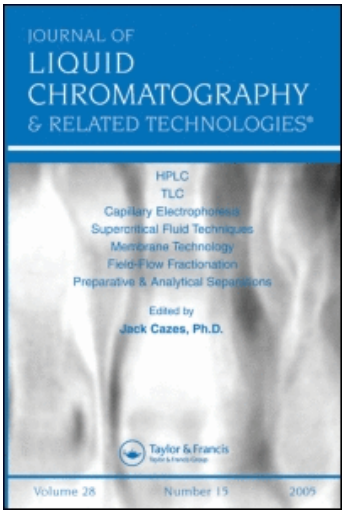

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AN EFFICIENT STRATEGY FOR OPTIMIZATION OF ISOCRATIC MOBILE PHASE CONDITIONS FOR HPLC SEPARATION OF A COMPLEX MIXTURE

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ABSTRACT

Chromatographic resolution of a complex mixture is often a trial and error process. An efficient strategy for the optimization of previously reported isocratic mobile phase conditions utilizes a factorial design and multivariate regression equations to model the dependence of elution time of each component on chromatographic parameters. Several parameters are varied simultaneously, with extreme and central values to allow interpolation in the modeling. Run order is scrambled to minimize time dependent errors. Reported here is the optimization of the separation of monoamine neurotransmitters and metabolites using isocratic reverse phase HPLC chromatography with serial oxidation and reduction electrochemical detection. The predicted retention times closely follow the experimental retention times for a set of conditions not included in the calculation of the model. The results enable the rational adjustment of parameters to meet subsequent chromatographic needs.

INTRODUCTION

Chromatographic resolution of a complex mixture, even working from published conditions, is often a trial and error process resulting in many chromatographic analyses before a satisfactory set of conditions is obtained. Although much has been reported on selection of the mobile phase (1,2) or column packing (3), perhaps the most frequent optimization is adaptation of a published method. With several chromatographic parameters, each optimized in turn, the number of runs is correspondingly increased. This common and intuitively obvious strategy is inefficient and ignores the complex interactions that may occur between parameters. Effort may be reduced and interactions between chromatographic parameters observed with an efficient optimization strategy. Here we report varying several parameters simultaneously in a full factorial design (where every combination of the chosen levels of two or more factors is used). Multivariate regression equations were used to model the chromatographic separation. The least squares regression analysis represents the significance and magnitude of the effects of each variable and their interactions in the form of an equation. (Numerous statistical texts discuss factorial designs and multivariate regression; ranging from an introduction (4) to a more complete discussion (5).

Although the optimization strategy is widely applicable, this study reports the optimization of the separation of monoamine neurotransmitters and metabolites using isocratic reverse phase HPLC chromatography with serial oxidation and reduction electrochemical detection. Methanol content and pH of the mobile phase were varied simultaneously. Multivariate regression equations and contour plots of retention times for each component of the mixture to be separated were obtained with the computer program Strategy (Edgework, Inc., Seattle, WA). The predicted retention times for each component could then be compared and the desired separation between components weighed against the length of the chromatographic run. We are able to routinely separate and identify 12 components, 11 monoamine neurotransmitters and metabolites along with an internal standard. Furthermore, the optimization study resulted in a better understanding of the manipulations which alter the separation, resulting in a sensitive (about 0.1 pmoles detection limit) and reliable separation method.

METHODS

Chromatographic

Monoamine neurotransmitter and metabolite standards (Sigma, St. Louis, MO) were separated and identified using isocratic reverse phase (25cm, 5 micron column; Vydac, Hesperia, CA) HPLC with electrochemical detection. The mobile phase, modified from Kilpatrick et al, (6), contained 0.09M sodium acetate, 0.039M citric acid (varied to adjust pH), 130μM EDTA, 400μM 1-octane sulfonate (as ion pairing agent), and methanol, 2-20% (v/v). For maximal selectivity, the detection used serial oxidation (+0.5V) then reduction (-0.5V) with an ESA Coulochem 5100A (Bedford, MA) with a 5021 conditioning cell and a 5011 analytical cell.

Optimization

The pH values of 3 and 5 were chosen as likely to be the extremes within which an optimum pH might be expected, since the method being modified used a pH of 4.35. A central value of pH 4 was used to allow the model to detect curvature in the relationship of pH and retention time. This placement of trial conditions at the extremes and a central value define a central composite design. Similarly, it was judged that extremes of 2 and 20% methanol and a central value of 11% methanol would be used to model the separation of the components. Both variables were changed in each trial, taking advantage of the redundancy of information in any one trial. The factorial design then results in 3 levels x 3 levels or 9 different combinations. Replicates of the trial for the central point were run to provide estimates of error. The final trial (the twelfth) was the best estimate for the optimal conditions (pH 4.35 and 4% methanol) and was not included in the calculations for the model to provide a comparison to the predictions of the model. The trial order was scrambled to block out the effects of uncontrolled factors. For each of the twelve trials, a set of 6 chromatographic runs of pairs of the components were made, to allow sure identification of each peak. Components with widely differing retention times under the best estimate of optimal conditions were chosen for each pair, so their retention times and identities would not be confused.

