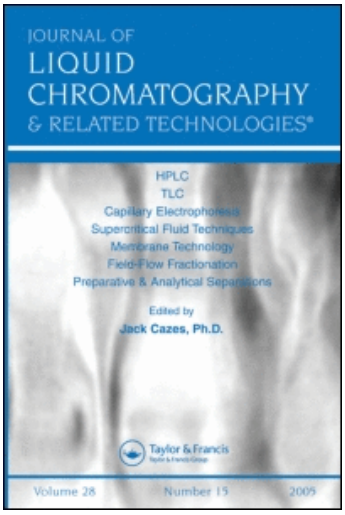

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Capillary Electrophoresis of Biologically Important Compounds: Optimization of Separation Conditions by the Overlapping Resolution Mapping Scheme

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CAPILLARY ELECTROPHORESIS OF BIOLOGICALLY IMPORTANT COMPOUNDS: OPTIMIZATION OF SEPARATION CONDITIONS BY THE OVERLAPPING RESOLUTION MAPPING SCHEME

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ABSTRACT

The use of a simple and rapid systematic optimization scheme is described. This scheme, known as the overlapping resolution mapping scheme (ORM), makes use of nine preliminary experiments to predict the optimum separation conditions. In this investigation, the validity of the ORM scheme is verified by choosing the optimum separation conditions predicted by the scheme in the separation of a group of twelve amino acids derivatised with the N-t-butoxycarbonyl functional group, and a group of seven anti-malarial drugs. The effect of the buffer composition on the separation is also discussed.

INTRODUCTION

Since the pioneering works of Jorgenson and Lukacs [1,2], capillary electrophoresis (CE) has witnessed tremendous advances and a variety of methods and modes of operation have been developed. In particular, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are now being considered as highly efficient and

sensitive microanalytical techniques. In these two modes of CE, pH and micellar concentration are regarded as the two most prominent factors to effect a separation. However, with the increasing complexity of samples to be analysed, pH and surfactant concentrations may not be selective enough to obtain optimum separations. Modifiers such as cyclodextrins [3,4], urea [5] and tetraalkylammonium salts [6] are added into the electrophoretic medium to improve the selectivity of the system.

With an increase in the number of modifiers in the buffer system, the optimization of these separations would be extremely complex and time consuming if trial-and-error type of approaches for optimization of separation are adopted. Therefore, there is a need to develop systematic schemes for CE separations.

A few approaches have been adopted for the optimization of CE separations. Foley [7] and Ghowsi *et al* [8] have evaluated and presented theoretical equations for the optimization of resolution (R_s) and resolution per unit time (R_s/t_R) for MEKC. In the work presented by Foley [7], the optimum retention factor ($k^{'}$) and the corresponding surfactant concentration to use can be predicted on the solute's property, the average partition coefficient of two solutes (P_{wm}) and the four parameters : t_0, t_{mc}, V and cmc. Electrokinetic equations for the resolution and migration time for micellar electrokinetic chromatography have also been derived by Ghowsi *et al* [8]. These equations permit the determination of optimum resolution for MEKC separation of neutral solutes in three cases, where the migration mobility of the micelle is negative, zero and positive. Nevertheless, these were theoretical treatments, and there was no attempt to verify them experimentally.

Systematic optimization of CE separations using the modified overlapping resolution mapping (ORM) schemes has been reported recently [9-11]. Widely used for the optimization of liquid chromatographic separations [12-14], the procedure has been modified for the CE separation of plant growth regulators [9], whereby a triangular experimental design was utilized for the optimization. Other modifications include a rectangular plot [10,11] which involves the optimization of the buffer composition involving two variables. An advantage of using the ORM scheme is that this scheme is suitable for separating complex samples containing many solutes of interest. In this work, the ORM scheme with the rectangular design was adopted for the CE separation of a group of N-t-butoxycarbonyl (Boc-derivatised) amino acids and a group of anti-malarial drugs. Both of these groups of compounds were selected as test substances to illustrate the applicability of systematic CE optimization to the analysis of biologically important species.

