the others. Second, the working ranges of the parameters should be defined with care. They should include the region where a basic separation has been achieved. In this sense, the ORM scheme is rather like a refining process and, therefore, simple diagrams based on migration times (as a function of the parameters) would be of much help to the success of the scheme.

The MEKC method developed showed good selectivity and sensitivity, which were either better than or comparable to those of the HPLC method. The method should be applicable to the determination of BBI alkaloids in plants. Such analysis is under investigation in our laboratory.

Table 5
Comparison of the limits of detection between the MEKC and HPLC methods

Compound	Limit of detection [a]			
	Concentration (ng/ml)		Amount (pg)	
	MEKC	HPLC	MEKC	HPLC
Colorflammine	35 000	240	35	480
Aromoline	24 000	110	24	220
Homoaromoline	29 000	120	29	240
Berbamine	16 000	110	16	210
Obamegine	33 000	140	33	280
Isotetrandrine	31 000	100	31	200
Thalrugosine	31 000	140	31	270
Tetrandrine	29 000	150	29	290

[a] At $S/N = 3$.

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