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Comparison of theory-based and empirical modeling for the prediction of chromatographic behavior in the ion-pairing separation of benzodiazepine-derived pharmaceutical compounds

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Abstract

Two approaches were examined for predicting chromatographic behavior during the reversed-phase ion-pairing separation of benzodiazepine-derived pharmaceutical compounds. The capacity factor for olanzapine and its resolution from a closely related compound, desmethylolanzapine, were studied as a function of the percentage of acetonitrile, the ion-pairing reagent concentration and the buffer pH. In the first approach, the results were analyzed using the theory-based software package DryLab I/mp. In the second approach, statistical analysis was used to derive empirical equations to predict the dependence of the chromatographic behavior on each of the experimental variables. At the lowest ion-pairing reagent concentration, DryLab I/mp was found to be a poor predictor of resolution. For this complex separation, the empirical equations derived from the statistical analysis were found to predict better the chromatographic behavior over the ranges tested. These equations were used to generate response-surface plots to evaluate the method ruggedness.

1. Introduction

The desire to speed up chromatographic method development has led to the design and implementation of computer modeling software [1]. Software packages have been described for both isocratic [2] and gradient separations [3]. Recent applications of this approach to chromatographic method development have included separations of drug substances [4] and phenolic pollutants [5]. Software designed to study multi-parameter effects (pH, temperature, percentage of organic modifier and buffer concentration) have also been described [6–8]. DryLab I/mp is a chromatographic simulation package capable of predicting the optimum conditions for performing multi-parameter separations, including ion-pairing assays [6].

Statistical analysis using factorial designed studies to model multi-parameter chromatographic behavior has been used as an alternative approach [9–11]. This approach has been successfully used to optimize the ion-pairing separations of alkaloids [9] and monoamine neurotransmitters [10], along with the isocratic reversed-phase separation of hormonal steroids [11].

Olanzapine, an investigational new drug for the treatment of schizophrenia, and related compounds are in the benzodiazepine class of phar-

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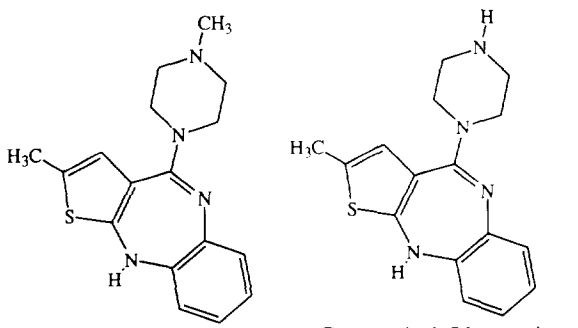

Fig. 1. Structures of olanzapine and desmethylolanzapine.

maceuticals. These compounds can be characterized as having low water solubility, high basicity and aromaticity. The structures of olanzapine and a closely related compound, desmethylolanzapine, are shown in Fig. 1. Note that the only difference between these two compounds is the presence of a methyl group on the distal nitrogen of the piperazine ring. The development of a chromatographic method capable of separating these highly basic and similar compounds was not trivial.

Despite the similarities in these compounds, a reversed-phase ion-pairing method was developed that could perform the separation. The eluent was composed of acetonitrile and sodium phosphate buffer with sodium dodecyl sulfate (SDS) as the ion-pairing reagent. During method development, accurate preparation of the mobile phase, i.e. percentage of acetonitrile, concentration of SDS and pH, was determined to be critical for method reproducibility. The purpose of this investigation was to compare the applicability of DryLab I/mp and statistical analysis for modeling this multi-parameter chromatographic separation. The models derived would be used to determine the method ruggedness.

