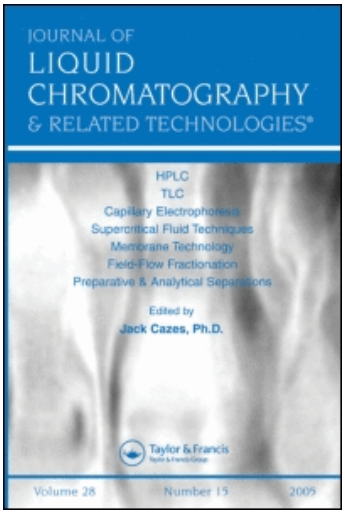

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Comparison of Solvent Modulation with Premixed Mobile Phases for the Separation of Corticosteroids by Liquid Chromatography

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COMPARISON OF SOLVENT MODULATION WITH PREMIXED MOBILE PHASES FOR THE SEPARATION OF CORTICOSTEROIDS BY LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation of eight common corticosteroids is optimized by solvent modulation and by conventional premixed solvents. The separation is achieved on an octadecylsilica column using aqueous acetonitrile and methanol mobile phases. The optimized separations are compared with respect to accuracy, total analysis time, critical resolution, and overall quality of the separation. The solvent modulation approach compares favorably to premixed solvents in all of these respects and, hence, is demonstrated to be a very promising optimization strategy.

INTRODUCTION

In liquid chromatography, solute retention and selectivity are controlled primarily by varying the composition of the mobile phase. Various theoretical models have been developed to predict the effect of

mobile phase composition on retention. These include classical thermodynamic models, such as those based on regular solution theory (1-5) and solvophobic theory (6-9), as well as statistical thermodynamic models (10-14). While these models can elucidate trends and provide qualitative information, they have limited accuracy for predicting quantitative information about the chromatographic separation. This limitation is due to the complex nature of retention processes such that a complete and rigorous theoretical treatment is elusive.

Empirical and semi-empirical models are used extensively to predict and to optimize chromatographic separations. These models can be generally classified into three categories: simultaneous, sequential, and interpretive (15). In the simultaneous or grid search methods, all experiments are performed concurrently and the conditions that yield the most desirable separation are selected as the optimum. These methods typically require a large number of experiments in order to ensure that the global optimum is identified. In the sequential methods, a few initial experiments are performed and, based on their outcome, the conditions are chosen for subsequent experiments. These methods utilize an iterative search technique such as simplex (16,17), Hooke–Jeeves directed search (18), or Box–Wilson steepest ascent path (19) to progressively approach the optimum conditions. Thus, the sequential methods may require fewer experiments to reach the optimum. An important advantage of both the simultaneous and sequential methods is that they do not rely on any predictive model to identify the optimum conditions. In the interpretive or regression methods, a few preliminary experiments are performed and the data are fitted to a predefined

mathematical function. After calculation of the regression coefficients, the separation can be predicted at intermediate conditions to construct a complete response surface, from which the optimum is identified.

Among these methods, the regression approach has proven to be the most widely used in liquid chromatography. This method is attractive because, by using a linear or quadratic model (20-23), only a few experiments are required to optimize the mobile phase composition. However, these models implicitly assume that molecular interactions are ideal, so that solutes interact independently with each component of the mobile phase. Unfortunately, molecular interactions are not completely independent of one another within a solvent mixture, especially for the polar solvents used in reversed-phase liquid chromatography. This non-ideal behavior limits the accuracy with which solute retention can be predicted by using these models (24,25).

Recently, solvent modulation was introduced as a practical alternative to premixed mobile phases for liquid chromatography (26-28). In this technique, individual solvent zones are introduced sequentially onto the chromatographic column. Because the zones are spatially and temporally separated, solutes undergo interaction independently within each solvent zone. Thus, the overall retention of the solute is a simple time-weighted average of its capacity factor in the individual solvent zones. Consequently, optimization of the chromatographic separation is more accurate and requires fewer preliminary experiments by using solvent modulation than by using premixed mobile phases (28).

In this work, solvent modulation and premixed mobile phases are used to optimize the separation of eight common corticosteroids. The

separation is achieved on an octadecylsilica column using aqueous methanol and acetonitrile mobile phases. Based on these results, the optimization methods are compared with respect to accuracy, total analysis time, critical resolution, and the overall quality of the separation.

