Frontiers in Medical Science Research
ISSN 2618-1584 Vol. 7, Issue 2: 97-102, DOI: 10.25236/FMSR.2025.070214

Exploration of Capillary Electrophoresis Separation Mechanism for Alkaloids of Sophora Flavescens by Computer-aided Molecular Modeling

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Abstract: *Capillary electrophoresis (CE) shows remarkable separation performance and has advanced to a mature technique for all aspects of pharmaceutical analysis. In this study, triangle coupled with tetrahedron optimization method was used to develope a simple CE method for establishing the CE fingerprints of Alkaloids of Sophora flavescens (ASF). Computer-aided molecular modeling study was first performed to investigate the separation mechanism of the diastereomers in ASF. Experimental results and molecular modeling studies both support that significant differences in the complexation stabilities of the alkaloids with borate and citric acid is mainly responsible for the separation of matrine and sophoridine.*

Keywords: *capillary electrophoresis, molecular modeling, separation mechanism, Alkaloids of Sophora flavescens (ASF), triangle coupled with tetrahedron optimization method*

1. Introduction

Alkaloids of Sophora flavescens (ASF) contains many alkaloids with similar structure and properties. For example, matrine, oxymatrine, and sophoridine own the same basic tetracyclic skeleton, and matrine differs from sophoridine only in H-conformation at C-5 (Figure 1B). ASF quality control is typically based on the total alkaloid content using a titration method [1], or on the determination of one or several quinolizidine alkaloids by HPLC [2] and capillary electrophoresis (CE)[3]. However, none of these reports have done the comprehensively holistic quality control nor discussed the possible separation mechanism. With the development of herbal medicines, there is an emergent need to have insight into the mechanism of separation, which would help clarifying the influence of conditions and developing new separation methods more rapidly and efficiently. Thus, proposing a method to develop fingerprints for comprehensively assessing the quality consistency of Alkaloids of Sophora flavescens (ASF) and exploring the separation mechanism are of great importance.

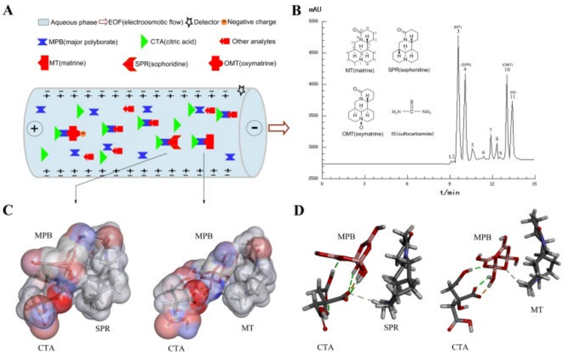


Figure 1 (A) Schematic diagrams of separation mechanisms. (B) The electropherogram of ASF under the optimal conditions. (C) The molecular electrostatic potential surface of the optimized conformation of SPR-complexe and MT-complexe. Red color stands for the maximum negative region and blue color stands for the maximum positive region. (D) Geometries of the most favourable complexes of SPR-complexe and MT-complexe. C:grey, H:white, O:red, N:blue,B:pink.

CE is a potential separation method of low consumption and environmental friendliness. Though remarkable progress has been achieved in separating mixtures of enantiomers [4], utilization of CE for separation of diastereomers and multi-components is still challenging. The ligand exchange principle is widely used in enantioseparations [5], in which Cu^{2+}, Zn^{2+}, borate ions, or other metal ions are usually used as central ions [6]. In the present work, CZE was used to develop the CE fingerprint for Alkaloids of Sophora flavescens, and computer-aided molecular modeling study was first performed to investigate the separation mechanism of the diastereomers in the phytochemical compositions based on the complexation among three moleculars (Figure 1), in which boron anion was used as central ion.

