Journal of Chromatography, 485 (1989) 585–596
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 940

COMPUTER-ASSISTED OPTIMIZATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION FOR CHLORPROMAZINE AND THIRTEEN METABOLITES

JEFFREY S. KIEL[a] and STEPHEN L. MORGAN*
Department of Chemistry, University of South Carolina, Columbia, SC 29208 (U.S.A.)
and
RUTH K. ABRAMSON
William S. Hall Psychiatric Institute, Columbia, SC 29201 (U.S.A.)

SUMMARY

Experimental design and response surface modeling of retention behavior were employed to optimize the reversed-phase high-performance liquid chromatographic separation of chlorpromazine and thirteen biological metabolites. A single-factor screening design and window diagram identified a region of mobile phase pH yielding the best relative retention for the worst separated pair of peaks. The effects of three mobile phase factors (pH, methylamine concentration and methanol concentration) were evaluated by a factorial design and modeled by regression analysis. Chromatographic conditions were then selected on the basis of relative retention, peak symmetry and retention time using a series of pseudo-three-dimensional window diagrams.

INTRODUCTION

A major reason for the widespread applicability of high-performance liquid chromatography (HPLC) is that mobile and stationary phase combinations may be tuned to select conditions for optimum separations of a wide variety of solutes. A particular mode of HPLC (*e.g.*, reversed-phase) may be selected for its suitability for separating the sample. The chemical nature of the solutes and their polarity relative to the stationary phase and the allowable combinations of mobile phase components guide the initial choice of a chromatographic system. Having specified these discrete choices, however, appropriate mobile phase conditions that will optimize the chromatographic quality must be chosen. In reversed-phase separations, these continuously variable mobile phase factors include concentrations of organic modifiers or other modifying agents, pH and ionic strength. Some of these factors may be varied independently of one another (*e.g.*, pH or the concentration of an organic modifier), while other factors may be related by constraints (*e.g.*, percentages of methanol, acetonitrile and water totalling 100%).

[a] Present address: Applied Analytical Industries, Route 6, Box 55, Wilmington, NC 28403, U.S.A.

0021-9673/89/$03.50

Numerous strategies have been employed for optimization of HPLC separations[1,2]. Common experimental designs include mixture designs[2], factorial designs[3,4] and sequential simplex optimization[5–9]. Although mixture designs typically may require only a small number of experiments, they are usually restricted to situations involving constrained variables such as volume percentages of solvents in a solvent mixture. Factorial designs combined with window diagrams or minimum alpha plots[10–12] can be extended to optimize more than one independent experimental factor[13–15]. Factorial designs also have the advantage that several responses can be evaluated simultaneously, permitting the chromatographer to judge acceptable tradeoffs between these responses after the experimentation is completed. Sequential simplex optimization requires a single response function; although multi-criteria response functions can be formulated, their application and interpretation can be difficult. Factorial designs, followed by regression modeling of solute retention, can deal with reversals in solute elution order (which are not easy handled by certain other techniques, including simplex). Multi-dimensional window diagrams of the chromatographic behavior of the solutes can then guide the selection of optimum conditions.

In this work, a preliminary screening design varying the mobile phase pH was employed to select an initial promising region of pH; a factorial design varying three mobile phase factors (pH, organic modifier concentration and methanol concentration) was then employed to characterize the chromatographic performance of the system. Reversed-phase separations of amine-containing solutes have been of interest to our laboratory for several years and the chromatographic problem selected for this study was the separation of the antipsychotic drug chlorpromazine and thirteen biological metabolites (Fig. 1). This mixture of solutes includes primary, secondary and tertiary amines, N-oxides and sulfoxides and hydroxylated compounds. Although gradient separations are possible, isocratic elution conditions were desired for their simplicity in application and for robustness with respect to long-term column stability. The polarities of the different solutes are varied and several of the amines tend to exhibit poor chromatographic behavior on silica-based columns, necessitating careful optimization of the isocratic mobile phase conditions for the best separation.