2. Experimental

2.1. Reagents and materials

HPLC-grade acetonitrile, phosphoric acid (85%, w/w) and sodium hydroxide (50%, w/w) aqueous solutions were obtained from EM Science (Gibbstown, NJ, USA). Electrophoresis-grade SDS was obtained from Eastman Kodak (Rochester, NY, USA). Water for mobile phases was purified with a Milli-Q system from Millipore (Milford, MA, USA). The aqueous component of each mobile phase was prepared by adding 5.0 mL of phosphoric acid per 1500 ml of water and dissolving the appropriate amount of SDS to obtain the specified concentration. The pH of each aqueous component was adjusted to the desired value by dropwise addition of sodium hydroxide solution. The appropriate volume of acetonitrile was combined with each aqueous component to yield the desired mobile phase composition. Olanzapine and desmethylolanzapine were obtained from Eli Lilly (Lafayette, IN, USA). Samples were prepared at concentrations of ca. 0.2 mg/ml (olanzapine) and 0.1 mg/ml (desmethylolanzapine) in the mobile phase. Chromatographic separations were performed using a 250 mm × 4.6 mm I.D. column of 5-μm particle size Zorbax Rx/SB-C_8 (MacMod, Chadds Ford, PA, USA).

2.2. Apparatus

The HPLC system consisted of a Model 600E multi-solvent delivery system (Waters, Bedford, MA, USA) equipped with a column oven set at 35°C, a Model 728 autoinjector (Alcott, Norcross, GA, USA) equipped with a 20-μL fixed-loop injection valve (Valco, Houston, TX, USA) and a Model 757 single-wavelength UV detector operated at 220 nm (Applied Biosystems, Ramsey, NJ, USA). The mobile phase flow-rate was 1.5 ml/min for all experiments. An in-house data acquisition system was used to record all chromatograms. The software packages, DryLab I/mp (LC Resources, Walnut Creek, CA, USA), JMP (SAS Institute, Cary, NC, USA) and Excel (Microsoft, Redmond, WA, USA) were operated on a Macintosh IIci computer (Apple Computer, Cupertino, CA, USA).

2.3. Procedure

Table 1 contains a summary of the experiments performed to model the chromatographic behavior. All separations were performed iso-

Table 1
Experimental design

Experiment No.	Acetonitrile (%)	SDS (m*M*)	Buffer pH
1	53	10	3.0
2	43	50	3.0
3	43	10	3.0
4	53	10	2.0
5	53	50	2.0
6	43	10	2.0
7	43	50	2.0
8	53	50	3.0
9	53	30	2.0
10	43	30	3.0
11	53	70	2.0
0	48	30	2.5

cratically. Experiments 1–8 represent a factorial design study using every possible combination of either 43 or 53% acetonitrile, 10 or 50 m*M* SDS and a pH of 2.0 or 3.0. Experiments 9–11 were performed to make additional predictions based on variations in SDS concentration. Experiment 0 was performed each day as an equipment check, using the center points of each of the variable ranges.

2.4. DryLab I/mp

DryLab I/mp required results from two or three initial runs, in which all conditions were held constant except the variable being studied to make predictions of chromatographic behavior. Results from only two initial runs were needed to make predictions for the percentage of organic component. For example, results from the paired experiments 6, 4 and 7, 5 could be used for this purpose. In both cases the ion-pairing reagent concentration and pH were held constant, but the percentage of acetonitrile was varied. Several other combinations of experiments could also be treated in this way. Results from three initial experiments were required to make predictions for ion-pairing reagent concentration. In experiments 4, 5, 9 and 11 the percentage of acetonitrile and pH were held constant and the ion-pairing reagent concentration was varied.

Use of DryLab I/mp was straightforward. For each study, the retention times and peak areas of the analytes in the respective two or three experiments were entered. DryLab I/mp then made predictions of resolution (R_s) and capacity factor (k') over a selected range of the variables being studied. Computerized plots of the predicted chromatograms could be made. The peak areas are input so that the relative peak sizes could be appropriately plotted. The experimental values for resolution or the number of theoretical plates (N) could be input for one run to fine-tune further the predictions. The actual equations used by DryLab I/mp to predict resolution and capacity factor have been described [7, 8]. The equation for resolution is based on chromatographic theory, which assumes Gaussian band broadening. DryLab I/mp uses a quadratic fit of the data to make predictions for capacity factor values.

2.5. Statistical analysis

JMP was used to perform a statistical analysis of the experimental results. Empirical equations were derived which modeled the chromatographic behaviors (capacity factor and resolution). To compare directly the magnitude of the dependences of each behavior on each of the variables, the variables had to be normalized by converting them to integer values. For example, the percentage organic values of 43, 48 and 53% were actually represented as $-1, 0$ and 1. The first step in using JMP was to determine the best linear fit of the experimental variables to the behavior being modeled. By considering the value of each variable and the experimental results obtained, JMP predicted which variables or combination of variables (interaction and higher order terms) were significant. JMP derives a coefficient for each term which was found to be significant, along with an intercept term. The intercept, coefficients and experimental variables comprise an equation that models each chromatographic behavior. A more detailed discussion of the operation of JMP is beyond the scope of this paper; more information can be found in the JMP operator's handbook.

