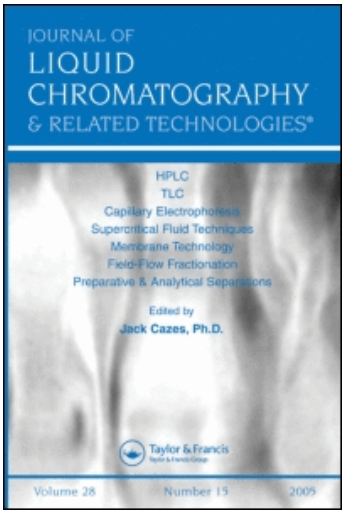

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Mathematical Modeling of Various Amines in the Ion-paired Liquid Chromatographic Analysis of Bilirubin

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MATHEMATICAL MODELING OF VARIOUS AMINES IN THE ION-PAIRED LIQUID CHROMATOGRAPHIC ANALYSIS OF BILIRUBIN

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ABSTRACT

The use of amines as ion-paired agents for bilirubin is explored. The effect of amine type, amine concentration, hydrogen ion concentration, and methanol concentration are determined and modeled by equations. The ability to adjust the capacity factor using multiple variations of the mobile phase should be useful in optimizing the separation of bilirubin from other components in various biological and theoretical studies of this interesting bile pigment.

INTRODUCTION

Bilirubin is a key bile pigment which plays an important role in jaundice, and other pathological conditions. The variation of

the concentration of bilirubin and its metabolites forms an important diagnostic tool. Because of the unique chemistry involved in its photodecomposition(1) and protein binding, analytical methods which are flexible enough to handle the differing problems posed by this molecule are needed. High performance liquid chromatography(2), with its high resolution and specificity, has been used (in a number of forms) to analyze bilirubin, its metabolites, and its photolytic decomposition products.

Although there is no lack of HPLC systems used for the determination of bilirubin, the complexity of the analysis has apparently not allowed optimization of a system which is capable of wide use. Early systems required either complex gradients or lacked the ability to resolve all components. Because of its unique conformation, bilirubin has solubility characteristics which render it insoluble in water despite its obvious polar nature. Bilirubin is sometimes referred to as being lipophilic, but because of its internal hydrogen bonding the solubility in water is low. Its solubility in other solvents are not consistent with lipophilicity.

The polar nature of bilirubin and of the conjugates which occur in biological systems make the use of ion-paired chromatography an obvious technique to use in the development of analytical systems. A large number of systems have been utilized, but little systematic exploration of the effects of mobile phase on retention time has been reported. Several workers(3),(4),(5) have used a system consisting of methanol or acetonitrile, DMSO, and an ammonium acetate buffer. This system shows some evidence of an ion-pair mechanism operating in addition to the expected reverse phase aspects. A number of workers(6),(7),(8) have used

tetrabutylammonium ion as an ion-pair agent using either acetonitrile alone or in combination with DMSO.

Development of optimized systems requires systematic evaluation of mobile phase effects on the retention of bilirubin. Moreover, modeling of the behavior by either mechanistic or mathematical techniques allows one to define the parameter space needed for optimization of the analysis. An additional benefit may be the development of isocratic conditions rather than the need for complex solvent programming.

EXPERIMENTAL

The chromatograph consisted of components from different manufacturers. A Reodyne 7225 injector (Rheodyne Inc., Cotati, CA) equipped with a 20 microliter sample loop was employed. The pump consisted of a Spectra-Physics SP8810 (Spectra-Physics, San Jose, CA) operated at 1.0 ml per min. A Waters 481 Lc spectrophotometer (Waters Assoc., Milford, MA) was used as the detector and was operated at 450 nm. The chromatographs were recorded on a Hewlett-Packard 3396A Integrator (Hewlett-Packard Company, Avondale, PA). A Whatman Partisphere 5 C18 (110 x 4.7 mm id) column (Whatman, Inc., Clifton, NJ) was used throughout the study and was equilibrated with the test solvent for 20 minutes prior to any injections.

Dihexylamine and tributylamine were obtained from Aldrich Chemical, while bilirubin was obtained from Sigma Chemical. These chemicals were used without further purification. Water used for the production of buffers was obtained from a Corning Mega-Pure systems. The pH's were measured on the aqueous portion of the buffer with a Corning model 125 digital pH meter calibrated with three standard commercial buffer solutions. The amine was added

to the water and the pH was adjusted with acetic acid. No attempt was made to measure the pH of the mobile phase after adding the methanol. The concentration of the amine was adjusted to account for the dilution with methanol making all amine concentrations expressed in terms of the final mobile phase composition. Solutions were degassed prior to use either by passing helium through the solution or exposing the solutions to ultrasonic energy for a period of ten minutes.