2. Basic principles of triangle coupled with tetrahedron optimization method

The BGE is a critical factor that has an important influence on the CZE analysis. As described in Figure 2, we firstly used triangle coupled with tetrahedron optimization method in the optimization of background electrolytes (BGE), which is easier to use compared with other methods [7]. The triangle and tetrahedron optimization methods belong to the methods of direct searching optimal value. The basic process for the triangle optimization method is as follows. First, we have to choose an equilateral triangle △ABC (Figure 2a). The three vertexes, named as A, B and C, represent three most representative and commonly used buffers including sodium borate, sodium dihydrogen phosphate, and disodium hydrogen phosphate, respectively. Then, one chooses the BGE conditions constituted by points No.1–No.3 for the electrophoresis and then determines which BGE is suitable for the separation of the sample according to the separation results. If the separation conditions for the substance present no big differences under the previous three conditions, the BGE of points No.4 - No.10 can be selected for testing and the zones with ideal test results, for example, new triangles △HIJ and △KLM can be used for further testing. The BGE composed of vertexes A, B, and C can improve its separation by adding organic solvents like methyl alcohol and acetonitrile, or by adjusting the pH with dilute phosphoric acid or sodium hydroxide to obtain ideal analytical results. If the separation of the sample solution under the BGE conditions constituted by points in △ABC is not ideal, the tetrahedron optimization method should be considered, i.e., the use of △ABC and point D (representing H3BO3) to form a tetrahedron (Figure 2b). Thus, △ABD, △ACD and △BCD (Figure 2c), or a random triangle (△EFG) obtained from the tetrahedron (Figure 2b), can be adopted for the optimization according to the triangle method so as to obtain the ideal BGE. The purpose of adding point D is to enhance the separation of the components that can form coordination complexes with boron atoms. Since the ionization of H3BO3 is quite weak, it has little influence on the ionic strength.

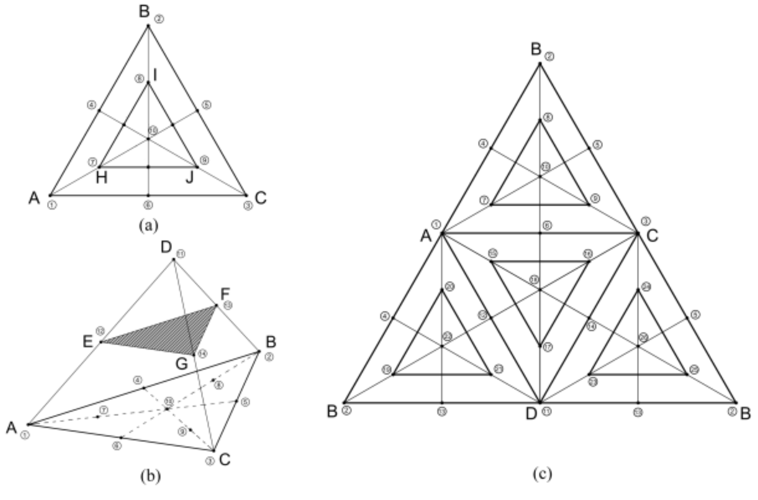


Figure 2 Triangle coupled with tetrahedron optimization method for the selection of BGE.

3. Results and discussion

3.1. Optimization of CE conditions

As shown in Figure 2, three vertices of triangle, named as A, B, C, represented 50 mmol/L sodium tetraborate, 150 mmol/L sodium dihydrogen phosphate, and 50 mmol/L disodium hydrogen phosphate, respectively. First, the experiment was carried out under the conditions of A, B and C. However, the separation was not satisfactory. Then, a total of 23 other types of BGE (shown in Table1) were tested in terms of triangle coupled with tetrahedron optimization method. It was found that the BGE (No.12) composed of sodium tetraborate and boric acid was most suitable for sample separation in all the systems. We guessed it might be related to boron anion complexaion with certain components. Thus, a series of BGE composed of sodium tetraborate and boric acid with different concentrations were prepared and tested. The results demonstrated that the aqueous double-borate solution composed of 50 mmol/L sodium tetraborate and 500 mmol/L boric acid favors the sample separation and the pH value of the above solution was measured to be 8.03.