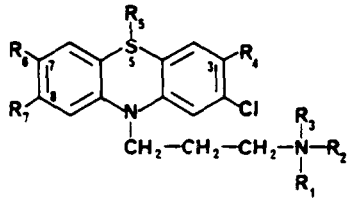
EXPERIMENTAL

Apparatus

Experiments were performed using a Waters Assoc. (Milford, MA U.S.A.) HPLC system equipped with a Model 441 UV absorbance detector (operated at 214 nm), a Model 6000A pump, a Model 710B autoinjector, a Model 720 integrator and a Model 730 system controller. A Supelcosil C_8 reversed-phase column (250 × 4.6 mm I.D. with a 5-μm packing), fitted with a C_8 guard column (50 × 4.6 mm I.D. with a 40-μm packing) was used (Supelco, Bellefonte, PA, U.S.A.). The column was heated to 40°C in a heating block coupled to a Haake FJ (Saddle Brook, NJ, U.S.A.) water-bath. A Model 601A ion analyzer (Orion Research, Cambridge, MA, U.S.A.) fitted with a combination pH electrode (Corning, Corning, NY, U.S.A.) was used to determine the apparent pH of the mobile phases.

Reagents

HPLC-grade methanol and phosphoric acid were obtained from Fisher



Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7
1. Chlorpromazine	CH_3	CH_3	--	H	--	H	H
2. Nor_1	CH_3	H	--	H	--	H	H
3. Nor_2	H	H	--	H	--	H	H
4. N-Oxide	CH_3	CH_3	O	H	--	H	H
5. Sulfoxide	CH_3	CH_3	--	H	O	H	H
6. Nor_1 Sulfoxide	CH_3	H	--	H	O	H	H
7. Nor_2 Sulfoxide	H	H	--	H	O	H	H
8. 7-OH Sulfoxide	CH_3	CH_3	--	H	O	OH	H
9. N-S Dioxide	CH_3	CH_3	O	H	O	H	H
10. 7-OH	CH_3	CH_3	--	H	--	OH	H
11. Nor_1 7-OH	CH_3	H	--	H	--	OH	H
12. Nor_2 7-OH	H	H	--	H	--	OH	H
13. 3-OH	CH_3	CH_3	--	OH	--	H	H
14. 8-OH	CH_3	CH_3	--	H	--	H	OH

Fig. 1. Structures of chlorpromazine and thirteen metabolites.

Scientific (Atlanta, GA, U.S.A.) and analytical-reagent grade methylamine hydrochloride and dibasic sodium phosphate from Aldrich (Milwaukee, WI, U.S.A.).

Experimental design and modeling

Two different factorial designs were used to examine the effect of three different factors (pH, methanol concentration and methylamine concentration) on the chromatography of the fourteen solutes investigated. Earlier work had shown that mobile phase pH and methylamine concentration greatly affect the chromatography of a similar group of compounds[6]. Initial mobile phase conditions of 250 m*M*

methylamine, 60% methanol and 25 m*M* dibasic sodium phosphate were chosen, as used in our previous work[12,15].

Defining the optimum pH region

To define the effects of mobile phase pH on the retention of the different solutes, a single-factor screening design was employed. In this series of experiments the mobile phase methanol concentration was fixed at 60%, the methylamine concentration at 250 m*M* and the sodium ion concentration at 50 m*M* (as sodium phosphate). The retention time for each solute was determined at mobile phase pH levels of 7.8, 7.4, 7.0, 6.0, 5.0, 4.0 and 3.0 and the capacity factor (k') were calculated. Matrix least squares[16,17] was used to fit a cubic polynomial to the capacity factor data for each solute *versus* mobile phase apparent pH:

$$k' = \beta_0 + \beta_1 x_1 + \beta_{11} x_1^2 + \beta_{111} x_1^3 \quad (1)$$

where β_0, β_1, β_{11} and β_{111} are the polynomial model parameters and x_1 is the pH. A plot of each of the fitted models over the pH range examined was produced using a Hewlett-Packard HP-7225B digital plotter interfaced to a Hewlett-Packard HP-85 microcomputer.