The retention times were transferred to a Lotus 1,2,3 spreadsheet and the capacity factors were calculated by the spreadsheet using the void volume of the column determined by the manufacturer. This data was displayed using the followint commercially available programs; Grapher for two dimension plots, and Surfer for three dimension plots. The platform for these functions was a Zenith 386 based microcomputer equipped with a Hewlett-Packard Laserjet series II as a plotting device. Numerical analysis were carried out using the SAS system and either proc reg or proc nlin. The SAS system was operated on the Higher Education Computing Network (North Dakota) and was run on the Soulbourne 5/802 computer system. A SAS system was also run on the Zenith 386 system.

RESULTS AND DISCUSSION

Experimental Design

The experimental design was selected to model the effect of the three mobile phase variables namely, ion-pair concentration, pH, and methanol concentration. Consequently, one variable was varied while the other two were held constant. Each variable was

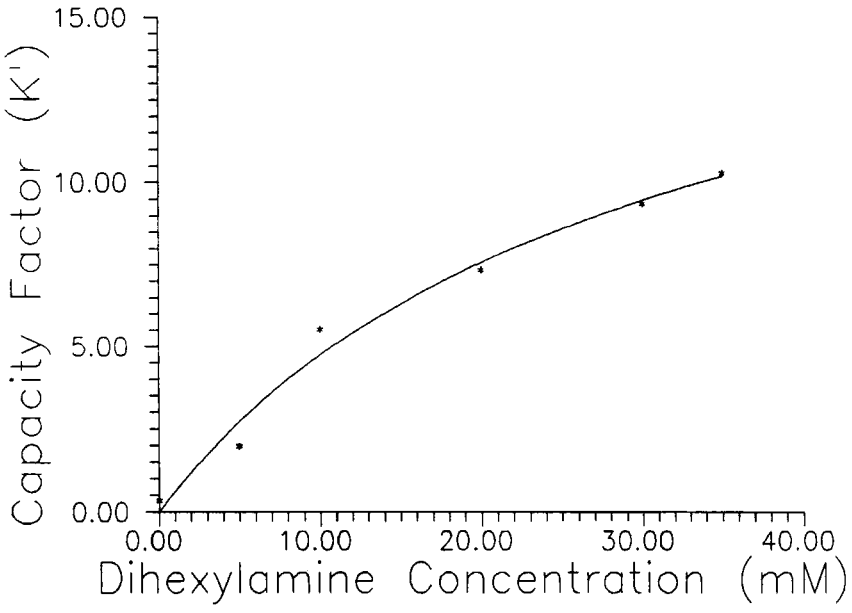

Figure 1. The capacity factor of bilirubin as a function of dihexylamine concentration at pH 7 and 80 % methanol. The line represents the nonlinear regression fit of the data resulting in the equation:

$$K' = \frac{18.94}{1 + 29.86/[amine]}$$

studied at two points in the design matrix, for example the pH was varied at two independent sets of amine and methanol concentrations. This design produced an efficient exploration of the effect of each variable being studied.

Dihexylamine

The capacity factor of bilirubin as a function of dihexylamine showed the usual nonlinear behavior (figure 1). This behavior can be mathematically modeled in a number of ways, most

commonly as a power function or as a hyperbolic function. Either of these models predict a rapid initial rise followed by either a limit (hyperbolic function) or a greatly attenuated rise (power). We chose to use the hyperbolic function to model our data since this behavior can be deduced from a mechanistic consideration(9).

$$K' = \frac{(k_1)}{(1 + k_2/[amine])}$$

Nonlinear regression analyses provides the coefficients for the equation. A good fit was obtained for the hyperbolic function using 6 data points at a methanol concentration of 80% and a pH of 7. The function indicated a maximum K' of 18.9 and the concentration at half saturation was 30 mM. The lipophilicity of dihexylamine limited our study to about 35 mM. This experimental limitation restricted the experimental design and precluded higher amine concentrations.

The effect of pH on the capacity factor of bilirubin using dihexylamine as an ion-pair agent is shown in figure 2. A mechanistic model(10) showed a good fit. For data measured at different pH's at 90% methanol and 5 mM amine concentration, nonlinear regression gave a K' of 0.29 at high pH's and 8.34 at lower pH's.

$$K' = \frac{(K'_1 + K'_2 \cdot Hion/Ka)}{(1 + Hion/Ka)}$$

The curve indicated that an ionizable group with a pKa of about 4.85 was influencing retention time. This is in the range expected for the carboxyl groups of bilirubin. What is not expected is the increase in capacity factor as the pH is lowered,