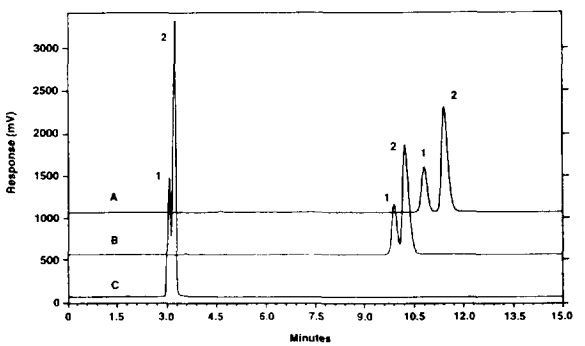

Fig. 2. Chromatograms demonstrating the effect changes in mobile phase composition on the separation of olanzapine from desmethylolanzapine. Peaks: 1 = desmethylolanzapine; 2 = olanzapine. The following mobile phase compositions are represented: (A) 43% acetonitrile–10 m*M* SDS–phosphate buffer (pH 2.0); (B) 48% acetonitrile–30 m*M* SDS–phosphate buffer (pH 2.5); (C) 53% acetonitrile–10 m*M* SDS–phosphate buffer (pH 2.0). All other conditions as described in the text.

3. Results

Representative chromatograms demonstrating the separation of olanzapine from desmethylolanzapine using various mobile phase conditions are shown in Figs. 2 and 3. As would be expected, the retention times and peak resolutions were dependent on the mobile phase. The results obtained from each experiment for the capacity factor of the olanzapine peak and the resolution between the olanzapine and desmethylolanzapine peaks are summarized in Table 2. As shown, the capacity factor for olanzapine ranged from a minimum of 1.0 (experiment 1) to a maximum of 31.8 (experiment 7). Using the conditions chosen for experiment 1, olanzapine and desmethylolanzapine would be expected to elute with minimum retention because the percentage of acetonitrile was at a maximum and the ion-pairing reagent concentration was at a minimum. Further, the pH of the buffer used in experiment 1 (3.0) was the highest value tested. Olanzapine and desmethylolanzapine would be expected to be less protonated (less cationic) at the higher pH. Therefore, their ability to form ion pairs with SDS was diminished using the conditions of experiment 1. Conversely, these compounds would be expected to elute with maximum retention using the conditions chosen for experiment 7. Experiment 7 represented the combination of highest concentration of ion-pairing reagent and lowest percentage of acetonitrile. Further, at the pH of the buffer used in experiment 7 (2.0), these compounds would be in their most protonated, or cationic, forms. Note in Table 2 that the peak elution orders reversed during experiment 2. Desmethylolanzapine eluted later than olanzapine during experiment 2. This produced a negative value for the calculated resolution obtained for experiment 2. Also note in Table 2 that these compounds co-eluted during experiments 8 and 10.

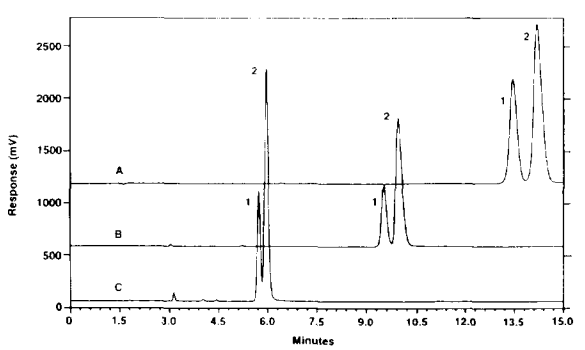

Fig. 3. Chromatograms demonstrating the effect of changes in SDS concentration on the separation of olanzapine from desmethylolanzapine. Peak identities as in Fig. 2. Each mobile phase contained 53% acetonitrile and phosphate buffer (pH 2.0). The SDS concentrations were as follows: (A) 70; (B) 50; (C) 30 m*M*. All other conditions as described in the text.