THEORETICAL CONCEPTS AND OPTIMIZATION STRATEGIES

Premixed Solvents

In reversed-phase liquid chromatography, a mixture of aqueous and organic solvents is often employed to effect the separation of solutes. Various approaches have been utilized to optimize the composition of these mixed solvents (29-31), among which the commercially available optimization program known as DryLab I™ is one of the most popular and successful (32-37). By using the regression approach, this program combines semi-empirical models of chromatographic retention and dispersion with a few initial experiments for the solutes of interest in order to optimize their separation. In this approach, a linear model is employed to relate the solute factor (k) and the mobile phase composition (ϕ)

$$\log k = \log k_w - s\phi \qquad [1]$$

where the slope (s) is a constant that is characteristic of each solute within the chromatographic system, and the intercept ($\log k_w$) is the logarithm of the solute capacity factor using pure water as mobile phase. The solute capacity factor is calculated from the experimental data as

$$k = \frac{(t_r - t_o)}{t_o} \qquad [2]$$

where t_r and t_o are the elution times of a retained and nonretained solute, respectively.

Practical application of this technique requires that solute retention be measured by using at least two solvent compositions. This enables the estimation of the coefficients s and k_w for each solute by linear regression. Based on the values of these coefficients, the solute capacity factor can be predicted at other mobile phase compositions by means of Equation [1]. From the predicted capacity factors, the resolution between adjacent solutes can be calculated as follows

$$R_S = \frac{N^{1/2}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{1+k}\right) \qquad [3]$$

where N is the number of theoretical plates, α is the selectivity factor, and k is the capacity factor. The quality of the separation is evaluated by means of the resolution between the least-resolved solute pair, called the critical resolution (R_{crit}). In order to optimize the separation, the critical resolution is mapped as a function of the solvent composition (20,36,38). From this resolution map, the mobile phase that yields the highest value of the critical resolution can be determined by visual inspection.

Solvent Modulation

The general concept and theory of solvent modulation have been discussed previously by Wahl *et al.* (26-28). In this technique, the overall retention of solute i is given by

$$k_i = \frac{\sum_{j=0}^{n} x_j \left(\frac{1+k_{ij}}{k_{ij}}\right)}{L} - 1 \qquad [4]$$

where k_{ij} is the capacity factor of solute i in solvent j, x_j is the solvent zone length, and the limit of summation n represents the number of solvent zones required to elute the solute from a column of length L. Thus, in solvent modulation, the overall retention is varied by means of the type, sequence, and length of the solvent zones applied to the column.

The strategy for optimization by this technique requires preliminary measurement of solute capacity factor in each solvent of interest. Based on these measurements, the overall capacity factor under the conditions of solvent modulation can be estimated by means of Equation [4]. Next, the resolution between adjacent solutes i and i+1 is calculated as

$$R_{i,i+1} = \frac{N^{1/2}}{2} \left(\frac{k_{i+1} - k_i}{2 + k_i + k_{i+1}} \right) \qquad [5]$$

The quality of the separation is then assessed by using a modified form of the multivariate function known as the Chromatographic Resolution Statistic (CRS) developed by Schlabach and Excoffier (39)

$$CRS = \left(\sum_{i=1}^{m-1} \left(\frac{R_{i,i+1} - R_{opt}}{R_{i,i+1} - R_{min}} \right)^2 \frac{1}{R_{i,i+1}} + \sum_{i=1}^{m-1} \frac{(R_{i,i+1})^2}{(m-1)\, R_{avg}^2} \right) \frac{t_f}{m} \qquad [6]$$

where m is the total number of solutes, t_f is the elution time of the final solute, R_{opt} is the optimum or desired resolution, R_{min} is the minimum acceptable resolution, and R_{avg} is the average resolution which is given by

$$R_{avg} = \frac{1}{m} \sum_{i=1}^{m} R_{i,i+1} \qquad [7]$$

The first term of the CRS function is a measure of the extent of separation between each pair of adjacent solutes in the chromatogram. This term

approaches zero when the individual resolution elements approach the optimum value, and approaches infinity when the individual resolution elements approach the minimum value. The second term of the CRS function reflects the uniformity of spacing between solutes, and approaches a minimum value of unity when the individual resolution elements are equal to the average value. The final term of the CRS function is intended to minimize the analysis time, and may be neglected if this is not a primary goal of the optimization.