Multifactor optimization

The effects of mobile phase pH, methylamine concentration and methanol concentration on the chromatographic behaviour of the fourteen solutes were investigated with a 4 × 3 × 3 factorial design (Fig. 2). Based on results from the previous pH screening design, experiments were performed at four different mobile phase pH values (6.0, 6.6, 7.2 and 7.8), three different methylamine concentrations

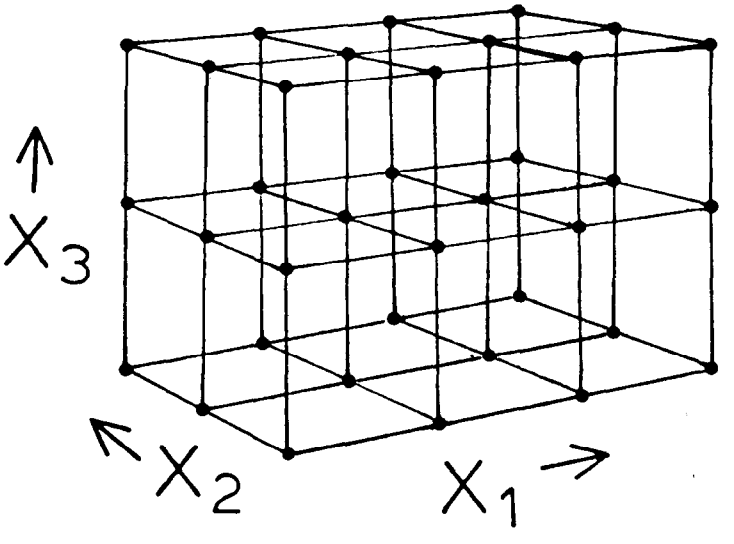


Fig. 2. The 4 × 3 × 3 factorial design. Factor x_1 represents the mobile phase pH, factor x_2 the mobile phase methylamine concentration and factor x_3 the mobile phase methanol concentration.

(100, 300 and 500 m*M*) and three different methanol concentrations (55, 60 and 65%). The total number of mobile phase combinations investigated was 36. The retention time for each solute peak was measured and the capacity factor calculated for each of the 36 mobile phase combinations. Matrix least squares was used to fit a full second-order model including all two-factor interactions to the capacity factor data as a function of the three factors (mobile phase pH, methylamine concentration and methanol concentration):

$$k' = \beta_0 + \beta_1 x_1 + \beta_{11} x_1^2 + \beta_2 x_2 + \beta_{22} x_2^2 + \beta_3 x_3 + \beta_{33} x_3^2 + \\ + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (2)$$

where β_0, β_1, β_{11}, β_2, β_{22}, β_3, β_{33}, β_{12}, β_{13} and β_{23} are the polynomial model parameters, x_1 is pH, x_2 is methylamine concentration and x_3 is methanol concentration.

This response surface model (k' response as a function of pH, methylamine concentration and methanol concentration) can best be represented as three-dimensional slices of this four-dimensional surface. To define the regions of maximum relative retention, a series of plots were produced by fixing the methanol concentration at 55, 57.5, 60, 62.5 and 65% and plotting the relative retention for the worst separated pairs of peaks as a function of mobile phase pH and methylamine concentration. The relative retention for the worst separated pair of peaks was calculated at 900 different points (a 30 × 30 grid) over the factor space examined by comparing the relative retention for each of the peak pair combinations of the fourteen fitted models. The smallest relative retention at each of the 900 points of factor space was plotted by a pseudo-three-dimensional plotting program[18].

RESULTS

The relationship between mobile phase apparent pH and capacity factor for each

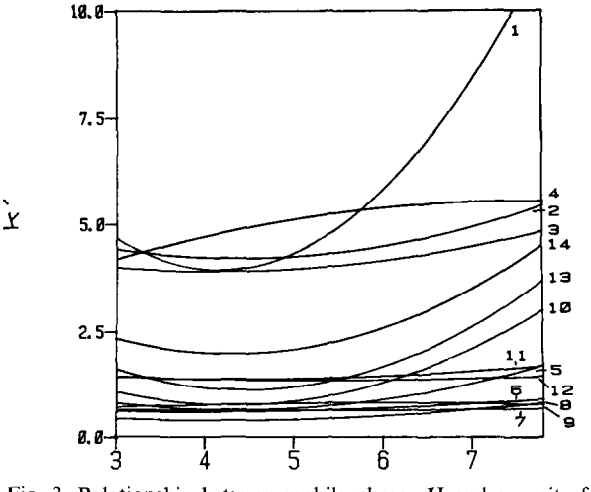


Fig. 3. Relationship between mobile phase pH and capacity factor (k') for the fourteen solutes. Peak identities as in Fig. 1.