Table 1 Compositions and concentrations of BGEs

No.	composition	A (mmol/L)	B (mmol/L)	C (mmol/L)	D (mmol/L)
1	A	50			
2	B		150		
3	C			50	
4	A:B(1:1, v/v)	25	75		
5	B:C(1:1, v/v)		75	25	
6	A:C(1:1, v/v)	25		25	
7	A:B:C(4:1:1, v/v/v)	33.33	25	8.33	
8	A:B:C(1:4:1, v/v/v)	8.33	100	8.33	
9	A:B:C(1:1:4, v/v/v)	8.33	25	33.33	
10	A:B:C(1:1:1, v/v/v)	16.67	50	16.67	
11	D				500
12	A:D(1:1, v/v)	25			250
13	B:D(1:1, v/v)		75		250
14	C:D(1:1, v/v)			25	250
15	A:D:C(4:1:1, v/v/v)	33.33		8.33	83.33
16	A:D:C(1:4:1, v/v/v)	8.33		8.33	333.33
17	A:D:C(1:1:4, v/v/v)	8.33		33.33	83.33
18	A:D:C(1:1:1, v/v/v)	16.67		16.67	166.67
19	A:D:B(4:1:1, v/v/v)	33.33	25		83.33
20	A:D:B(1:4:1, v/v/v)	8.33	25		333.33
21	A:D:B(1:1:4, v/v/v)	8.33	100		83.33
22	A:D:B(1:1:1, v/v/v)	16.67	50		166.67
23	C:D:B(4:1:1, v/v/v)		25	33.33	83.33
24	C:D:B(1:4:1, v/v/v)		25	8.33	333.33
25	C:D:B(1:1:4, v/v/v)		100	8.33	83.33
26	C:D:B(1:1:1, v/v/v)		50	16.67	166.67

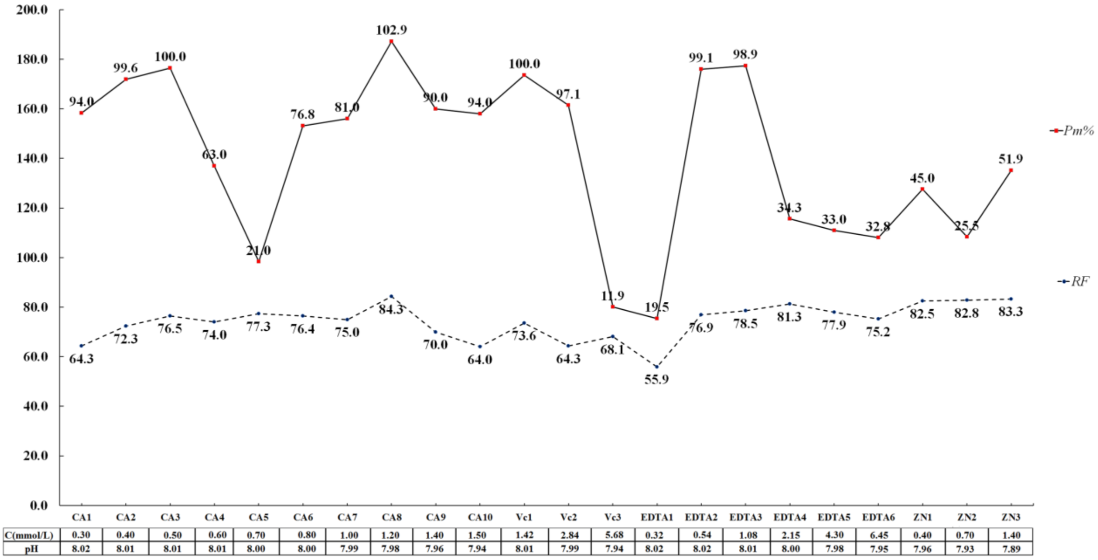
The selection of pH values, organic modifiers (including methanol, acetonitrile, isopropanol and hexane), and sodium dodecyl sulfate (SDS) was then investigated, but the results were unsatisfactory, especially the peaks of MT and SPR were nearly coeluted. Interestingly, the addition of acid additives (including citric acid, zinc sulfate heptahydrate, vitamin C and EDTA) to the BGE markedly improved sample resolution. So different types and concentrations of acid additives were investigated as listed in Figure 3. The resolution index (*RF*) and the macro quantitative similarity ($\boldsymbol{P}_m$) [8] were employed as double indexes to evaluate the conditions. The ***RF*** (Eq. 1) reflects the resolution, effective signal amount, and degree of fingerprint signal uniformity, while $\boldsymbol{P}_\mathrm{m}$ (Eq. 2) measures the total contents of all common peaks. The higher the *RF* and $\boldsymbol{P}_\mathrm{m}$ values, the better the test conditions. Figure 3 shows that the BGE composed of sodium borate (50 mmol/L), boric acid (500 mmol/L) and citric acid (1.2 mmol/L) is optimal because a better resolution is achieved with maximum ***RF*** and $\boldsymbol{P}_m$ values of 84.3 and 102.9, respectively. Other conditions were further investigated. Sodium dihydrogen phosphate (50 mmol/L) was selected as the sampling solvent due to its maximum ***RF*** value. A detection wavelength of 210 nm led to a maximum ***RF*** value of 60.2 with higher sensitivity than other detection wavelengths. A running voltage of 12 kV was selected to achieve high resolution and an appropriate analytical time. The injection time was set to 20 s with the appropriate response value of the sample, but no peak broadening. To our knowledge, this is the first time ASF has been separated with 10 peaks of components by CE.