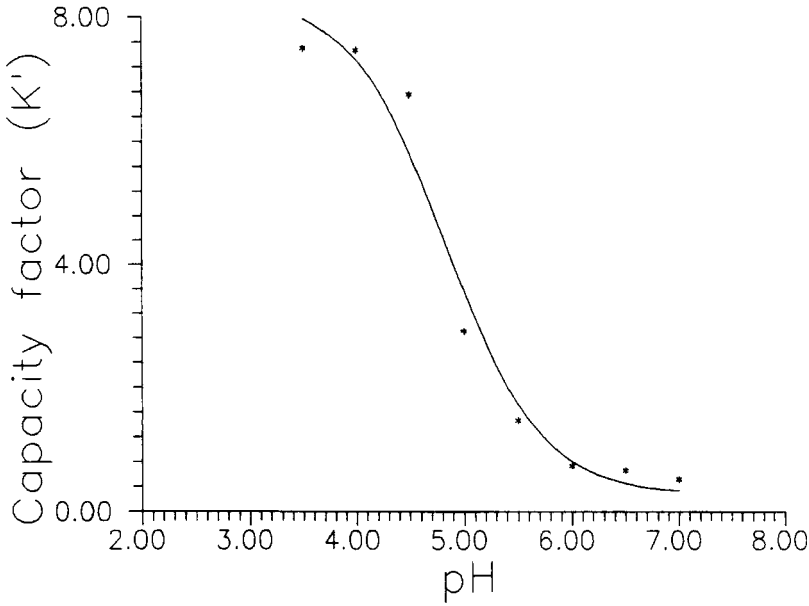

Figure 2. The capacity factor of bilirubin as a function of pH at a dihexylamine concentration of 5 mM and 90 % methanol. The line represents the nonlinear regression fit of the data resulting in the equation:

$$K' = \frac{0.29 + 8.34 \cdot [hion]/0.000014}{1 + [hion]/0.000014}$$

indicating a new complex forms between bilirubin and the amine. Other workers have observed this increase in capacity factor with decreases in pH with other ion pairing agents. Bilirubin has a number of hydrogen bonding sites in both the ionized and neutral molecule which could form a lipophilic complex with an ion pairing reagent thus explaining the experimental results.

Changes in the capacity factor of bilirubin as a function of methanol concentration are quite normal and can be modeled well by the usual exponential function.

$$K' = k_1 e^{(-k_2[methanol])}$$

$$\ln K' = k_1 - k_2[methanol]$$

The increase in the capacity factor is extremely rapid and limits the range which can be studied to 75 per cent or greater even at moderate dihexylamine concentrations. Although it is generally assumed that the relationship of log(K') with per cent methanol is linear, Jandera and Kubat(11) studying wide ranges of methanol concentration have noted the need for a quadratic relationship to fit the slightly curved data. These workers also pointed out the difficulties in obtaining accurate values for k_1, which represents the retention time at zero methanol concentration. This constant is particularly meaningless when a narrow range of high methanol concentrations is studied. When the range of methanol concentrations is small the relation of log(K') and per cent methanol may be regarded as linear. The fit shown in figure 3 at 20 mM dihexylamine and pH 7 has the intercept at 9.11E7 and the slope of 0.20.

Tributylamine

The use of tributylamine as an ion-pair reagent caused the retention time of bilirubin to shift in a manner analogous to the behavior when dihexylamine was the ion-pair reagent. The difference between the two reagents can be attributed to the lower lipophilicity of tributylamine. Bilirubin was eluted with a much lower methanol concentration with tributylamine. Tributylamine offers an opportunity to work at lower methanol concentrations than analyses using dihexylamine which could be useful in some analyses.

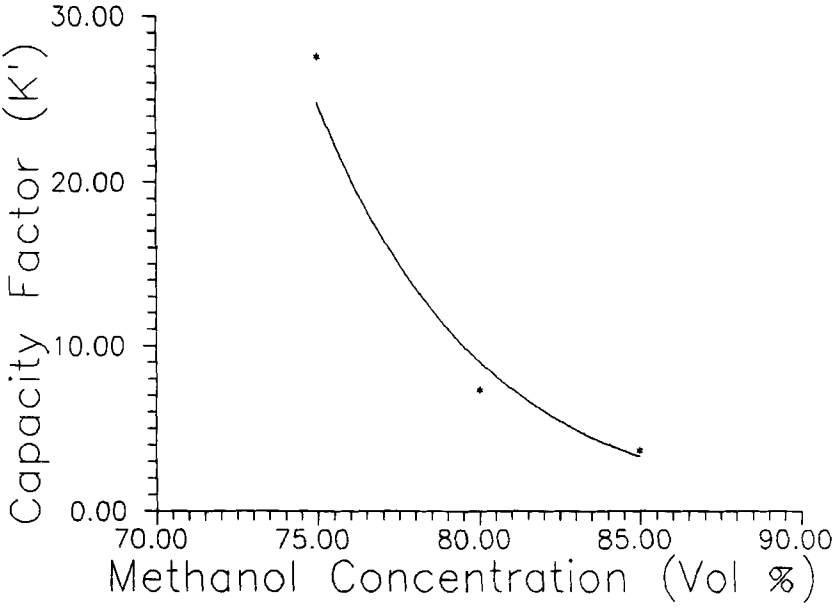

Figure 3. The capacity factor of bilirubin as a function of methanol concentration at pH 7 and a dihexylamine concentration of 20 mM. The line represents the regression fit of the data resulting in the equation:

$$K' = 9.11 \cdot 10^{7} \cdot e^{(-0.202 \cdot [methanol])}$$

The effect of the change in tributylamine on the capacity factor is shown in figure 4. The general shape of the curve which indicates a saturation or near saturation as the concentration of the ion-pair reagent reaches high values. Again the hyperbolic model proves useful in fitting the data and understanding the basic relationships involved. At a methanol concentration of 70 per cent and pH of 7 nonlinear regression analysis indicated a capacity factor of 15.6 and the concentration of the amine at half maximum capacity factor was 20 mM. These figures are similar to those found for dihexylamine at an alcohol concentration of 80 per cent. The effect of alcohol concentration on the maximum K' which