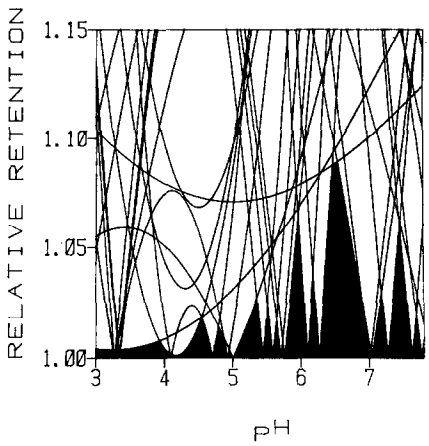


Fig. 4. Window diagram of relative retention as a function of pH for all 14 solutes. Peak identities as in Fig. 1.

of the fourteen solutes is shown in Fig. 3. The effect of pH on k' is substantial for many of the solutes, especially for the tertiary amines (solutes 1, 5, 8, 10, 13 and 14). There are several peak inversions and coelutions as the mobile phase pH is varied from 3 to 7.8. The area of maximum relative retention can be readily seen from the window diagram plot in Fig. 4. The highest "window" of relative retention for the worst separated pair of peaks occurs at a mobile phase pH of 6.5. A chromatogram produced at this optimum mobile phase pH (with the other chromatographic conditions being 60% methanol and 250 mM methylamine) is shown in Fig. 5. The separation under the

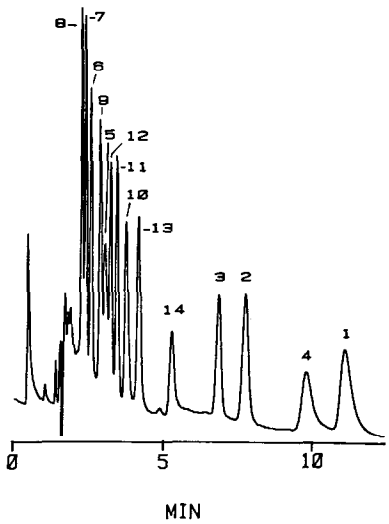


Fig. 5. Chromatogram obtained under the optimum mobile phase conditions defined by the window diagram in Fig. 4. Peak identities as in Fig. 1.

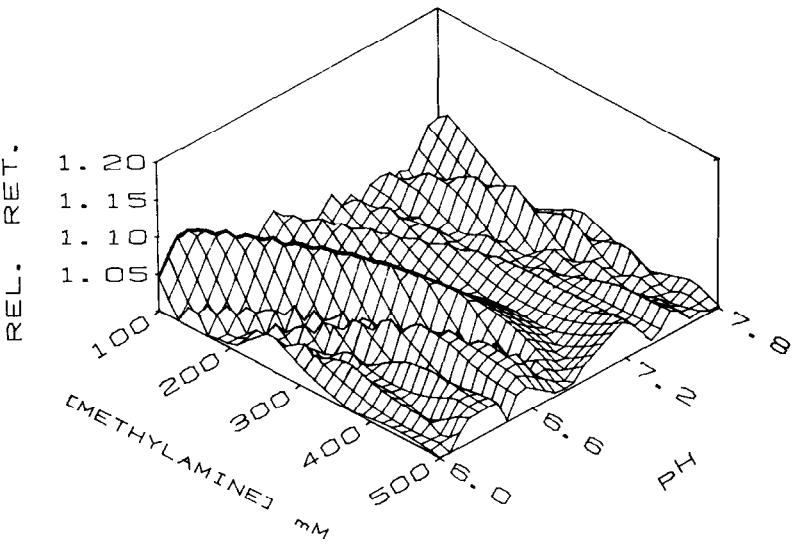


Fig. 6. Window diagram of relative retention of the worst separated pair of peaks as a function of mobile phase pH and methylamine concentration with 55% methanol in the mobile phase. The diagonal ridge defining optimum conditions is emphasized.

optimum mobile phase conditions indicated by this single-factor optimization did not provide an adequate separation of the early eluting solutes.