3.1. Modeling the chromatographic behavior with DryLab I/mp

Although the variables could be examined in many different combinations, the procedure for the analysis of the data using DryLab I/mp and the success of the package may be exemplified by illustrating only two of those studies. The chromatograms generated for experiments 6 and 4 are shown in Fig. 2. Experimentally, the observed resolution changed significantly with a change in the percentage of acetonitrile. Using 43% acetonitrile (experiment 6) resulted in a

Table 2
Experimental results for olanzapine capacity factor and resolution between the olanzapine and desmethylolanzapine peaks

Experiment No.	Acetonitrile (%)	SDS (m*M*)	pH	Experimental k'	Experimental R_s
1	53	10	3.0	1.0	1.2
2	43	50	3.0	22.6	−0.9
3	43	10	3.0	4.9	1.3
4	53	10	2.0	1.8	1.0
5	53	50	2.0	5.6	1.3
6	43	10	2.0	6.6	1.7
7	43	50	2.0	31.8	1.6
8	53	50	3.0	3.2	0.0
9	53	30	2.0	3.0	1.1
10	43	30	3.0	14.2	0.0
11	53	70	2.0	8.5	1.7
0	48	30	2.5	6.0	1.0

resolution of 1.68. Using 53% acetonitrile (experiment 4), the resolution was 0.96. The results for retention times from these two experiments were entered into the DryLab I/mp software program. The predicted values for resolution and capacity factor are shown in Table 3.

As shown in Table 3, the resolution between olanzapine and desmethylolanzapine was predicted to change only slightly over the range of percentage acetonitrile studied when compared with the actual experimental results. A net change in resolution of 0.7 was observed experimentally. DryLab I/mp did not accurately predict the observed behavior for resolution. In fact, no change was predicted for values of 43 and 53% acetonitrile. The predictions for the olanzapine capacity factor were reasonable, however. The predictions made by DryLab I/mp for the dependence of resolution on the percentage of acetonitrile using the data from the other possible combinations of SDS concentration and pH were similar to those shown in Table 3.

The chromatograms generated for experiments 4, 5, 9 and 11 are shown in Figs. 2 and 3. Experimentally, the resolution was observed to increase with increasing SDS concentration. Using 10, 30, 50 and 70 m*M* SDS resulted in resolution values of 0.96, 1.11, 1.29 and 1.65, respectively. The peak retention times from these experiments were entered into the DryLab

Table 3
DryLab I/mp predictions made from results of experiments 6 and 4 (10 m*M* SDS, pH 2.0, percentage of acetonitrile varied)

Acetonitrile %	Predicted R_s	Predicted k'	Experimental R_s	Experimental k'
43	1.4	5.7	1.7	6.6
44	1.5	4.7		
45	1.5	3.9		
46	1.6	3.3		
47	1.6	2.7		
48	1.6	2.3		
49	1.6	1.9		
50	1.6	1.6		
51	1.6	1.3		
52	1.5	1.1		
53	1.4	0.9	1.0	1.2

Table 4
DryLab I/mp predictions made from results of experiments 4, 5, 9 and 11 (53% acetonitrile, pH 2.0, SDS concentration varied)

SDS (m*M*)	Predicted R_s	Predicted k'	Experimental R_s	Experimental k'
10	1.4	0.9	1.0	1.2
16	1.2	1.4		
22	1.0	1.9		
28	1.0	2.5		
30	1.0	2.7	1.1	3.0
34	1.0	3.1		
40	1.0	3.7		
46	1.0	4.4		
50	1.1	4.8	1.3	5.6
52	1.1	5.1		
58	1.2	5.8		
64	1.2	6.5		
70	1.3	7.3	1.7	8.5

I/mp software program. The predicted values for resolution and capacity factor are shown in Table 4.