In order to optimize the separation, the solvent zone lengths which yield the minimum value of the CRS function must be determined. This minimum CRS value may be determined in two ways: 1) by varying the length of each solvent zone systematically to produce the complete response surface from which the optimum is determined by visual inspection, or 2) by using an sequential search routine such as the simplex method (17,40). The former method is time consuming, but provides a detailed view of the complete response surface. The latter method is more efficient but, because the surface may contain many local maxima and minima, care must be taken to ensure that the global optimum is identified. Consequently, a combination of these approaches is desirable.

EXPERIMENTAL METHODS

Chromatographic System

A chromatographic pump equipped with two 40-mL syringes (Model 140, Applied Biosystems, Foster City, CA, USA) is used to deliver the

mobile phase at 0.5 mL/min. Sample introduction is achieved by using a 10-μL injection valve (Model EQ 60, Valco Instruments Co., Houston, TX, USA). The chromatographic column (47 cm × 0.46 cm i.d.) is packed with octadecylsilica material (Spheri-5 RP-18, 5 μm, Applied Biosystems) to have a total plate number (N) of approximately 10,000 for the solutes of interest. Solute detection is accomplished by using a variable-wavelength UV-visible absorbance detector (240 nm, 0.005 AUFS, Model 166, Beckman Instruments, San Ramon, CA, USA).

Materials and Methods

The following corticosteroids are utilized in this investigation: cortisone (17α,21-dihydroxy-pregn-4-ene-3,11,20-trione), hydrocortisone (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione), tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione), tetrahydrocortisol (3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one), prednisone (17α,21-dihydroxy-pregna-1,4-diene-3,11,20-trione), prednisolone (11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione), methylprednisolone (11β,17α,21-trihydroxy-6α-methyl-pregna-1,4-diene-3,20-dione), and dehydrocorticosterone (21-hydroxy-pregn-4-ene-3,11,20-trione). These corticosteroids shown in Figure 1 are obtained from the Sigma Chemical Company (St. Louis, MO, USA) and are used without further purification. Standard solutions are prepared in methanol at 10^{-3} M concentration for tetrahydrocortisone and tetrahydrocortisol, and at 10^{-6} M concentration for all other steroids. Organic solvents are high-purity, distilled-in-glass grade (Baxter Healthcare, Burdick & Jackson Division, Muskegon, MI, USA); water is deionized and double distilled in glass (Model MP-3A, Corning Glass Works, Corning, NY, USA).