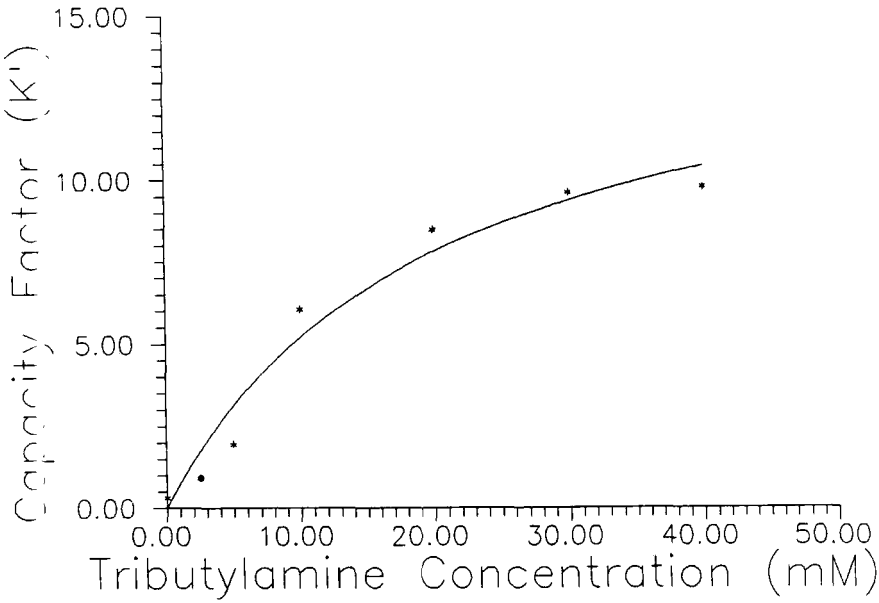

Figure 4. The capacity factor of bilirubin as a function of tributylamine concentration at pH 7 and 70 % methanol. The line represents the nonlinear regression fit of the data resulting in the equation:

$$K' = \frac{15.62}{1 + 20.0/[amine]}$$

is obtained at any pH is exponential, indicating a significant difference between dihexylamine and tributylamine in the lipophilicity of their complexes with bilirubin.

The change in capacity factor resulting from pH changes are shown in figure 5. The curve at lower pH's is not easily assessable because the capacity factors would become too large. The curvature of the data in this type of plot is too sharp to be fitted well by exponential, power, or quadratic functions. However, the mechanistic equation relating the change in capacity

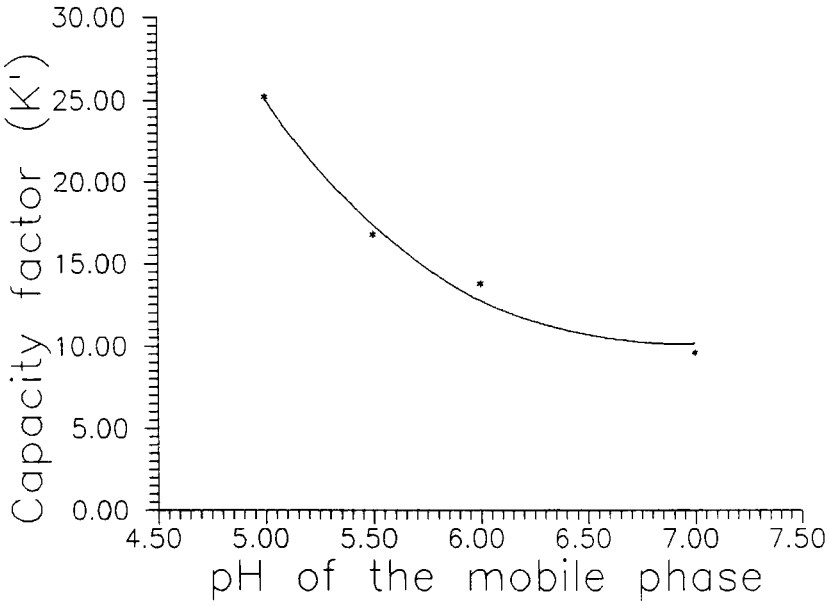

Figure 5. The capacity factor of bilirubin as a function of pH at a tributylamine concentration of 30 mM and 70 % methanol. The line represents the nonlinear regression fit of the data resulting in the equation:

$$K' = \frac{9.83 + 38.9 \cdot [hion]/0.000009}{1 + [hion]/0.000009}$$

factor to pH, and the use of the ionization constant of the acid is useful in fitting the data. Nonlinear regression analysis of the data shown in figure 5 for a concentration of tributylamine of 30 mM and a methanol concentration of 70 per cent gave a capacity factor of 9.83 at high pH's and and 38.9 at low pH's. The pKa determined in this analysis was 5.0, and is in good agreement with the pKa determined with dihexylamine. The acidity constant reflected in these analyses is derived from the ionization of bilirubin, since the amines would not change their ionization in the pH range studied.