As shown in Table 4, the resolution between olanzapine and desmethylolanzapine was predicted to be constant from 22 to 46 m*M* SDS. The resolution was predicted to increase slightly from 46 to 70 m*M* SDS. The resolution was also predicted to increase with decreasing SDS concentration from 22 to 10 m*M*. The greatest resolution was predicted to occur at 10 m*M* SDS. Experimentally, the trend for resolution from 30 to 70 m*M* SDS agreed with that which was predicted. However, the actual resolution observed at 70 m*M* SDS was significantly larger than the predicted value. The experimental resolution at 10 m*M* SDS was actually slightly less than that observed at 30 m*M* SDS. The predicted increase in resolution at 10 m*M* SDS was not observed experimentally. This prediction was opposite to the observed experimental results. The predicted capacity factors were in reasonably good agreement with those observed experimentally.

3.2. Modeling of chromatographic behavior by statistical analysis

It was apparent from the results obtained using DryLab that the experimental data did not fit the model used by the software package for making predictions of resolution at low SDS concentration. Further, DryLab did not accurately predict the dependence of resolution on changes in percentage of acetonitrile at constant SDS concentration. Additional modeling of the data was performed using the statistical analysis software JMP. Using the coded values for each variable and the data generated from experiments 1–10 and 0, empirical equations were derived. The equations derived by JMP for capacity factor and resolution are as follows:

$$k' = 5.97 - 6.94[\mathrm{ACN}] + 6.19[\mathrm{SDS}] - 4.53[\mathrm{ACN}][\mathrm{SDS}] - 1.61(\mathrm{pH}) + 3.43[\mathrm{ACN}][\mathrm{ACN}] \quad (1)$$

$$R_s = 0.92 - 0.39[\mathrm{SDS}] + 0.17[\mathrm{ACN}][\mathrm{SDS}] - 0.51(\mathrm{pH}) + 0.27[\mathrm{ACN}](\mathrm{pH}) - 0.46[\mathrm{SDS}](\mathrm{pH}) \quad (2)$$

Table 5 shows the experimental results for capacity factor and resolution, along with those values calculated using the equations derived from the statistical analysis. By comparing the experimental and calculated values, it was concluded that the resolution equation provided an excellent representation of the observed chromatographic behavior. With the exception of experiment 1, the capacity factor equation also provided a reasonably good estimate of the experimental behavior. The data from experi-

Table 5
Comparison of experiment results and those predicted by statistical analysis for capacity factor and resolution

Experiment No.	Experimental k'	Calculated k'	Experimental R_s	Calculated R_s
1	1.0	−0.8	1.2	1.4
2	22.6	25.4	−0.9	−0.9
3	4.9	4.0	1.3	1.2
4	1.8	2.4	1.0	0.9
5	5.6	5.7	1.3	1.4
6	6.6	7.2	1.7	1.8
7	31.8	28.7	1.6	1.6
8	3.2	2.5	0.0	0.0
9	3.0	4.1	1.1	1.2
10	14.2	14.7	0.0	0.1
11	8.5	7.5	1.7	1.6
0	6.0	6.0	1.0	0.9

ment 11 were not used to derive Eqs. 1 and 2. However, the equations provided an excellent prediction of the results observed from that experiment.

Discussion

4.1. Ruggedness of the separation

For our purpose, the most desirable assay conditions would produce the highest resolution between the olanzapine and desmethylolanzapine peaks, yet still provide reasonable capacity factors and method ruggedness. Method ruggedness is defined here as insensitivity to small variations in the mobile phase preparation and long column lifetimes. The sensitivity of the capacity factor and resolution to small changes in each variable can be estimated from the value of the coefficients (slope terms) shown in the empirical equations. The larger the value of the coefficient, the greater is the effect of small changes in the variable. This is most easily demonstrated by generating graphical presentations of each equation.

The response-surface plots for capacity factor and resolution are shown in Figs. 4–9. By comparison of Figs. 4, 6 and 8, it can be concluded that k' for olanzapine exhibits the same dependence on the percentage of acetonitrile and SDS concentration over the range of buffer pH tested (2.0–3.0). As shown by the slopes of these plots, the sensitivity of k' to small changes in the percentage of acetonitrile was dependent on the concentration of SDS chosen. Likewise, the sensitivity of k' to small changes in SDS concentration was dependent on the percentage of acetonitrile chosen. At larger SDS concentrations and smaller percentages of acetonitrile, the slopes of the response-surface plots