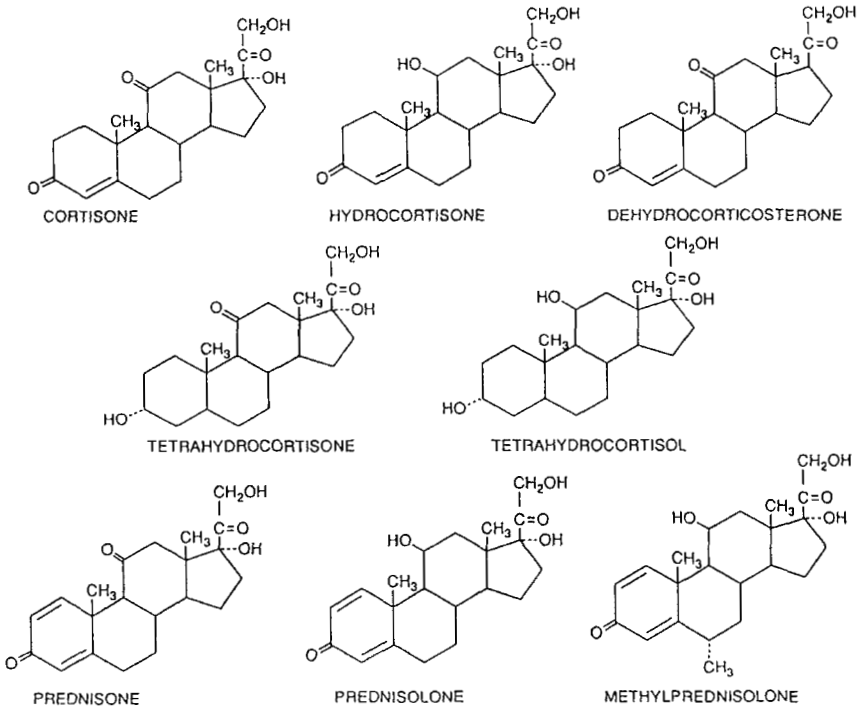

FIGURE 1: *Structure of corticosteroids.*

Computer-Assisted Optimization Programs

The optimization program for premixed mobile phases, DryLab I™ Isocratic HPLC Simulation/Optimization Program (LC Resources Inc., Lafayette, CA, USA), is executed on an IBM-compatible computer with 80486 microprocessor. From the initial measurement of solute retention times, this program uses linear regression to calculate the capacity factor as a function of the mobile phase composition. In addition, the total analysis time, selectivity factor, and critical resolution are calculated from Equations [1] to [3], assuming a plate number of 10,000. The optimum

conditions are identified from a graph of the critical resolution as a function of mobile phase composition (38).

The optimization program for solvent modulation is written in the Fortran 77 language and executed on a VAX Station 3200 computer (Digital Equipment, Maynard, MA, USA) (26-28). From the initial measurement of solute capacity factors, the overall capacity factor of each solute is calculated under the conditions of solvent modulation by using Equation [4]. The resolution of each solute pair is calculated by using Equation [5], and the overall quality of the separation is assessed by means of the CRS function in Equation [6], where the selected values for the optimum and minimum acceptable resolutions are 1.5 and 0.5, respectively. The optimum conditions are identified by two methods. In the topographic mapping method, these calculations are performed while systematically incrementing each solvent zone length within a prescribed range. By graphing the resulting CRS values as a function of the solvent zone length, a complete response surface is constructed. The minimum CRS value is then located by visual inspection of this response surface (28). In the sequential search method, the modified simplex algorithm of Nelder and Mead (40) is employed. This algorithm permits expansion and contraction of the simplex during the search and will converge at the optimum position. In order to ensure the identification of the global optimum, both the size and the location of the initial simplex are varied systematically in 200 independent searches (28). For each initial simplex, the calculations are performed according to Equations [4] to [6] at each successive vertex, and the best conditions are continuously updated and stored in a file. This file contains the solvent zone lengths, the solute

elution order, the predicted capacity factors, the predicted resolutions, and the corresponding CRS value for the separation.

RESULTS AND DISCUSSION

In this work, computer-assisted optimization programs using solvent modulation and premixed solvents are compared to optimize the separation of eight corticosteroids. The practical utility of both techniques requires that solute retention be measured in solvents of different compositions, from which the optimum conditions for the separation can be predicted. The capacity factor of each corticosteroid was measured on an octadecylsilica column using 35% and 50% acetonitrile as well as 60% and 75% methanol. Table I summarizes the retention measurements for the corticosteroids.

In the methanol mobile phases, all of the corticosteroids are well separated except for prednisolone and hydrocortisone. On the other hand, in the acetonitrile mobile phases, the corticosteroids are separated with the exceptions of prednisone and hydrocortisone as well as methylprednisolone and tetrahydrocortisone. Thus, the least-resolved solute pairs vary with the type of organic modifier.

To optimize the separation of corticosteroids using premixed solvents, the DryLab I™ program is utilized. Based on the preliminary measurements of the capacity factors in Table 1, this program calculates the retention of the corticosteroids at other solvent compositions according to Equation [1]. In order to determine the optimum solvent composition,

TABLE 1

Capacity Factors for Corticosteroids on Octadecylsilica Stationary Phase using Methanol and Acetonitrile Mobile Phases

CORTICOSTEROIDS	CAPACITY FACTOR (k)			
	Methanol–Water		Acetonitrile–Water	
	60%	75%	35%	50%
Prednisone	1.57	0.41	1.66	0.50
Cortisone	1.73	0.48	1.83	0.58
Prednisolone	2.14	0.55	1.40	0.41
Hydrocortisone	2.18	0.56	1.69	0.50
Dehydrocorticosterone	2.97	0.69	4.09	1.39
Methylprednisolone	3.79	0.86	2.53	0.70
Tetrahydrocortisol	4.29	0.96	1.94	0.50
Tetrahydrocortisone	5.17	1.08	2.53	0.63

the critical resolution of the least-resolved solute pair is mapped as a function of the mobile phase composition. The resolution maps for the aqueous acetonitrile and methanol mixtures are shown in Figures 2 and 3, respectively. For the acetonitrile mixtures, the composition of 30% acetonitrile is predicted to yield the highest value of the critical resolution. The least-resolved solutes are prednisone and hydrocortisone, with a predicted resolution of 0.3. Because this region of the resolution map is irregular, slight variations in the mobile phase composition may result in a large change in the critical resolution. For the methanol mixtures, however, a more rugged optimum region is observed between 55% and 60% methanol. The least-resolved solutes are prednisolone and hydrocortisone, with a predicted resolution of 0.3. Because this region is