The dependence of the retention time of each of the components upon percent methanol and pH was then modeled with least squares multivariate statistical analysis. The dependence upon percent methanol and pH of the minimal difference in retention time seen between any two components and the dependence of the total run time (the retention time for the slowest eluting component) were also modeled. In each case, the sum of squares of the deviation from the model was minimized. The multivariate regression analyses with response surface or contour plots were performed with the computer program Strategy (Edgework, Seattle, WA) but could be done with many statistics programs (including the widely available program SAS, Cary N.C.).

For each dependent variable (individual component retention time, minimal retention time difference between peaks, or total run time) an equation was generated that had an intercept (b_0), one linear term for the main effect of each controlled variable (pH and percent methanol), and a cross-product term representing the effect of the interaction of the two controlled variables. Quadratic terms were not significant and were dropped. An example is:

retention time (5HIAA) =

15.1 - 0.13*pH - 0.39* percent methanol - 0.039*pH* percent methanol

Based upon the variance for the 3 replicate trials at pH 4 and 11% methanol, 95% confidence limits were calculated for each of these coefficients. The residuals (the difference of the observed from the predicted dependent variable for each set of runs) were plotted against the trial order to expose any time dependence of error. The residuals were also plotted against the predicted retention times to examine the uniformity of the standard deviation, and against pH and against percent methanol to assure that the variation was independent of the scale of the measurement. A response surface or contour plot of each dependent variable (individual component retention time, minimal retention time difference or total run time) versus pH and percent methanol was made, based upon the equation with its least squares fitted coefficients.

RESULTS

Chromatographic conditions chosen to give a central composite factorial design (extremes and central values for each factor and all possible combinations of the factors) resulted in widely varying retention times for each component (metabolite or neurotransmitter). Regression analysis then gave a unique equation with linear, cross-product and interaction terms to predict retention times for interpolated pH and percent methanol values for each component. The contour plot of the multivariate analysis equation's predicted retention times for the neutral noradrenaline metabolite 3-methoxy-4-hydroxyphenyl glycol (Figure 1A.) shows minimal effect of percent

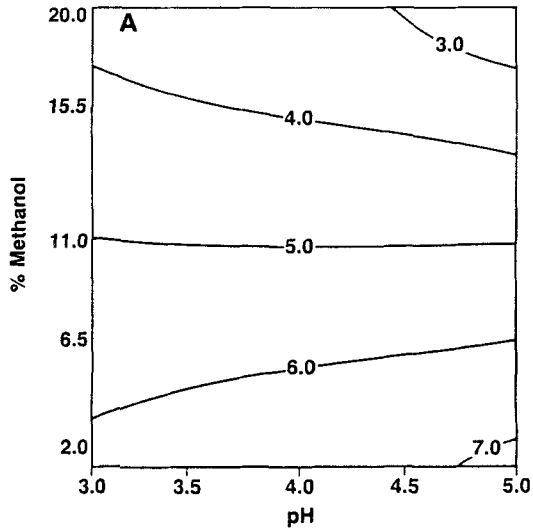

FIGURE 1.A.

Contour plot of the interpolated retention times versus pH and percent methanol for the neutral noradrenaline metabolite 3-methoxy-4-hydroxyphenyl glycol. The retention times (in minutes) on the contour lines are the predictions of the multivariate analysis model. Changes in percent methanol have minimal effect resulting in wide vertical spacing of the contour lines. The effect of pH (the slopes of the contour lines) varies with percent methanol.

methanol on predicted retention times (retention time changes by only 4 minutes from 2 to 20% methanol). The contour plot for the acidic serotonin metabolite 5-hydroxyindole acetic acid (Figure 1B.) shows that increasing percent methanol and pH each decrease the predicted retention time. The predicted retention times can be obtained from each component's equation or may be read off the contour plots. Retention times for the conditions not used in the calculation of the model were very close to the model's predicted retention times (see Figure 2.).