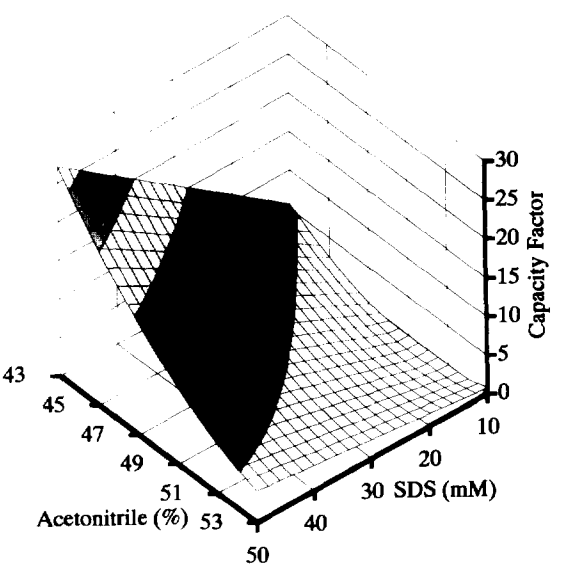

Fig. 4. k' vs. SDS concentration and percentage of acetonitrile at pH 2.5.

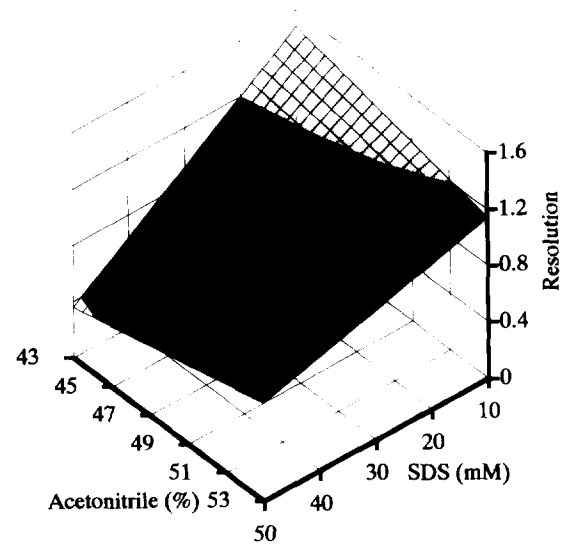

Fig. 5. Resolution vs. SDS concentration and percentage of acetonitrile at pH 2.5.

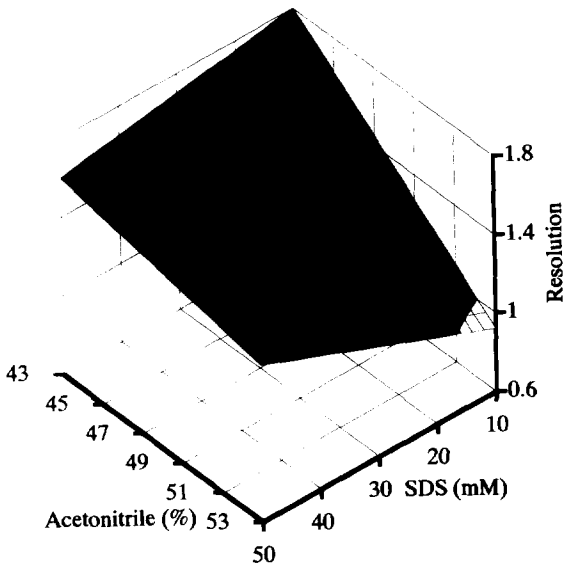

Fig. 7. Resolution vs. SDS concentration and percentage of acetonitrile at pH 2.0.

are greatest and the separation is the least rugged with respect to k'. In this same region the analysis times become excessively long. Conversely, at lower SDS concentrations and the largest percentage of acetonitrile, the slopes are not as great. However, in this region k' is too small and the separation capacity is greatly diminished. It was concluded that k' exhibits the best combination of ruggedness (relatively small slopes) and adequate compound retention around the center point regions of each plot.