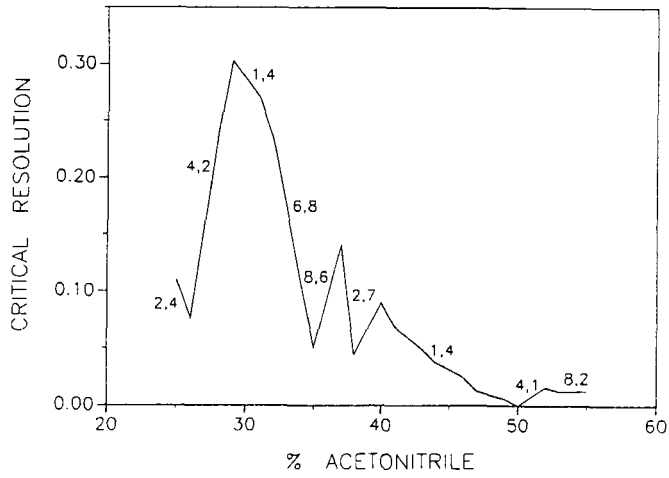

FIGURE 2: Critical resolution as a function of the mobile phase composition for aqueous acetonitrile mixtures. Column: 47 x 0.46 cm i.d., packed with octadecylsilica material. Solutes: (1) prednisone, (2) cortisone, (3) prednisolone, (4) hydrocortisone, (5) dehydrocorticosterone, (6) methylprednisolone, (7) tetrahydrocortisol, (8) tetrahydrocortisone.

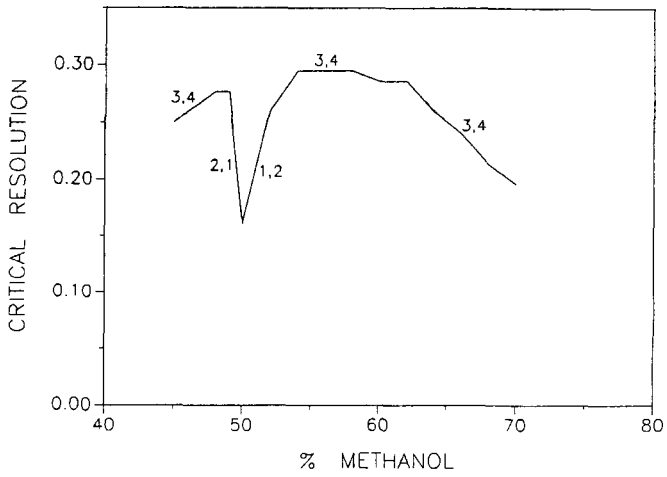

FIGURE 3: Critical resolution as a function of the mobile phase composition for aqueous methanol mixtures. Experimental conditions as given in Figure 2.