EXPERIMENTAL

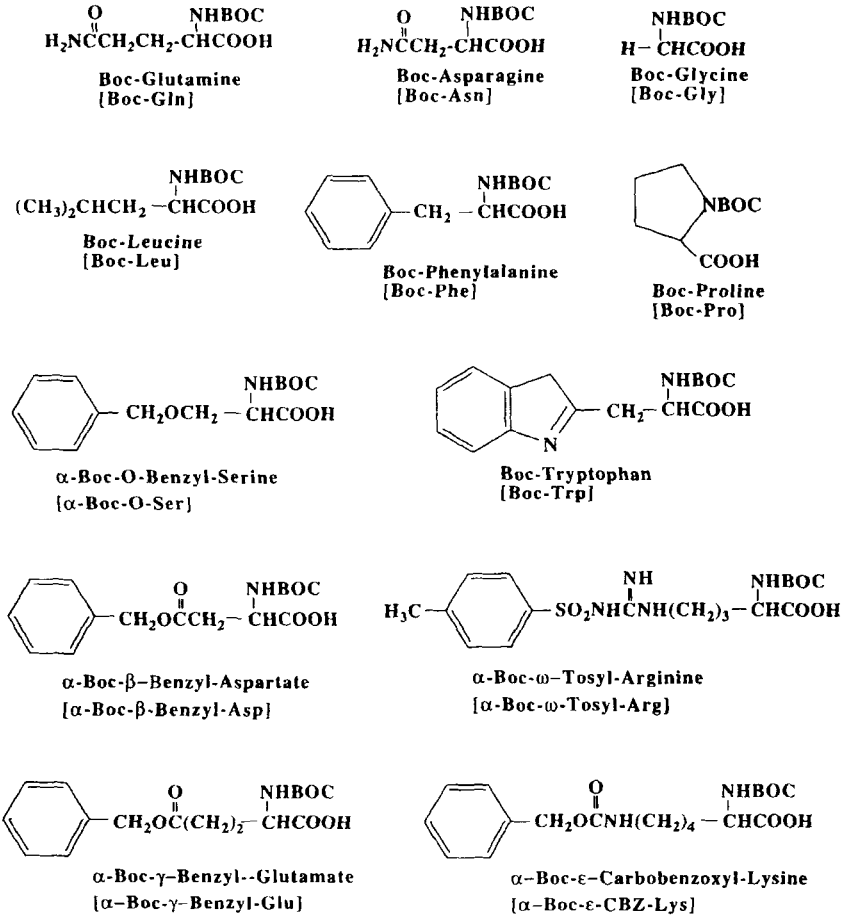
Capillary electrophoretic separations were conducted on a laboratory-built CE system. Detection of peaks was performed on a microUVis20 UV detector (Carlo Erba Instruments, Milan, Italy) with wavelengths of 190 nm and 240 nm set for the detection of boc-amino acids and anti-malarial drugs respectively. High voltage was achieved through the use of a Spellman (Plainview, NY, USA) Model RM15P10KD power supply capable of delivering up to 15 kV. Fused silica capillary columns of 50 cm x 50 μm ID were obtained from Polymicro Technologies (Phoenix, Az, USA). Chromatographic data were collected and analysed using a Shimadzu Chromatopac CR6A (Kyoto, Japan) integrator.

All chemicals used were of analytical grade or better. Sodium dodecyl sulphate (SDS), sodium tetraborate and sodium dihydrogen phosphate were obtained from Fluka (Buchs, Switzerland), while tetrabutylammonium bromide (TBA), which was used as a modifier, was purchased from Tokyo Kasei Kogyo Company Ltd (Tokyo, Japan). All boc-derivatised amino acids and the anti-malarial compounds, sulfadiazine and pyrimethamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dapsone, quinacrine and primaquine were purchased from Fluka, while quinine and chloroquine were obtained from the Department of Pharmacology, National University of Singapore. The structures of the boc-amino acids and the anti-malarial drugs are shown in Figures 1(a) and 1(b) respectively. Solutions were filtered through a Millipore filtration system. All standards were prepared in the concentration range of 100-200 ppm in methanol. Samples were injected hydrodynamically for 5 sec at a height of 5 cm. Each injection volume was estimated to be 1 nL [15].

RESULTS AND DISCUSSION

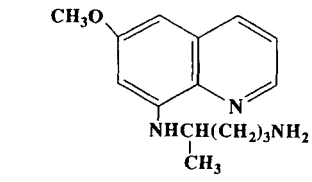
The Overlapping Resolution Mapping (ORM) scheme is a statistical experimental model used to define the region of interest in which the optimum condtions reside. The scheme predicts the optimum operating conditions such as the pH of electrophoretic medium and β-cyclodextrin [10] or pH and a micellar solution containing SDS [11] from nine preliminary experiments.