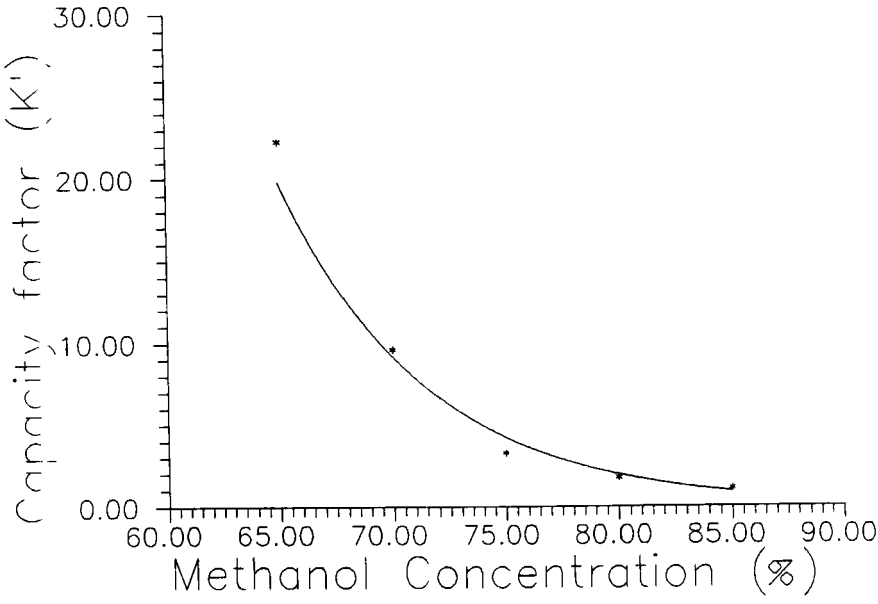

Figure 6. The capacity factor of bilirubin as a function of methanol concentration at pH 7 and a tributylamine concentration of 30 mM. The line represents the regression fit of the data resulting in the equation:

$$K' = 4.39 \cdot 10^5 \cdot e^{(-0.154 \cdot [methanol])}$$

Finally, the effect of methanol concentration on capacity factor is shown in figure 6. The exponential function is clearly observable. Comparison with the data from dihexylamine indicates that the slope and the intercept for tributylamine are both less than for dihexylamine. Although the data are measured at different concentrations of amine, the slope and intercept indicate tributylamine will allow higher water concentration in mobile phases in the development of an assay. A variety of ion-pair reagents which function at different methanol concentration allow flexibility in assay development.

Combination of Factors

While the fit of equations relating the change in a single mobile phase component to the retention time is quite straight forward, combining of multiple factors into single equations and the determination of the parameters for these equations become increasingly complex as the number of factors increase. Nonlinear relationships complicate matters even more. Currently, the models range from empirical models to mechanistic models. A number of techniques may be used to fit the models; appropriate techniques frequently are dependent on the model. For example, multiple linear regression is not suitable for models which can not be expressed in a linear form. Although some smoothing techniques such as cubic spline fitting do not depend on a mechanistic model, a mathematic form is assumed in the calculations.

Multiple linear regression is a popular method for computing and examining the effect of multiple factors on chomatagraphic retention times. The advantage of this method is the speed and simplicity of the calculations, the availability of measures of the appropriateness of fit, and the powerful diagnostics which can be used to examine any weaknesses of the models. The disadvantage is the need for the problem to be linear. Although some nonlinear systems can be made linear, care must be used to avoid inappropriate transformation of variances leading to violation of the basic assumptions of the linear regression process. A more serious problem of this technique is that some equations are inheritently nonlinear and can not be transformed into a linear form. Despite the potential problems, this method has been applied numerous times in HPLC and it is particularly successful providing the range of the variables which can be fit by linearized terms(12). The use of polynomal forms such as

quadratic forms, including all cross products, has been particularly useful. However, some hypersurfaces can not be fit by polynomals. One technique which appears to work well(13) is to use some type of interpolation. The adaptability of this procedure to different retention mechanisms represents its major advantage. These techniques suffer from two problems; they may follow the data too closely providing an unrealistic graph with many hills and valleys which don't exist, or they may smooth out curvature which actually exists thereby creating an inaccurate model.