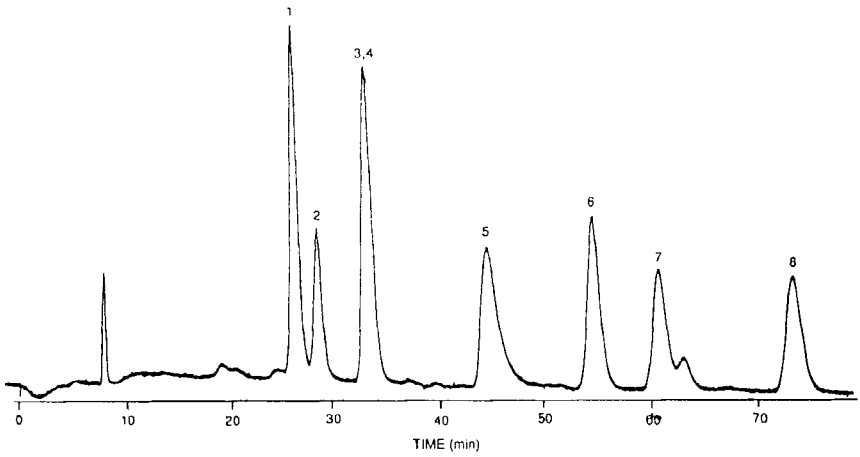

FIGURE 4: Experimental chromatogram of corticosteroids obtained under the predicted optimum conditions for premixed mobile phases. Column: 47 × 0.46 cm i.d., packed with octadecylsilica material. Mobile phase: 56% methanol, 0.5 mL/min. Detector: UV-visible absorbance detector, 240 nm, 0.005 AUFS. Solutes: (1) prednisone, (2) cortisone, (3) prednisolone, (4) hydrocortisone, (5) dehydrocorticosterone, (6) methylprednisolone, (7) tetrahydrocortisol, (8) tetrahydrocortisone.

relatively broad and flat, slight variations in the mobile phase composition will not be as detrimental. Thus, 56% methanol was chosen as the optimum mobile phase composition and was used to obtain the separation shown in Figure 4. From this chromatogram, it is apparent that prednisolone and hydrocortisone are completely overlapped ($R_S \approx 0.3$), whereas all other solutes are fully resolved. The experimentally measured capacity factors are in good agreement with the theoretically predicted values from Equation [1], as summarized in Table 2, with an average relative error of ±3.22%.

To optimize the separation of corticosteroids using solvent modulation, the four solvent systems shown in Table 1 are utilized.

TABLE 2

Comparison of Experimental and Theoretical Capacity Factors under the Predicted Optimum Conditions for Premixed Mobile Phases

CORTICOSTEROIDS	CAPACITY FACTOR (k)		
	Theory†	Experiment	Relative Error (%)‡
Prednisone	2.46	2.25	–8.54
Cortisone	2.60	2.54	–2.31
Prednisolone	3.27	3.11	–4.89
Hydrocortisone	3.32	3.11	–6.32
Dehydrocorticosterone	4.62	4.55	–1.52
Methylprednisolone	5.84	5.80	–0.68
Tetrahydrocortisol	6.61	6.60	–0.15
Tetrahydrocortisone	8.08	8.19	1.36
Average			±3.22

† Calculated by using Equation [1].
‡ Calculated as 100 x (Experiment – Theory)/Theory.

Although there are twelve possible permutations of a two-solvent modulation sequence, the computer-assisted search routines provide a rapid and effective means to identify the most promising permutation. For each permutation, the sequential simplex method is used to determine the minimum CRS value on the complete response surface. The results of this preliminary search are summarized in Table 3.

From these results, the most promising solvent modulation sequence is identified to be 50% acetonitrile followed by 60% methanol (CRS_{min} = 1.9). The least-resolved solutes are cortisone and hydrocortisone, with a predicted resolution of 1.30. Although this permutation initially appears to

TABLE 3

Evaluation of the Permutations for a Two-Solvent Modulation Sequence

Solvent 1	Solvent 2	t_f (min)	R_{crit}	CRS_{min}
60% Methanol	75% Methanol	29.3	0.20	95
75% Methanol	60% Methanol	28.8	0.19	96
35% Acetonitrile	50% Acetonitrile	32.3	0.15	113
50% Acetonitrile	35% Acetonitrile	33.9	0.15	104
60% Methanol	35% Acetonitrile	50.3	0.72	26
35% Acetonitrile	60% Methanol	51.9	0.99	3.6
60% Methanol	50% Acetonitrile	30.3	0.86	8.1
50% Acetonitrile	60% Methanol	31.1	1.30	1.9
75% Methanol	35% Acetonitrile	18.9	0.20	97
35% Acetonitrile	75% Methanol	19.3	0.18	98
75% Methanol	50% Acetonitrile	18.6	0.18	100
50% Acetonitrile	75% Methanol	18.6	0.18	101

be very promising, a more detailed inspection by the topographic mapping method reveals that the response surface is highly irregular. Variations in the solvent zone length as small as ±1.0 cm alter the identity of the least-resolved solute pair and cause a significant change in the critical resolution.