The first step of the ORM scheme involved determining the criteria for optimization [10,11]. The criteria used in the present investigation are firstly, all peaks should be baseline separated and secondly, all peaks should elute within a suitable migration time window [10,11]. For hydrophobic compounds, which involves the use of SDS, a typical migration

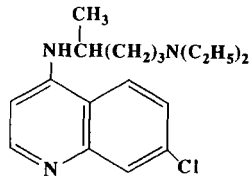


* : BOC denotes the derivatising group : $(CH_3)_3COC(=O)-$

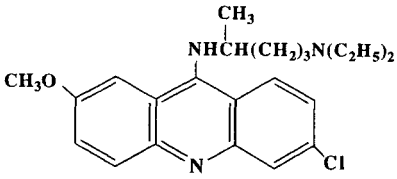
FIGURE 1(a). Structures of the twelve boc-derivatised amino acids used in this investigation.



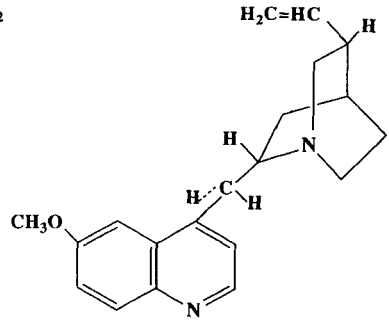
Primaquine



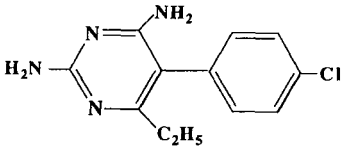
Chloroquine



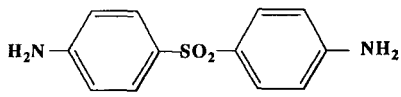
Quinacrine



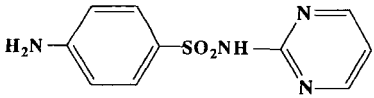
Quinine



Pyrimethamine



Dapsone



Sulfadiazine

FIGURE 1(b). Structures of the seven anti-malarial compounds used in this investigation.

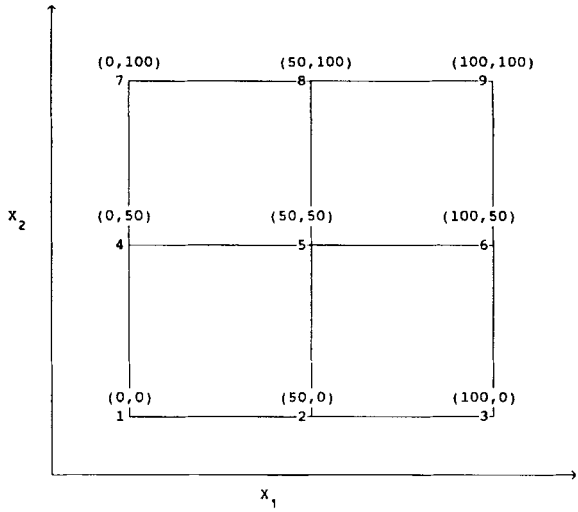


FIGURE 2. The locations of the nine experiments chosen from the rectangular plot for the boc-amino acids (x_1 = SDS concentration, x_2 = pH), and the anti-malarial drugs (x_1 = TBA concentration, x_2 = pH). The conditions are represented in terms of percentages with the minimum and maximum values of pH and SDS or TBA at the respective apices set at 0 and 100 % respectively.

time would be 30 min. On the other hand, for hydrophilic or ionic compounds, a shorter migration window of 15 min should be assigned.

Choice of the two variables

After setting the criteria, the operating conditions at the nine positions shown in Figure 2 were chosen. The ORM is flexible in that it allows the four experiments at the vertices of the rectangular plot (experiments 1, 3, 7 and 9 in Figure 2) to cover as wide as possible the working range of the system. Preliminary CZE experiments conducted on the group of boc-amino acids showed that they attained a partial negative charge within the pH range of 6-8 due to ionization of the carboxylic hydrogen. Since they are relatively hydrophobic and do not appear to exhibit differential migration velocities under the influence of pH, no satisfactory separation would be expected by varying pH alone. The addition of SDS to the buffer would serve to solubilize the more hydrophobic boc-amino acids. Hence, pH and SDS concentrations were used in the optimization scheme.