A three-factor factorial design was used to optimize the three mobile phase factors pH, methylamine concentration and methanol concentration. Five slices of the

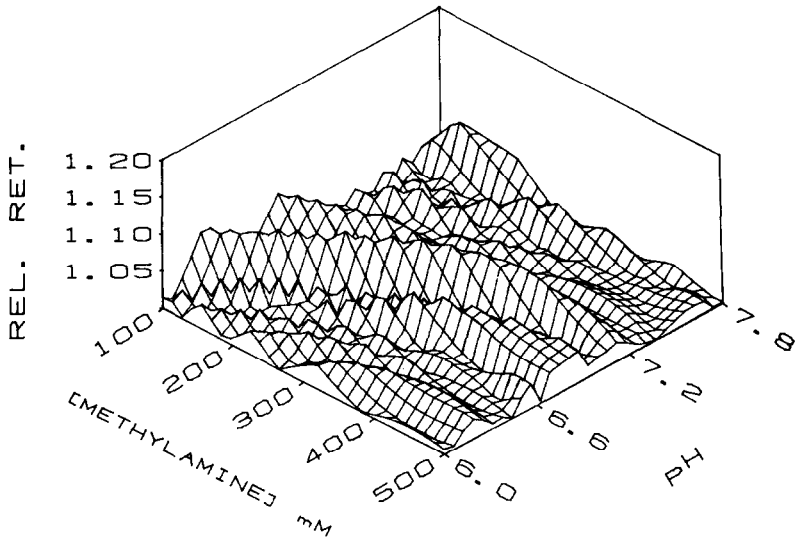


Fig. 7. Window diagram of relative retention of the worst separated pair of peaks as a function of mobile phase pH and metylamine concentration with 57.5% methanol in the mobile phase.

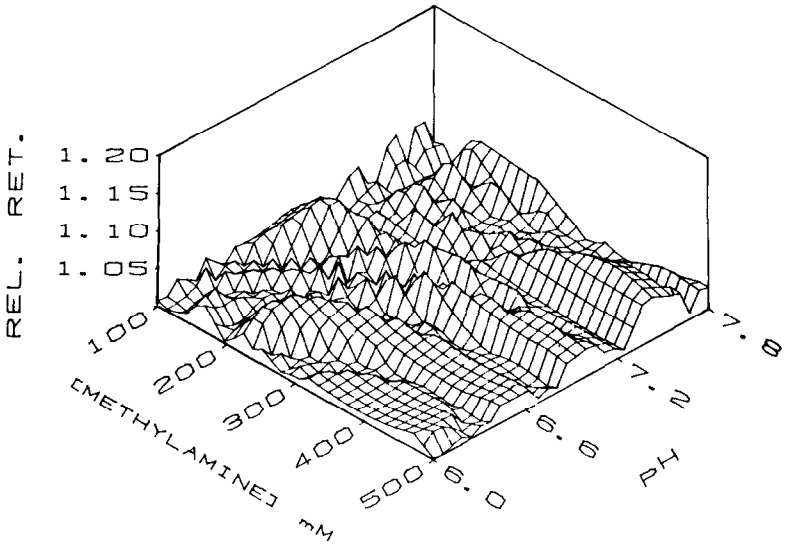


Fig. 8. Window diagram of relative retention of the worst separated pair of peaks as a function of mobile phase pH and methylamine concentration with 60% methanol in the mobile phase.

four-dimensional response surface were plotted by fixing the methanol concentration at 55, 57.5, 60, 62.5 and 65%. The relative retention of the worst separated pair of peaks as a function of pH and methylamine concentration is shown in Figs. 6–10. Examination of these plots reveals several local optima with the global optimum appearing along a ridge in the 55% methanol plot (Fig. 6). This ridge runs diagonally