As a consequence of this limitation, the next most promising permutation of 35% acetonitrile followed by 60% methanol (CRS_{min} = 3.6) is selected for further study. The chromatograms corresponding to each of these solvents are shown in Figure 5. The least-resolved solutes in 35% acetonitrile are methylprednisolone and tetrahydrocortisone, whereas those in 60% methanol are prednisolone and hydrocortisone. Although

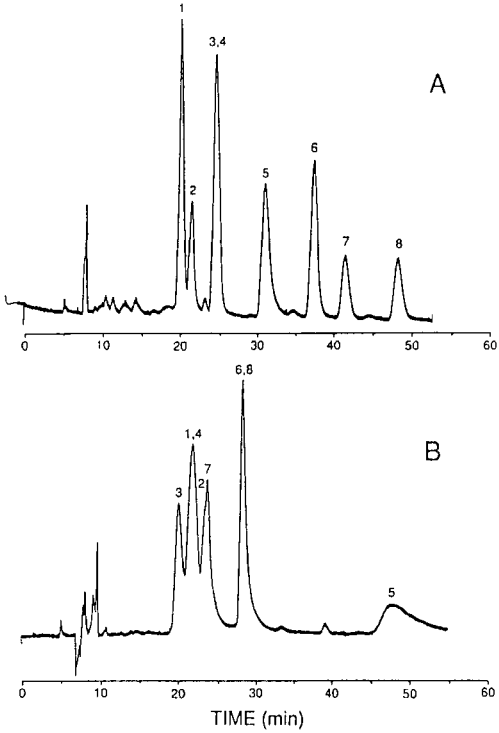

FIGURE 5: Separation of corticosteroids in individual mobile phases for solvent modulation. Mobile phase: (A) 60% methanol, (B) 35% acetonitrile, 0.5 mL/min. All other experimental conditions as given in Figure 4.

the separation of all solutes is not achievable in either of these solvents individually, the results in Table 3 suggest that the modulation of these solvents may provide a more beneficial separation. In order to determine the optimum conditions for solvent modulation, the complete response surface was constructed by the mapping method. The topographic and contour maps of the CRS response surface are shown in Figure 6 as a function of the solvent zone length. When expressed in terms of the

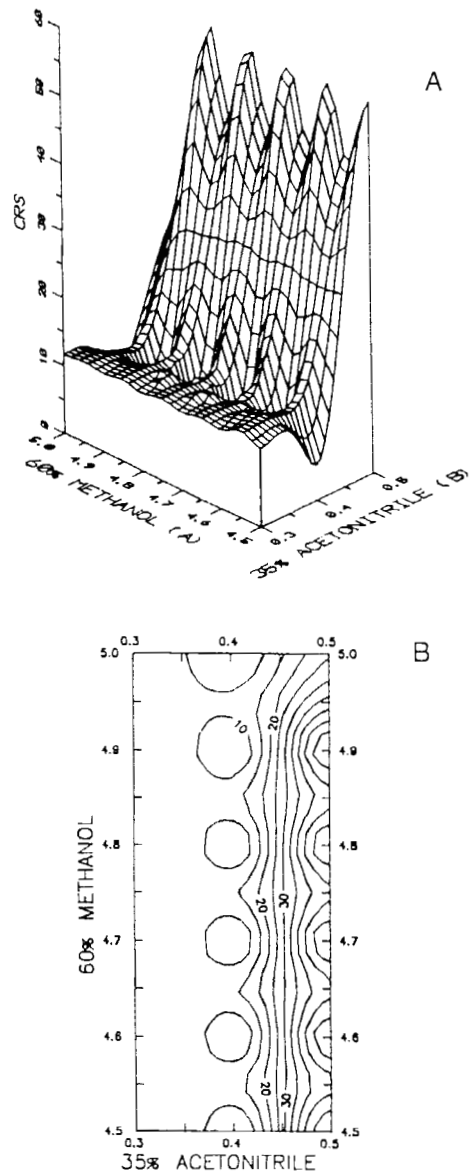

FIGURE 6: Topographic (A) and contour (B) maps of the CRS response surface as a function of the fractional zone lengths for 35% acetonitrile and 60% methanol.