The use of multivariate analysis of the dependence of each component's retention time on chromatographic conditions predicted a

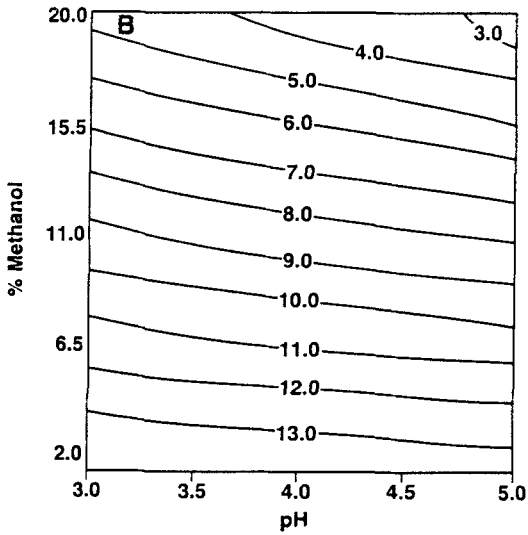

FIGURE 1.B.

Contour plot of the interpolated retention times versus pH and percent methanol for the acidic serotonin metabolite 5-hydroxyindole acetic acid. The retention times (in minutes) on the contour lines are the model's predictions. Changes in percent methanol strongly affect the retention times (resulting in close vertical spacing of the contour lines). Increasing percent methanol and pH each decrease the retention time.

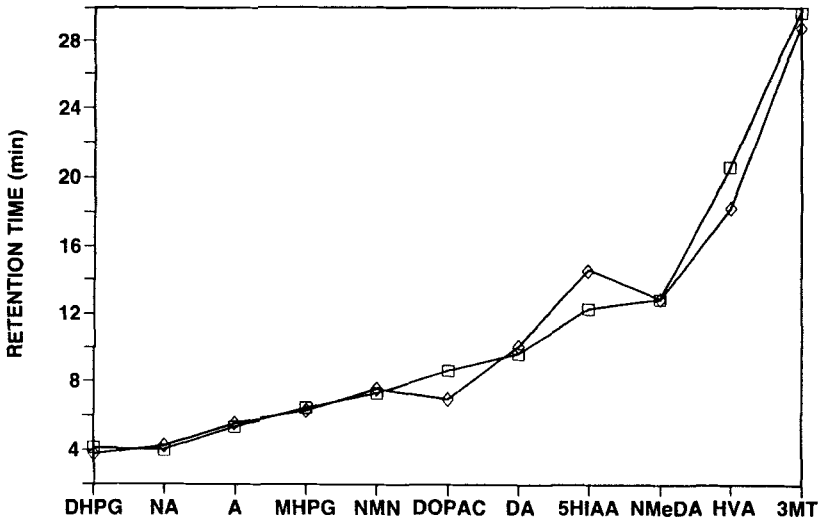

FIGURE 2.

Comparison of the model's predictions (squares) to the actual retention times (diamonds) for the conditions withheld from the calculation. The x axis shows the abbreviations for the components. DHPG, dihydroxyphenyl glycol; NA, noradrenaline; A, adrenaline; MHPG, 3-methoxy-4-hydroxyphenyl glycol; NMN, normetanephrine; DOPAC, dihydroxyphenyl acetic acid; 5HIAA, 5-hydroxyindole acetic acid; NMeDA, N-methyldopamine (an internal standard); HVA, homovanillic acid; 3MT, 3-methoxytyramine.

maximal separation of monoamine neurotransmitters and metabolites with a pH of 5 and no methanol, conditions extrapolated beyond the extremes values of percent methanol utilized in constructing the model. A very similar choice of high pH and low percent methanol for maximal separation resulted from the modeling based on the difference in the retention time between the two closest peaks rather than on the individual retention times. As might be expected, the maximal separation required a long total run time. A superimposition of the contour plot for the predicted total run time upon that for the minimal

TABLE 1.

Experimental design varying both pH and percent methanol simultaneously while randomizing run order for maximum efficiency and minimum error. The last run was the best estimate for resolution prior to the optimization. It was withheld from the calculations to check the accuracy of the model.

Trial Order	pH	% Methanol
1	4	11
2	4	20
3	4	2
4	3	11
5	5	11
6	5	2
7	5	20
8	3	20
9	3	2
10	4	11
11	4	11
Not included in calculations:		
12	4.35	4

separation between the two closest peaks (Figure 3.) allows selection of conditions that provide separation within a chosen total run time.