Five of the seven anti-malarial compounds, primaquine, chloroquine, quinacrine, quinine and pyrimethamine have basic functional groups [17] (the heterocyclic nitrogen as well as the side chain nitrogen, as shown in Figure 1(b)). Thus, they would be expected to be positively charged while dapsone and sulfadiazine are neutral and negatively charged respectively within the buffer pH range of 6-8. The positively charged compounds would tend to be adsorbed onto the wall of the capillary column. Thus, a modifier, tetrabutylammonium bromide (TBA), which could prevent adsorption by ion-pairing with the negative charges on the wall of the capillary and also serve to enhance separation efficiency was used [16]. Accordingly, pH and the concentration of TBA were chosen as the parameters to vary in the optimization scheme.

Calculation of resolution values

Once all the conditions for the nine experiments were determined, the experiments were carried out. The resolution R, between the individual pairs of adjacent peaks regardless of their identities and positions in the nine electropherograms were calculated using equation (1)

$$R = \frac{2(t_2-t_1)}{W_1+W_2} \qquad (1)$$

where t_1 and t_2 are the migration times of the two adjacent peak pair, W_1 and W_2 are the widths of the peak pair. These values were then fitted into a polynomial equation :

$$R = a_0 + a_1x_1 + a_2x_2 + a_{12}x_1x_2 + a_{11}x_1^2 + a_{22}x_2^2 + a_{112}x_1^2x_2 + a_{122}x_1x_2^2 + a_{1122}x_1^2x_2^2 \qquad (2)$$

where x_i's correspond to the proportions (in percentages) at the respective axes and a_i's are the coefficients. By substituting the resolution values obtained from equation (1) from the nine preliminary experiments into equation (2), the coefficients (a_i's) for each peak pair were determined. With the known coefficients, the R values for all experimental conditions within the maximum range selected could be calculated.

With the 12 boc-amino acids, eleven resolution plots were generated, each resolution plot representing a particular peak pair. Similarly, for the seven anti-malarial drugs, six resolution plots were obtained. By overlapping all the resolution plots for the twelve boc-amino acids

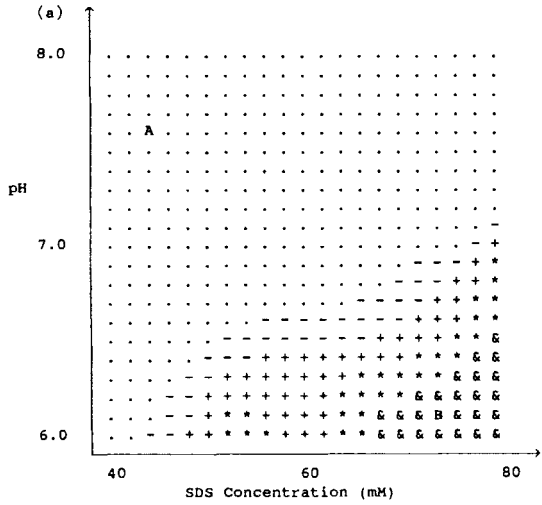


FIGURE 3(a). Final overlapped resolution plot for all the pairs of peaks for the boc-amino acids.
Notation : .., R < 0.4; --, 0.4 ≤ R < 0.8; ++, 0.8 ≤ R < 1.2; **, 1.2 ≤ R < 1.6; &&, R ≥ 1.6.

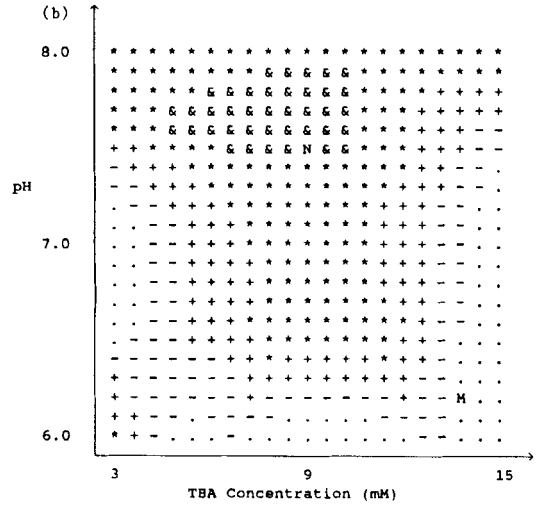


FIGURE 3(b). Final overlapped resolution plot for all the pairs of peaks for the anti-malarial compounds
Notation : .., R < 0.2; --, 0.2 ≤ R < 0.4; ++, 0.4 ≤ R < 0.6; **, 0.6 ≤ R < 0.8