Attempts to apply multiple linear regression to our data gave only partially successful results. Attempts to utilize a quadratic design with all cross products gave high correlations (R-square 0.97) but some of the terms were statistically not significant. When the problem was studied using stepwise regression techniques with a wide selection of transformed independent variables (log, reciprocal, cross products, squares) and the best equation with all terms significant at the 0.05 level, the equations showed lower R-squares and definite shortcomings in their fit. Usually poor fit gave large residuals for a group of data such as the points at certain pH's or methanol concentrations. A probable cause for poor fits observed is the behavior of the capacity factor with changes in pH, which can't be linearized.

Nonlinear regression is a powerful technique which can fit any model in which the data is accurate and the data points have been selected using an appropriate design. The first problem faeed is the means of combining the mathematical models which were successfully fit in the single parameter studies into a multiple parameter model. This problem is compounded by the combinations

TABLE 1.

Parameters Derived from Nonlinear Regression Analysis of Capacity Factor as a Function of Mobile Phase Composition[a].

Parameter	Dihexylamine	Tributylamine
k_1	345 (46)	27.8 (5.6)
k_2	0.263 (0.015)	0.195 (0.028)
k_3	47.6 (10.6)	7.62 (6.24)
k_4	84.5 (21.8)	72.8 (21.5)
k_5	0.124 (0.032)	0.093 (0.025)
K_a	$1.55X10^{-4}$ ($3.9X10^{-5}$)	$7.60X10^{-5}$ ($3.1X10^{-6}$)

[a] The number in parenthesis represents the asymptotic standard error associated with the parameter estimate.

becoming inheritently nonlinear, and the general complexities introduced into the mechanistically based equations(14).

$$K' = \frac{k_1 \cdot e^{-k_2 \cdot Meth} / (1 + k_3 / amine) + \left(k_4 \cdot e^{-k_5 \cdot Meth}\right) \cdot Hion / Ka}{1 + Hion / Ka}$$

The above model was fit to the data for dihexylamine and tributylamine. Please notice the second term lacks the provision for the effects of amine on the capacity factor at low pH's. This factor was eliminated because our experimental design did not explore this behavior. An additional simplification was used by restricting the fit to the lowest methanol concentration utilized

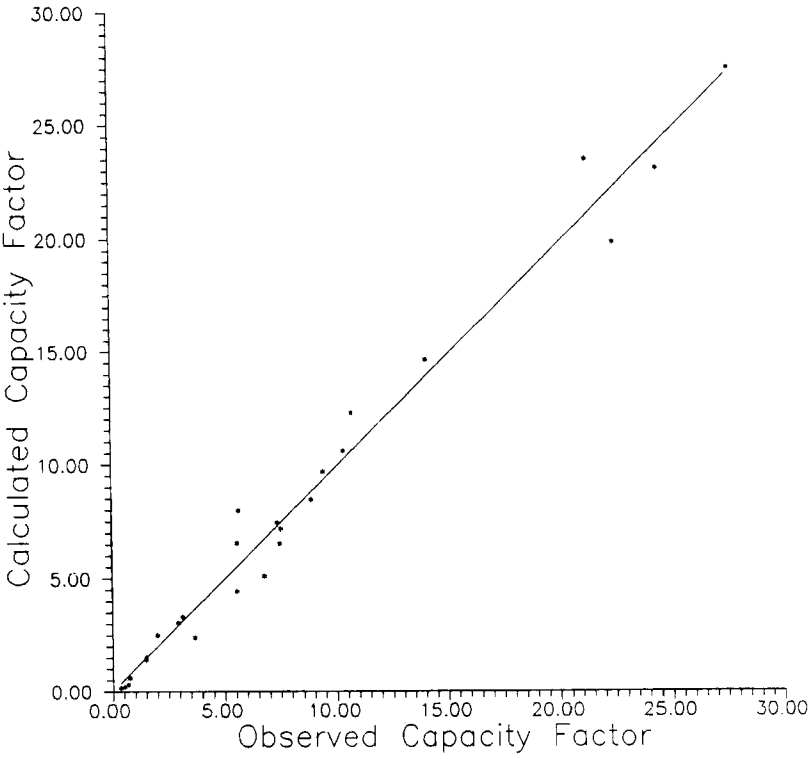

Figure 7. The relationship between observed capacity factors and those calculated from the nonlinear regression equations in table 1 for dihexylamine.

in the experimental data. This was accomplished by simply subtracting the lowest methanol concentration from all methanol concentration. This in effect moves the axis from zero per cent methanol to the lowest methanol concentration used in the experiments (70 per cent for dihexylamine and 65 per cent for tributylamine). This does not alter the fit of the data to the model, but does allow a more realistic estimate of the error in the parameters. The results are shown in table 1.