DISCUSSION

As compared to the trial and error process of optimizing a previously reported chromatographic separation, optimization with a factorial design for the chromatographic trials and modeling of the separation with multivariate regression analysis offers greater efficiency. Several features of the experimental design lead to its efficiency in creating the model. The simultaneous variation of two (or more) chromatographic conditions minimizes the number of trials needed to construct a model of the separation. Indeed, the advantages actually would be more pronounced as the number of levels or the number of factors increased since a balanced fraction of the total possible

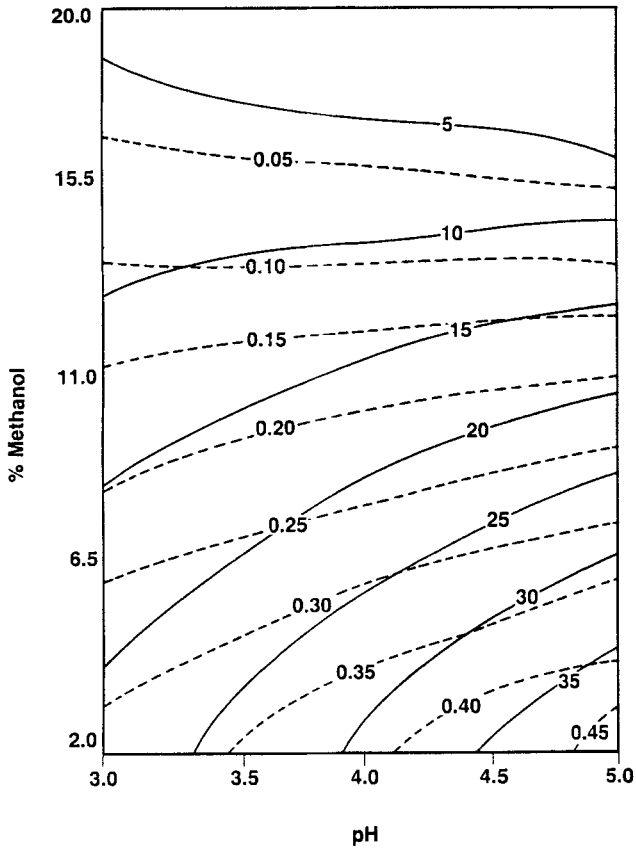

FIGURE 3.

Contour plot of the dependence of the minimal time between the two peaks with the closest retention times (solid lines) and the total run time (the time required for elution of the last peak) upon percent methanol and pH. The contour lines are labeled with the predicted retention times in minutes.

combinations can be selected to further increase the efficiency of the design. Thus, the inclusion of variation of a third controllable variable, such as the ion pairing reagent, might have improved the efficiency of the analysis and the completeness of the model. However, variation of the ion pairing reagent would have required much more time for column equilibration between trials.

It is important that the conditions chosen be widely spaced; as often happens, the maximal resolution of the example separation was predicted to occur at conditions outside those chosen for the trials. Such extrapolation beyond the data of the trials is clearly less reliable than interpolation within data from the trials. A non-linear relationship of the controllable parameter and retention time can be detected with an experimental design containing some additional trials in a symmetrical pattern between the chosen extremes (in this example, at the center). Replicates of some trial conditions are necessary for an assessment of confidence in the predictions. An additional trial with conditions not included in the calculation of the model can be used to check the accuracy of the predictions. Finally, a scrambled trial order minimizes the risk of error due to unforeseen time dependent variation.

Several aspects of the analysis are also significant. Multivariate regression analysis makes maximum use of the data since all the main effects and interactions are calculated from all the data. To more accurately predict the separation, an equation with linear, quadratic and interaction terms was used to model the possibly complex dependence of the separation on chromatographic conditions (pH and percent methanol).

The dependent variable can be chosen to provide maximum knowledge of the chromatographic response of each component or to provide a more direct prediction of optimal separation conditions for the mixture. In the first case, the dependent variable is each component's retention time (revealing in this example differences in the response of neutral versus acidic metabolites to pH and methanol as in Figure 1A and B). In the second case, the dependent variable may be the minimal time between the two peaks with the closest retention times (the identity of which may change with chromatographic conditions). Another calculation with the total run time (the time required for the

elution of the last peak) as the dependent variable allows selection of the optimal separation conditions within a criteria for total run time. All the data for the various dependent variables are available from the same 12 trials. In either case, to visualize the model's predictions, a contour plot of the dependence of the response (dependent variable) upon the chromatographic conditions is made from the equation resulting from multivariate regression analysis. If more than two chromatographic conditions are varied within the optimization experiment, additional contour plots could be generated for each additional parameter and its interaction with the other parameters.

The use of multivariate analysis and appropriately selected trial chromatographic conditions enabled optimization of the separation of monoamine neurotransmitters and metabolites. The simultaneous variation of two chromatographic conditions minimized the number of trials needed to construct a model of the separation patterns and allowed analysis of interaction of the parameters. Scrambling of the trial order minimized the risk of time dependent error. Modeling of the retention time for each component accurately predicted the separation for the trial not included in the calculation of the model. The improved predictability of the effects of manipulations should enable chromatographic alterations as needed. Modeling of the minimal separation between the two closest peaks and the total run time allows the selection of conditions meeting separation and time requirements. These concepts could be applied to optimization of any chromatographic separation.

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