and seven anti-malarial compounds, a final overlapping resolution map could be obtained each for these two groups of compounds and they represent the worst separation among all peak pairs. The final overlapped plots for the boc-amino acids and the anti-malarial drugs are shown in Figures 3(a) and (b). In these figures, the regions marked with "&" indicates the highest resolution between all peak pairs and these are regions where the optimum conditions are expected to be found. Satisfactory separation of all the peaks is expected to be obtained with experimental conditions selected from these regions.

To validate the ORM scheme, experimental conditions corresponding to points A and B in Figure 3(a) and M and N in Figure 3(b), were chosen from the regions represented by the symbols "." and "&" respectively. Points A and M represent experimental conditions for which poor resolution ($R < 0.4$ for boc-amino acids and $R < 0.2$ for the anti-malarial drugs) is expected, whereas points B and N represent experimental conditions which are expected to produce satisfactory separation with $R > 1.6$ for the boc-amino acids and $R > 0.8$ for the anti-malarial compounds. Typical electropherograms obtained using these conditions (A and B for the boc-amino acids and M and N for the anti-malarial compounds) are shown in Figures 4 and 5. From Figure 4(a), it can be seen that coelution of peaks occurred for experimental conditions corresponding to point A which lies outside the optimum region. On the other hand, using the experimental conditions corresponding to point B, all peaks are baseline separated, as shown in Figure 4(b). Thus, the optimum scheme has proven to be an efficient scheme where overall optimum conditions can be predicted with good accuracy. With two or more different optimum regions indicated in the final overlapped diagram, there is great flexibility in choosing the desired experimental conditions, especially when certain practical considerations have to be taken into account. For example, the constant use of high pH could be detrimental to the column or economically, high concentrations of added modifier would not be preferred. With the final overlapped resolution plot, however, alternative optimum conditions could be selected which would allow milder conditions or lower concentration of modifier to be used. From Figure 3(a), the optimum region found involved the use of low pH and high concentration of SDS. By considering the form of the final overlapped diagram, if experimental conditions involving a pH lower than 6 and a SDS concentration of above 80 mM are used, the resolution obtained would most likely fall within our desired range of above 1.6. However, with a simultaneous reduction in pH and increase in SDS concentration, the migration times of the solutes would be significantly increased. The use of the final overlapped diagram therefore, indicates the highest pH and lowest SDS