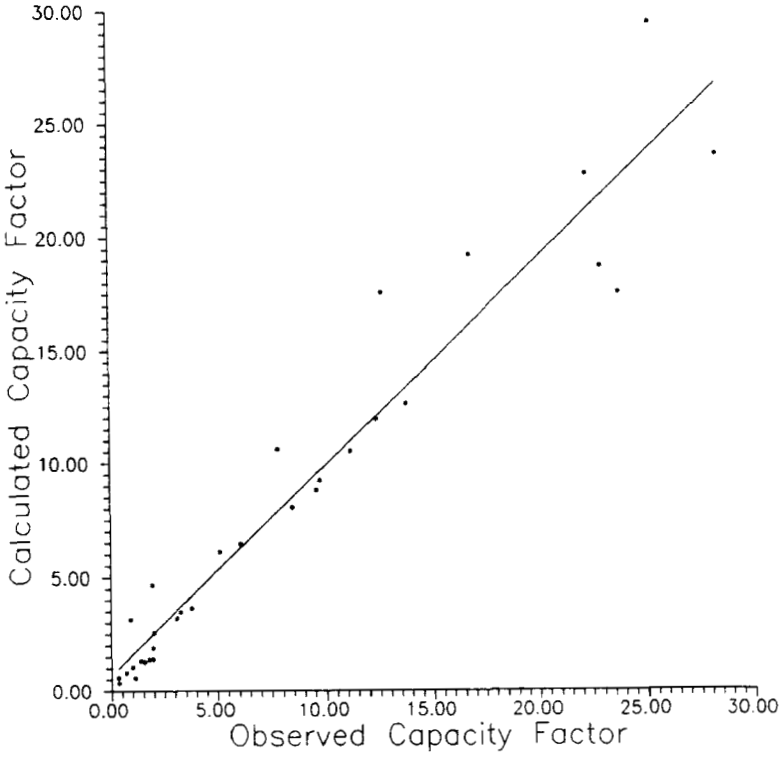

Figure 8. The relationship between observed capacity factors and those calculated from the nonlinear regression equations in table 1 for tributylamine.

The agreement between the calculated capacity factor and the observed capacity factors was good as shown in figure 7 for dihexylamine and figure 8 for tributylamine. Evaluation of the parameters show several interesting features. Dihexylamine shows a sharper increase in capacity factor when methanol concentration is reduced, demonstrating a difference which could be exploited if a separation required working at a certain methanol concentration in order to elute other peaks. This phenomena was present at both

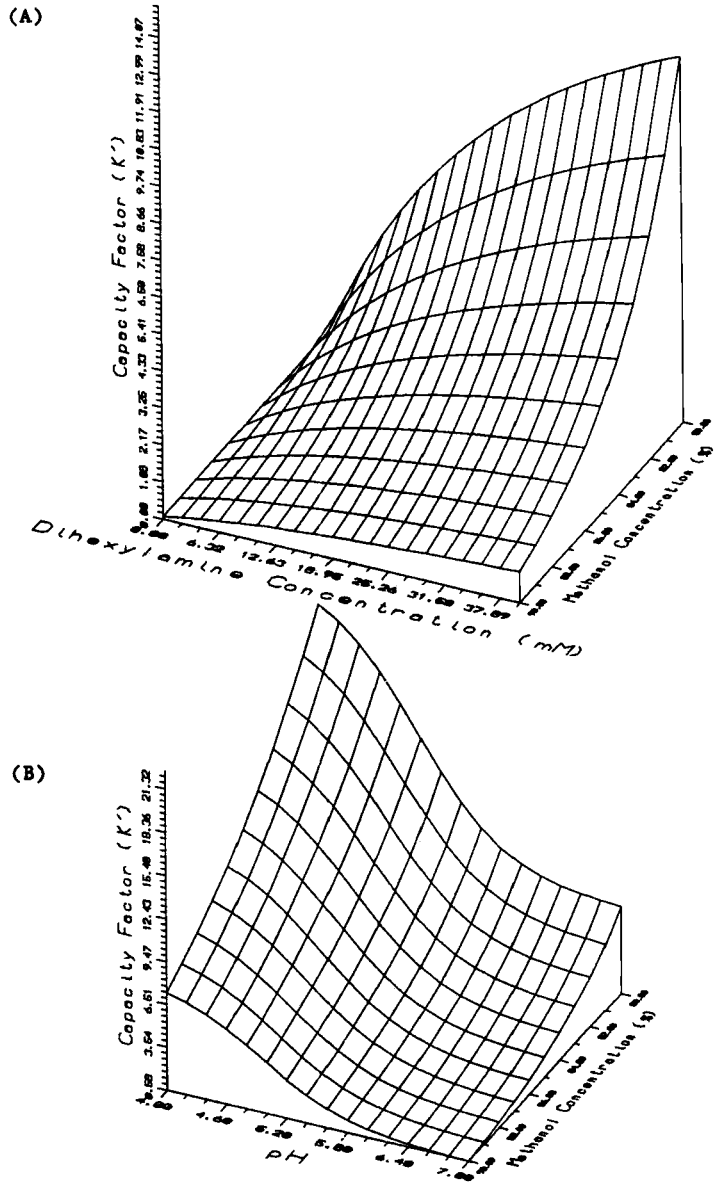

Figure 9. The relationship between capacity factor, dihexylamine and methanol concentration (A) and capacity factor, pH and methanol concentration (B) calculated from the nonlinear regression function.