$$\boldsymbol{RF} = \tau[\sum_{i=1}^{n-1} R_i \lg\left(\frac{A_i + A_{i+1}}{2}\right)_i + \frac{2(t_{R_n} - t_{R_1})}{(n-1)(W_1 + W_n)} \lg\left(\frac{A_1 + A_n}{2}\right)] \tag{1}$$

$$\boldsymbol{P}_m = \frac{1}{2}(C + P)\frac{m_{RFP}}{m_i} = \frac{1}{2}\left(\frac{\sum_{i=1}^{n} x_i y_i}{\sum_{i=1}^{n} y_i^2} + \frac{\sum_{i=1}^{n} x_i}{\sum_{i=1}^{n} y_i} S_F\right)\frac{m_{RFP}}{m_i} \times 100\% \tag{2}$$

3.2. Computer-aided molecular modeling study of the separation mechanism

We further explored the separation mechanism of alkaloids compositions based on complexation (Figure 1). The pH of the optimized BGE is measured to be 7.98, and the pKa values of MT, OMT and SPR are 8.24 [9], 6.98 [10], and 8.97 [11] ,respectively. So MT and SPR both exist in the form of molecular while OMT exists as an anion. The chemical constitution of the aqueous borate solutions mainly depends on the total boron concentration and pH in the solution [12]. Based on previous studies [13-16], we can conclude that the major polyborate is $B_4O_5(OH)_4^{2-}$ and that the minor chemical species are $B_5O_6(OH)_4^-$, $B_3O_3(OH)_4^-$,and $B(OH)_4^-$ in the BGE.



	CA1	CA2	CA3	CA4	CA5	CA6	CA7	CA8	CA9	CA10	Vc1	Vc2	Vc3	EDTA1	EDTA2	EDTA3	EDTA4	EDTA5	EDTA6	ZN1	ZN2	ZN3
C(mmol/L)	0.30	0.40	0.50	0.60	0.70	0.80	1.00	1.20	1.40	1.50	1.42	2.84	5.68	0.32	0.54	1.08	2.15	4.30	6.45	0.40	0.70	1.40
pH	8.02	8.01	8.01	8.01	8.00	8.00	7.99	7.98	7.96	7.94	8.01	7.99	7.94	8.02	8.02	8.01	8.00	7.98	7.95	7.96	7.93	7.89

*Figure 3 Stacked line chart of **RF** and $\boldsymbol{P}_m$ values under different experimental conditions*

Figure 3 showed that at the same pH, the separation performance was different among BGEs with different types and concentrations of acid additives. For example, citric acid (1.2 mmol/L) gave a better separation performance than EDTA (4.3 mmol/L) at pH 7.98. So the pH was not the crucial factor in the separation mechanism. We expect that the citric acid in the buffer would act as an ancillary ligand. By formation of transient reversible ternary complexes, the mobility differences can be effectively increased, thereby molecular recognition is obtained. Besides, the analytes with negative charge such as OMT would be eluted later.