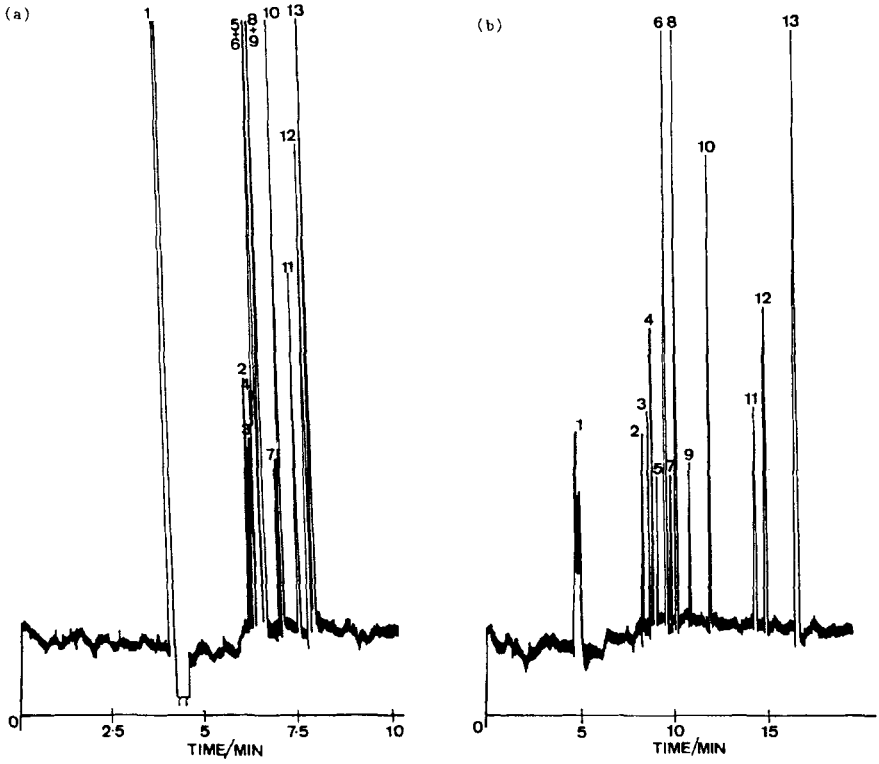


FIGURE 4(a). Electropherogram for the twelve boc-amino acids obtained using experimental conditions chosen from a point marked 'A', located outside the optimum region in Fig 3(a).
Peak identification : (1) Methanol; (2) Boc-Gln; (3) Boc-Asn; (4), (5), (6) Boc-Pro, Boc-Leu and Boc-Phe; (7) Boc-Gly; (8), (9) Boc-Ser and Boc-Trp; (10) Boc-Asp; (11) Boc-Arg; (12) Boc-Glu; (13) Boc-Lys
Electrophoretic conditions : 44 mM SDS in 0.025 M borate/0.05 M phosphate buffer; pH 7.6; separation tube : 45 cm x 50 μm ID; voltage : 15 kV; current : 41 μA; detection wavelength : 190 nm.

FIGURE 4(b). Electropherogram for the twelve boc-amino acids obtained using experimental conditions chosen from a point marked 'B', located in the optimum region in Fig 3(a).
Peak identification : (1) Methanol; (2) Boc-Gln; (3) Boc-Asn; (4) Boc-Pro; (5) Boc-Leu; (6) Boc-Phe; (7) Boc-Gly; (8) Boc-Ser; (9)Boc-Trp; (10) Boc-Asp; (11) Boc-Arg; (12) Boc-Glu; (13) Boc-Lys
Electrophoretic conditions : 74 mM SDS in 0.025 M borate/0.05 M phosphate buffer; pH 6.10; separation tube : 45 cm x 50 μm ID; voltage : 15 kV; current : 45 μA; detection wavelength : 190 nm.

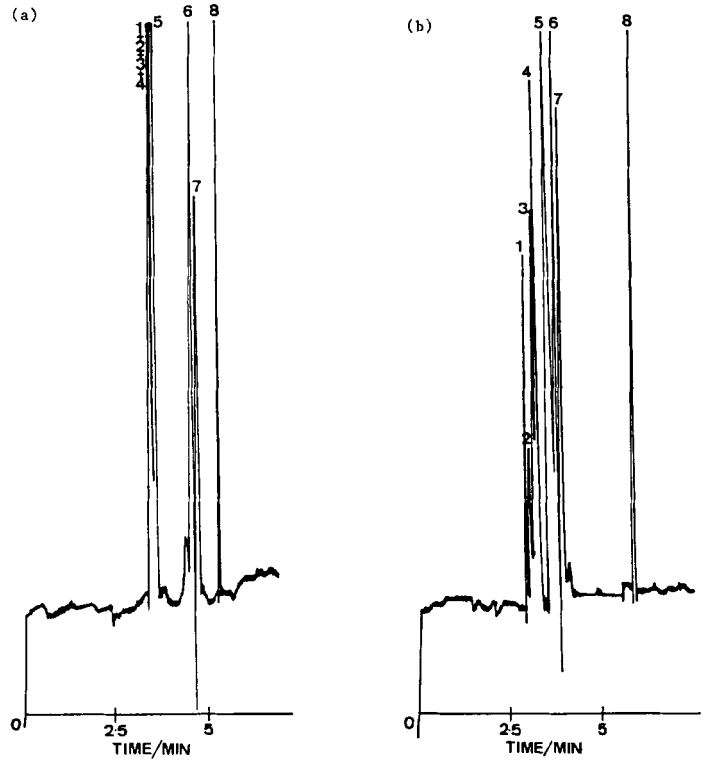


FIGURE 5(a). Electropherogram of the seven anti-malarial compounds with electrophoretic conditions corresponding to a point marked 'M' in Fig 3(b).
Peak identification : (1),(2),(3) and (4) primaquine, chloroquine, quinacrine, quinine; (5) pyrimethamine; (6) dapsone;
(7) methanol; (8) sulfadiazine
Electrophoretic conditions : 13.8 mM TBA in 0.025 M borate/ 0.05 M phosphate buffer; pH 6.20; separation tube : 45 cm x 50 μm ID; voltage : 15 kV; current : 39 μA; detection : 240 nm.