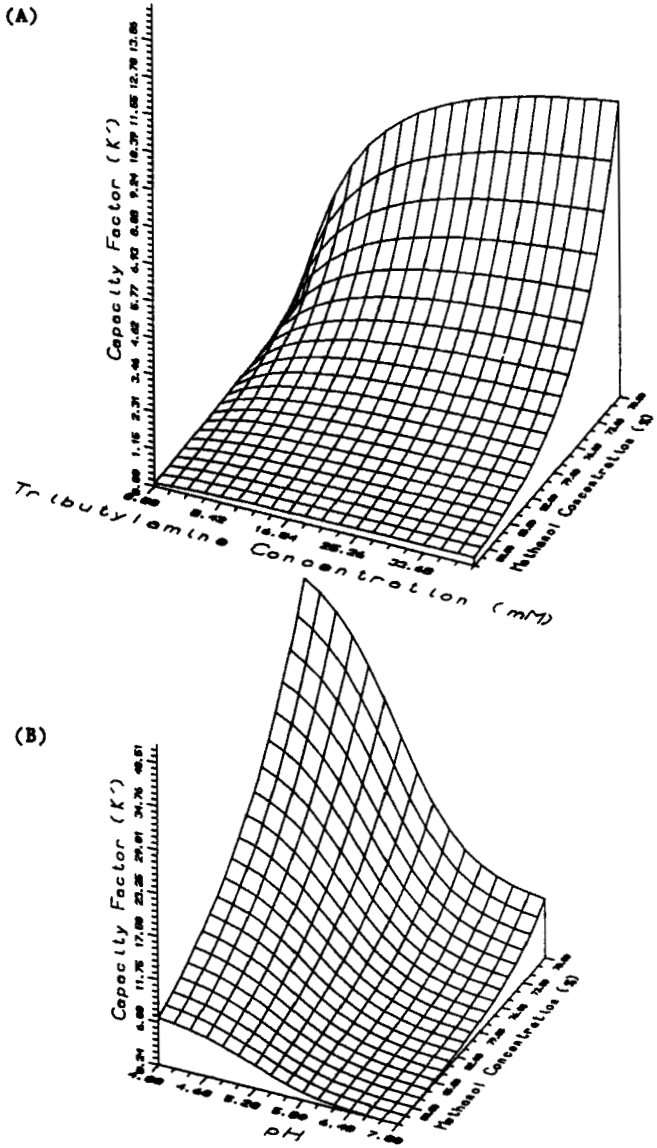

Figure 10. The relationship between capacity factor, tributylamine and methanol concentration (A) and capacity factor, pH and methanol concentration (B) calculated from the nonlinear regression function.

pH's. For both amines the change in capacity factor as a function of methanol concentration occured more slowly at low pH's. Although the K_a's appear quite different, this represents only a difference of 0.3 pKa units, although the values obtained from fitting all the data are lower than that obtained from the single parameter study.

The behavior of bilirubin retention using dihexylamine as an ion-pair reagent is shown in figure 9. The top graph shows the effect of amine and methanol and the bottom graph shows the effect of pH and methanol. The graph showing amine and methanol clearly demonstrates the interaction of the variables and appropriate combinations could be easily found to resolve mixtures of compounds showing different behavior. For example, separations involving bilirubin and a compound without a group reacting with the ion-pair reagent could be easily developed.

Similar graphs are shown in figure 10 for tributylamine. Comparison of figures 9 and 10 reveal generally similar behavior, although the graphs for tributylamine represent lower alcohol concentration.

CONCLUSIONS

The results of this study clearly indicate that either dihexylamine or tributylamine are useful as ion paired chromatographic agents for bilirubin. The concentration of the ion pair, methanol and the pH may be used to optimize any separations involving bilirubin from related material. A comparison of dihexylamine with tributylamine indicates that despite both of the molecules having twelve carbon atoms, dihexylamine seems to form a more lipophilic complex than

tributylamine. However, tributylamine does not require as much organic modifier for equivalent retention times. The differences between ion-pair reagents can be exploited in optimizing any assay procedures.

Our work also shows the shortcomings of using a factor design for the elucidation of the relation between mobile phase and capacity factor. Although this type of design is flexible it will not model all of the changes which are found in a broad range study. Factorial design and linear regression are useful in obtaining information over a limited range, and if the limitations are recognized this approach is quite useful. Nonlinear regression is more flexible and because the models do not have to be linear, the model can approach mechanistically and mathematically realistic forms. Differences between closely related agents are more easily seen and graphically accurate behavior can be easily and accurately visualized.

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