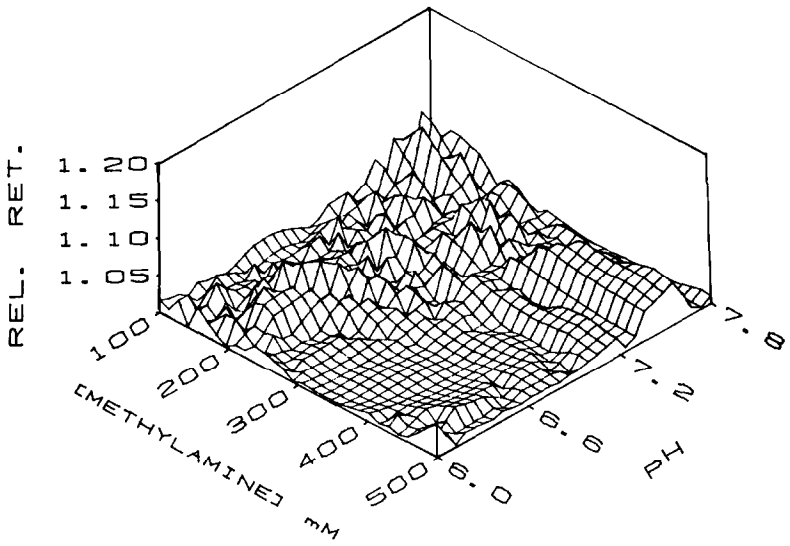


Fig. 9. Window diagram of relative retention of the worst separated pair of peaks as a function of mobile phase pH and methylamine concentration with 62.5% methanol in the mobile phase.

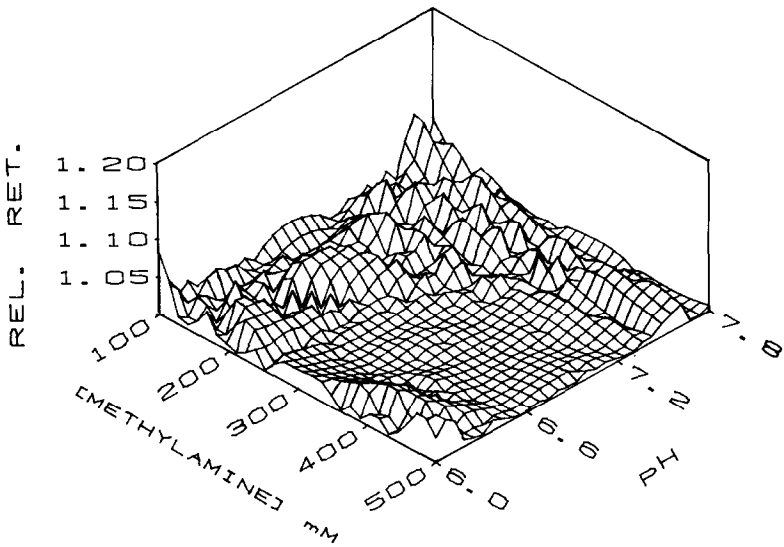
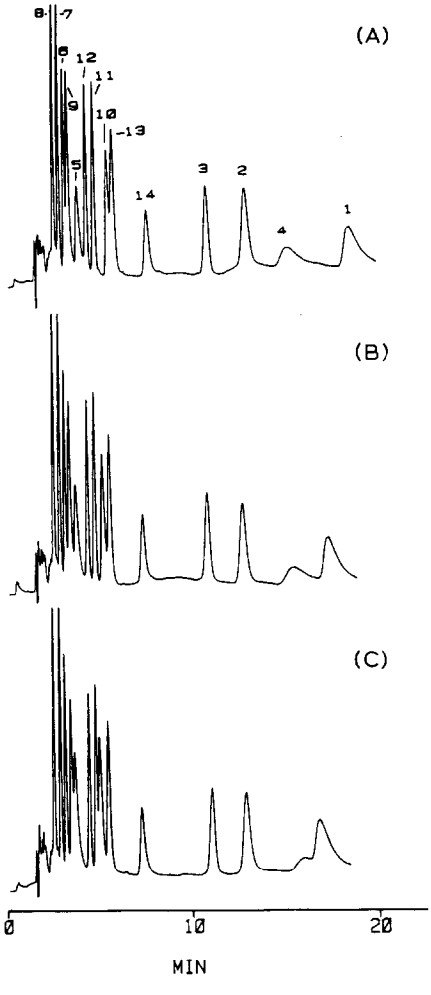


Fig. 10. Window diagram of relative retention of the worst separated pair of peaks as a function of mobile phase pH and methylamine concentration with 65% methanol in the mobile phase.

across the response surface. The highest relative retention for the worst separated pair of peaks is found at 100 m*M* methylamine and pH 6.2 (for 55% methanol). A chromatogram produced under these optimum conditions is shown in Fig. 11B. The separation of the solutes appears to be considerably improved in comparison with the conditions produced by the single-factor optimization, but several of the solutes show substantial peak asymmetry due to the low methylamine concentration and lower pH. Fig. 11 shows chromatograms produced at mobile phase pH above and below the optimum pH with a mobile phase methylamine concentration of 100 m*M*. The worst separated pair of peaks at mobile phase pH values slightly higher than the optimum is peaks 10 and 13. At lower pH, on the other side of the optimum ridge, the worst separated pair of peaks is peaks 9 and 5.