By comparison of the plots shown in Figs. 5, 7

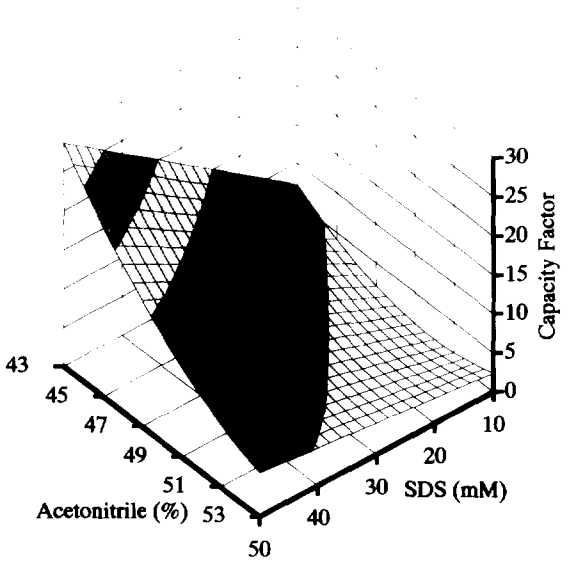

Fig. 6. k' vs. SDS concentration and percentage of acetonitrile at pH 2.0.

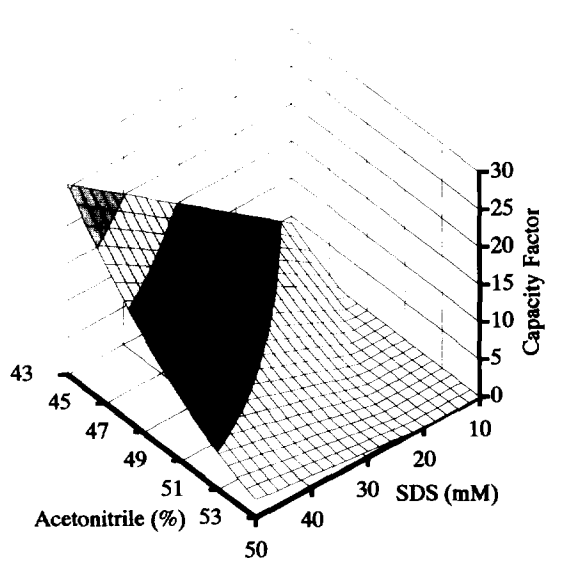

Fig. 8. k' vs. SDS concentration and percentage of acetonitrile at pH 3.0.

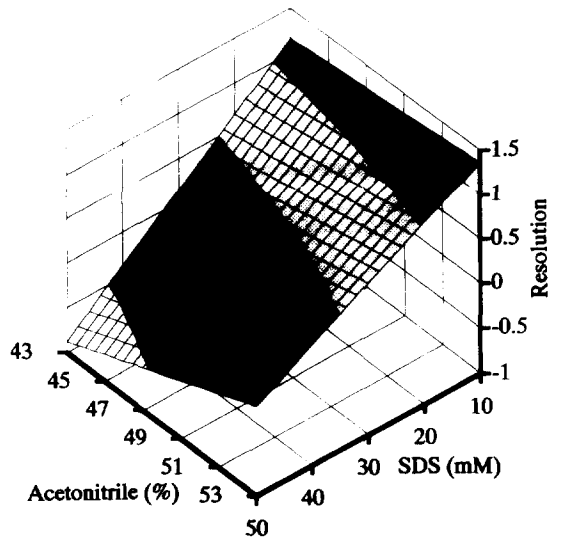

Fig. 9. Resolution vs. SDS concentration and percentage of acetonitrile at pH 3.0.

and 9, it can be concluded that the dependence of the resolution between olanzapine and desmethylolanzapine on the percentage of acetonitrile and SDS concentration is different with each buffer pH. At pH 2.0 (Fig. 7), the resolution ranges from 1.8 to 0.9, at pH 2.5 (Fig. 5) the resolution ranges from 1.5 to 0.4, and at pH 3.0 (Fig. 9), the resolution ranges from 1.4 to −0.9. The negative values for resolution observed at pH 3.0 correspond to a change in elution order between olanzapine and desmethylolanzapine. With respect to method ruggedness, at pH 2.5 (Fig. 5) the slopes are greatest at lower percentages of acetonitrile. At higher percentages of acetonitrile, the slopes are not as great. However, the combination of a low percentage of acetonitrile and a low SDS concentration resulted in the greatest resolution at pH 2.5. At pH 3.0 (Fig. 9), small changes in SDS concentration resulted in significant changes in resolution over the entire range of percentages of acetonitrile. At pH 2.0 (Fig. 7), the method is fairly rugged with respect to resolution, as evidenced by the relatively small slopes observed and the relatively large values for resolution across the entire surface.