FIGURE 5(b). Electropherogram of the seven anti-malarial compounds with electrophoretic conditions corresponding to a point marked 'N' in Fig 3(b).
Peak identification : (1) primaquine, (2) chloroquine, (3) quinacrine, (4) quinine; (5) pyrimethamine; (6) dapsone; (7) methanol; (8) sulfadiazine
Electrophoretic conditions : 9 mM TBA in 0.025 M borate/ 0.05 M phosphate buffer; pH 7.50; separation tube : 45 cm x 50 μm ID; voltage : 15 kV; current : 39 μA; detection : 240 nm.

TABLE I

The Nine Experimental Conditions Chosen for the Analysis of Boc-amino acids.

Experiment no.	pH	SDS concentration / mM
1	6	40
2	6	60
3	6	80
4	7	40
5	7	60
6	7	80
7	8	40
8	8	60
9	8	80

TABLE II

The Nine Experimental Conditions Chosen for the Investigation of Selected Anti-malarial drugs.

Experiment no.	pH	TBA concentration / mM
1	6	3
2	6	9
3	6	15
4	7	3
5	7	9
6	7	15
7	8	3
8	8	9
9	8	15

concentration which could be employed in the separation and yet satisfy our criteria of resolution and analysis time. This clearly illustrates the versatility of the ORM scheme.

As for the anti-malarial compounds, the first few peaks (peak numbers 1, 2, 3 and 4) migrated out at the same time under the experimental conditions corresponding to point M, as shown in Figure 5(a). However, Figure 5(b) shows the best separation of the seven compounds compared to any of the other nine preliminary experiments conducted (*electropherograms* not shown). Although the separation of the peaks is not complete, the fact that this set of conditions gave the best resolution value ($R > 0.8$) for the separation of anti-

malarial compounds indicates that without it, more experiments would have had to be done to conclude that no true optimum conditions (where $R > 0.8$) exist for this series of solutes. In this investigation, only the minimum number of experiments was carried out to determine the feasibility of separating the anti-malarial drugs, thus demonstrating the usefulness of the ORM scheme.

One particularly interesting trend observed in the separation of the anti-malarial drugs involved the neutral molecule, dapsone. Dapsone migrated out together with the solvent peak, methanol under CZE conditions since they are both neutral. However, with the addition of TBA, dapsone was observed to separate from the solvent peak. It was found that in the presence of TBA in the electrophoretic media, the migration time of dapsone decreased even though the electroosmotic flow was reduced (indicated by the increase in the migration time of the solvent, methanol) by the ion-pairing effect of TBA with the negative charges on the wall of the capillary. This indicates that some form of association between dapsone and TBA has occurred, resulting in dapsone attaining a partial positive charge. Subsequently, the electrophoretic mobility of dapsone was increased and a shorter migration time was observed. On the other hand, no significant ion-pairing with TBA was observed for sulfadiazine, which is the only anion in the mixture. If ion-pairing were to occur, the migration time of sulfadiazine would be expected to decrease. However, the opposite trend was observed. Ion-pairing could be hindered due to steric hindrance. Subsequently, the migration time of this solute increased as the electroosmotic flow was reduced.

CONCLUSION

A systematic optimization scheme was employed for the separation of boc-amino acids and anti-malarial compounds. Global optimum separation conditions were obtained for the separation of neutral as well as ionic compounds by optimizing the pH and SDS concentrations for the boc-amino acids, or pH and TBA concentrations for the anti-malarial compounds. The scheme was validated by using the experimental conditions predicted by it to obtain optimum separations. This procedure is fast and efficient in that only nine preliminary experiments need to be conducted to obtain global optimum separations. The results demonstrated that the ORM procedure, having proved its worth in HPLC work, can be readily and advantageously applied to CE separations with minor modifications.

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