Our prior experience with chromatography of amine-containing solutes[19] aided us in the selection of methylamine concentrations spanning a region of reasonable peak shapes. Varying methylamine concentration as a single factor at constant pH will produce conditions that provide symmetrical peaks; however, the amine modifier concentration also affects the relative retention. The diagonal ridge in the methylamine–pH factor space (Fig 6) implies that the optimum methylamine concentration for the best relative retention depends on the pH employed, *i.e.*, these two factors interact with one another. Lower pHs require a lower methylamine concentration and higher pHs require a higher methylamine concentration to maintain the highest relative retention for the worst separated pair of peaks. Varying the methylamine concentration at a constant pH will locate the ridge, but the local optimum found for the methylamine concentration would not be optimum at other pH values. With the ridge located by multifactor experimentation, it was recognized that the conditions could be shifted along the ridge to a higher mobile phase pH and methylamine concentration. Staying on the ridge maintained the relative retention of the worst separated pair of peaks as high as possible, while moving along the ridge produced



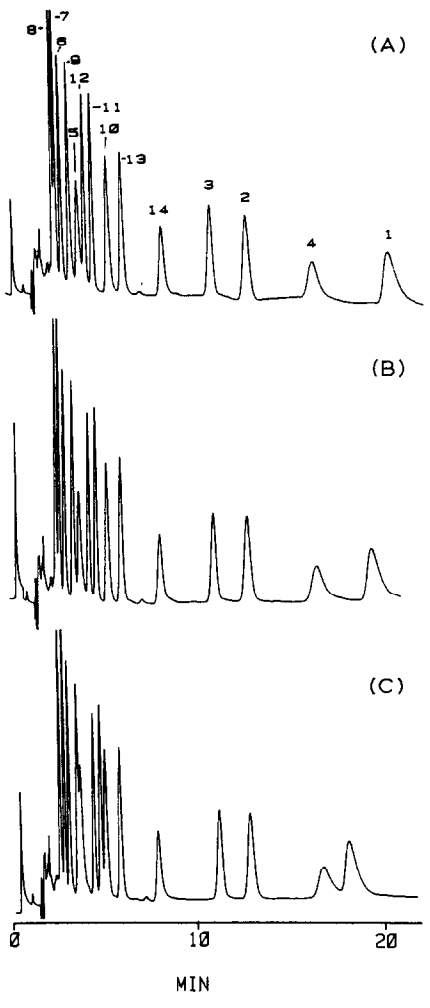


Fig. 11. Chromatograms obtained under mobile phase conditions of 55% methanol, 100 m*M* methylamine and (A) pH 6.4, (B) pH 6.25 (local optimum on ridge) and (C) pH 6.10. Peak identities as in Fig. 1.

Fig. 12. Chromatograms obtained under mobile phase conditions of 55% methanol, 275 m*M* methylamine and (A) pH 6.85, (B) pH 6.7 (local optimum on ridge) and (C) pH 6.55. Peak identities as in Fig. 1.

a better separation from the standpoint of peak symmetry. A better mobile phase combination was chosen along the optimum response surface ridge at pH 6.7, 275 m*M* methylamine and methanol concentration 55%. A chromatogram produced under these conditions is shown in Fig. 12B. This mobile phase combination provides both a good peak separation and symmetrical peaks. Peaks 8 and 7 are the worst separated at mobile phase pH values slightly above the optimum (Fig. 12A); peaks 9 and 5 are the worst separated pair of peaks at lower pH (Fig. 12C).