It is apparent from the above discussion on resolution that the pH of the buffer must be tightly controlled. Use of an improperly functioning and/or calibrated pH electrode could result in poor method ruggedness with respect to resolution. Performing the separation with a buffer pH greater than 2.5 would not be recommended owing to the relatively poor method ruggedness and decreased resolution observed. However, performing the separation with a buffer pH of less than 2.5 would not be recommended owing to the known instability of bonded phases on silica supports at low pH. A pH of 2.0 is the absolute minimum recommended for reversed-phase columns [12]. For these reasons, the center point values for percentage of acetonitrile, SDS concentration and buffer pH were concluded to be the best choice for performing the separation. Under these conditions, the desired separation will be obtained with adequate method ruggedness and column lifetimes.

4.2. Comparison of DryLab and statistical analysis

Owing to the nature of the model used by the DryLab I/mp program, it could not predict the apparent interactions between SDS, acetonitrile and pH in the resolution equation as derived by statistical analysis. Further, there is a deficiency in the DryLab software for entering ion-pair reagent information and pH while studying the effect of varying the percentage of organic component. Therefore, it is implied by DryLab that no such interactions should exist. The DryLab I/mp modeling equation for resolution does not truly consider the individual contributions to band broadening caused by the different equilibria that each analyte experiences, i.e., analyte and ion-pair reagent, analyte and stationary phase, etc. By assuming a Gaussian distribution to band broadening, DryLab predicted that the resolution would remain constant with changes in percentage of acetonitrile at constant SDS concentration and pH (see Table 3). In fact, a significant change in resolution was observed. Further, DryLab predicted that the resolution would increase with decreased SDS concentra-

Table 6
Experimental results for resolution, theoretical plates and peak asymmetry along with the resolution predicted by DryLab I/mp

Experiment No.	Theoretical plates	Peak asymmetry	Experimental R_s	Predicted R_s
4	6314	0.83	1.0	1.4
5	8485	1.56	1.3	1.2
6	12957	1.28	1.7	1.4
9	9969	1.36	1.1	1.0

tion below 20 m*M* (see Table 4). Behavior opposite to this prediction was observed experimentally. The capacity factor behavior was modeled reasonably well by both DryLab I/mp and statistical analysis. Therefore, the use of quadratic equations to predict chromatographic retention times during ion-pair separations are appropriate.

To understand further why the DryLab model failed to predict resolution accurately, the data from these experiments were examined more closely. The results for resolution, theoretical plates (N) and peak asymmetry obtained in experiments 4, 5, 6 and 9 along with the value of resolution predicted by DryLab are shown in Table 6. The peak shape of olanzapine was observed experimentally to change from tailing during experiments 5, 6 and 9 to fronting during experiment 4. Further, the smallest value for the number of theoretical plates was observed during experiment 4. It became apparent from examining these results that the contributions to band broadening and the retention mechanism were different during experiment 4. Perhaps the combination of low SDS concentration and high percentage of acetonitrile resulted in more interactions between the amine functional groups on these benzodiazepine-derived compounds and the silica support. If so, then the retention mechanism would no longer be strictly ion-pairing and reversed-phase in nature. Similar peak deterioration was observed by Lewis et al. [8] when modeling the effect of pH on the retention of 3,5-dimethylaniline. In that study, silanol interactions were also suggested as the cause of the observed behavior which led to erroneous predictions by DryLab.

5. Conclusions

The predictions made using theory-based software with respect to peak resolution may not be as accurate as those made from statistical analysis of factorial design studies for complex ion-pairing separations. The separation studied here did not fit the theory-based model at low SDS concentrations and high percentages of acetonitrile. The predictions made by DryLab I/mp can be obtained quickly and with a much smaller set of data than that required by factorial design studies. As part of method development, it is highly recommended that factorial design studies be considered to model the observed chromatographic behavior. This is especially true in the case of ion-pairing separations. The empirical equations derived are useful for gaining a better understanding of method ruggedness.

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