To have a thorough insight into the separation and to rationalize our experimental results, we performd computer modeling techniques on investigating the interaction among major polyborate, citric acid and diastereomers of MT and SPR at the density functional theory level with a B3LYP functional and 6–31G(d, p) basis set. The calculated binding energies (ΔE) associated with the formation complexes of diastereomer with borate and citric acid are obtained using following equation: $\Delta E = E_{complex} - (E_{free\text{-}borate} + E_{free\text{-}citric\ acid} + E_{free\text{-}diastereomer})$, where $E_{complex}$, $E_{free\text{-}borate}$, $E_{free\text{-}citric\ acid}$ and $E_{free\text{-}diastereomer}$ are the energies of ternary complexe, free borate, free citric acid and free diastereomer, respectively. The difference of ΔE of MT-complexe and SPR-complexe is calculated using following equation: $\Delta\Delta E = \Delta E_{MT/borate/citric\ acid} - \Delta E_{SPR/borate/citric\ acid}$. On account of the principle that the magnitude of the energy change indicates the tendency toward complexation, the more negative the binding energy change, the more thermodynamically favorable and the more stable the corresponding complex is. Compared the possible complexation geometries, the complexation energies are in favor of connection mode of complexation showed in Figure 1D. For this mode, $\Delta E_{\text{MT/borate/citric acid}}$ was -377.43 kJ/mol and $\Delta E_{\text{SPR/borate/citric acid}}$ was -417.04 kJ/mol. It is clear that SPR-complex are more stable than MT-complex with a formidable energy difference ($\Delta\Delta E$ = 39.61 kJ/mol). Considering the peak order in the previous CE experiments (Figure 1B), it was consistent with the inference that a more stable comlexe woud have longer migration time in capillary [17]. In addition, the migration behavior of MT and SPR in the presence of citric acid as a chiral additive confirms these theoretical calculation results, suggesting that the difference in the complexation stability of these compounds with citric acid lead to their separation.

The main forces that contribute to the stability of the ternary complexes are electrostatic forces and van der Waal's forces. The H-configurations at C-5 in MT and SPR are α-H and β-H, respectively, which lead to the different spatial conformations of the diastereomers. Compared with α-H, β-H is closer to carbonyl at C-15 in the spatial structure. As carbonyl has strong ability to absorb electrons, the different H-configurations of MT and SPR result in different electron-donating abilities. These differences gave rise to the difference of binding mode between diastereomer and borate. The hydrogen bonding also highly contributed to the stability of the complexes. Inspection of the optimized structures in Figure1D reveals that the presence of more hydrogen bonds add an additional stability to the SPR-complex.

4. Conclusions

In conclusion, we have developed a simple CE method to establish the CE fingerprints of ASF. Molecular modeling studies revealed for the first time that the separation of structurally similar alkaloids can be obtained due to the large differences in the complexation stabilities of ternary complexes. We expect the reported strategy of using borate and citric acid to separate phytochemical compositions will find more applications in separation research.

Acknowledgements

The authors wish to thank Yizhao Geng (Hebei University of Technology, Tianjin, China), Guoxiang Sun (Shenyang Pharmaceutical University, Shenyang, China) and Fangliang Yang (Shenyang Medical College, Shenyang, China) for their technical contribution to this manuscript.

This work was funded by High-level Talent Funding Project of Hebei Province:"333 Talent Project" Talent Training Funding Project (C20221100).

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