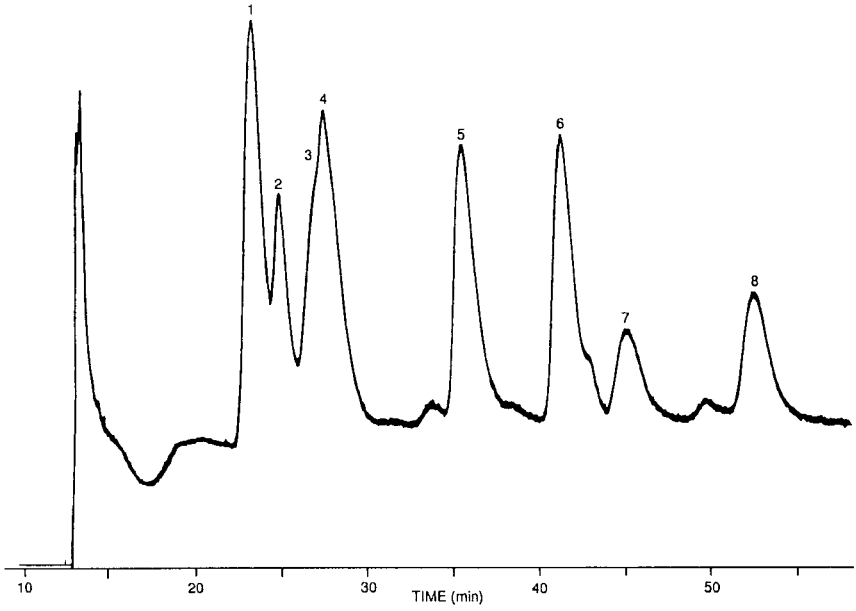

FIGURE 7: Experimental chromatogram of corticosteroids obtained under the predicted optimum conditions for solvent modulation. Mobile phase: solvent modulation sequence of 35% acetonitrile and 60% methanol in fractional zone lengths of 0.4 and 4.8, respectively, 0.5 mL/min. All other experimental conditions as given in Figure 4.

fractional length (x_j/L), these maps may be used to determine the optimum conditions independent of the column length. From these maps, the minimum CRS is predicted for zones of 35% acetonitrile and 60% methanol in fractional lengths of 0.4 and 4.8, respectively, which correspond to absolute lengths of 19 and 226 cm, respectively, for the 47 cm column utilized in this study.

The experimental chromatogram in Figure 7 shows good separation of all corticosteroids with the exception of the least-resolved solute pair, prednisolone and hydrocortisone ($R_{crit} \approx 0.7$). The experimentally measured capacity factors agree well with the theoretically predicted

TABLE 4

Comparison of Experimental and Theoretical Capacity Factors under the Predicted Optimum Conditions for Solvent Modulation

CORTICOSTEROIDS	CAPACITY FACTOR (k)		
	Theory†	Experiment	Relative Error (%)‡
Prednisone	1.59	1.58	–0.63
Cortisone	1.76	1.78	1.14
Prednisolone	1.93	2.08	7.77
Hydrocortisone	2.06	2.10	1.94
Dehydrocorticosterone	3.08	2.97	–3.57
Methylprednisolone	3.59	3.60	0.28
Tetrahydrocortisol	3.82	4.01	4.97
Tetrahydrocortisone	4.76	4.82	1.26
Average			±2.70

† Calculated by using Equation [4].
‡ Calculated as 100 x (Experiment – Theory)/Theory.

values from Equation [4], as summarized in Table 4, with an average relative error of ±2.70%.

The optimization methods used in this work may be compared on the basis of the following criteria: average relative error in predicted capacity factor, total analysis time, critical resolution, and overall quality of the separation assessed by the CRS function. The average relative error is ±2.70 for the solvent modulation technique, compared with ±3.22 for premixed solvents. When optimized with solvent modulation, the corticosteroid separation is achieved experimentally in 52 minutes with a critical resolution of 0.7, and a CRS value of approximately 26. When

optimized with premixed solvents by DryLab I™, the corticosteroid separation is achieved experimentally in 73 minutes with a critical resolution of 0.3, and a CRS value of approximately 146. On the basis of these criteria, the separation achieved by using solvent modulation is at least comparable to and, in some respects, significantly better than that achieved by using premixed solvents.

CONCLUSIONS

Solvent modulation is a practical alternative to premixed mobile phases in liquid chromatography. Because the solvent zones are spatially and temporally separated from one another, solute retention is a simple time-weighted average of the retention in each individual solvent. Consequently, optimization of the separation is more accurate and requires fewer preliminary experiments with solvent modulation than with premixed solvents. In this study, the separation of corticosteroids was optimized by each technique and compared with respect to accuracy, total analysis time, critical resolution, and overall quality of the separation. The solvent modulation approach compares favorably in all of these respects and, hence, is demonstrated to be a very promising optimization strategy.

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