CONCLUSION

Both single-factor experiments with varying pH and factorial designs in which the three experimental factors (pH, methylamine concentration, and methanol concentration) are varied can be interpreted by empirical modeling with computer display of the resulting minimum alpha values in window diagrams of one or more dimensions. In this work, optimization of the mobile phase conditions was sought for the separation of fourteen ionic solutes, some of which exhibited poor chromatographic peak shapes. Initial experiments were performed in which the mobile phase pH was varied over a broad region, holding all other factors constant, so as to identify the potential regions of highest overall separation. A window diagram was produced by plotting the calculated relative retention (alpha values) for every combination of two peak pairs of the fourteen solutes over the factor space examined. The region of highest relative retention for the worst separated pair of peaks (the highest window) was found at a mobile phase pH of 6.5 (see Fig. 3). The chromatogram produced at this optimum, however, showed a poor separation of the early eluting solutes (Fig. 4).

To improve the separation over that produced from the single-factor pH optimization, a $4 \times 3 \times 3$ factorial design was employed to explore the factor effects of pH, methylamine concentration and methanol concentration. A full second-order model was fitted to the retention data and a series of minimum relative retention plots were produced. The optimum relative retention was found on a ridge at 55% methanol, 100 m*M* methylamine and pH 6.2. The final chromatographic conditions, however, were chosen to improve the peak symmetry of the solutes by working along the same ridge but slightly off the global optimum with respect to relative retention. This optimized isocratic HPLC separation has since been coupled with a solid-phase extraction and used to determine levels of chlorpromazine and its metabolites in plasma from patients undergoing therapeutic treatment[20].

The approach applied here to HPLC mobile phase optimization is general and can be applied to other separation problems. With factorial designs, the chromatographer is not limited to related variables and can chose any response that is appropriate. In this study, three independent factors were investigated. Final mobile phase conditions were based on the best relative retention and peak symmetry of the solutes and retention time. Additionally, computer modeling and computer graphics allowed visualization of the effects of the experimental factors on the chromatographic behavior of the solutes and enabled appropriate regions for optimum separation of a complex solute mixture to be determined. Optimization intimately involves the decision-making ability of the chromatographer. Although completely automated optimization systems for HPLC can be envisioned, insight and judgement should not be neglected, particularly when multiple criteria such as resolution (or, as in this study, relative retention), retention time and peak shape are all important.

REFERENCES

1 J. L. Glajch and J. J. Kirkland, *Anal. Chem.*, 55 (1983) 319A.
2 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57.
3 S. L. Morgan and S. N. Deming, *Sep. Purif. Methods*, 5 (1976) 333.
4 W. Lindberg, E. Johansson and K. Johanson, *J. Chromatogr.*, 211 (1981) 201.
5 M. L. Rainey and W. C. Purdy, *Anal. Chim. Acta*, 93 (1977) 211.

6 V. Svoboda, *J. Chromatogr.*, 201 (1980) 241.
7 J. C. Berridge, *J. Chromatogr.*, 16 (1982) 172.
8 J. C. Berridge, *J. Chromatogr.*, 244 (1982) 1.
9 J. H. Nickel and S. N. Deming, *LC, Mag. Liq. Chromatogr. HPLC,* 1 (1983) 414.
10 S. N. Deming and M. L. Turoff, *Anal. Chem.*, 50 (1978) 546.
11 J. H. Nickel and S. N. Deming, *Am. Lab.*, 16 (1984) 69.
12 J. S. Kiel, R. K. Abramson, S. L. Morgan and J. C. Voris, *J. Liq. Chromatogr.*, 6 (1983) 2761.
13 B. Sachok, R. C. Kong and S. N. Deming, *J. Chromatogr.*, 199 (1980) 307.
14 B. Sachok, R. C. Kong and S. N. Deming, *J. Chromatogr.*, 199 (1980) 317.
15 J. S. Kiel, R. K. Abramson and S. L. Morgan, *J. Chromatogr.*, 383 (1986) 119.
16 S. N. Deming and S. L. Morgan, *Clin. Chem.*, 25 (1979) 840.
17 S. N. Deming and S. L. Morgan, *Experimental Design: A Chemometric Approach,* Elsevier, Amsterdam, 1987.
18 S. N. Deming, *Anal. Chem.*, 43 (1971) 1726.
19 J. S. Kiel, S. L. Morgan and R. K. Abramson, *J. Chromatogr.*, 320 (1985) 313.
20 C. S. Smith, S. L. Morgan, S. V. Greene and R. K. Abramson, *J. Chromatogr.*, 423 (1987) 207.