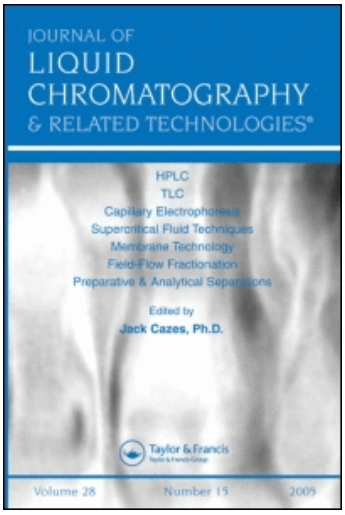

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Control of Retention Times in the Ion-Paired liquid chromatographic analysis of Bilirubin

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CONTROL OF RETENTION TIMES IN THE ION-PAIRED LIQUID CHROMATOGRAPHIC ANALYSIS OF BILIRUBIN

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ABSTRACT:

Fluctuations in bilirubin levels are biologically significant and the development of simple, reliable, and flexible chromatographic analyses are required for many research fields. Bilirubin's short retention time using reverse phase HPLC combined with the presence of acidic groups on bilirubin suggests ion-paired chromatography would produce the desired analytical procedures. Chromatography using dibutylamine as the ion-pair reagent proved to be successful and the capacity factor of bilirubin was reproducibly controlled by the concentration of dibutylamine, pH, and per cent of methanol in the mobile phase. The results, analyzed by a multilinear regression analysis, produced a mathematical model which calculated the capacity factor based on the dibutylamine concentration, methanol concentration and the hydrogen ion concentration. The analytical system was flexible, reproducible and easy to use. Manipulation of these factors should allow the facile separation of bilirubin from other similar compounds.

INTRODUCTION

Bilirubin is a bile pigment [1] formed from the breakdown of heme from hemoglobin. Hyperbilirubinemia occurs under certain pathological conditions making specific analyses of bilirubin desirable. In plasma and other biological fluids, bilirubin

exists as a mixture of free, mono, and di conjugates with various sugars [2] (beta-D-glucopyranuronic acid, beta-D-glucopyranose and beta-D xylopyranose). Because of the double bonds, bilirubin can exist in several isomeric forms. Although photo [3] and chemical [2] instability add to the complexities of the analysis of bilirubin, the biological importance of bilirubin necessitates that highly specific analytical methods are used. Development of reliable HPLC methods require a thorough exploration of the factors controlling the retention time of bilirubin.

The analysis of bilirubin by a number of chromatographic methods has recently been reviewed [4]. Many of the previously developed HPLC systems involve complex solvent gradients or unusual solvent systems. Most papers do not explicitly evaluate the relationship between mobile phase composition and bilirubin retention time or use too few points to establish meaningful relationships between the parameters.

Li, Lim and Peters [5] described the use of a system using acetonitrile, DMSO, and ammonium acetate buffer at pH 4.6 and demonstrated a pronounced effect of pH on the elution of bilirubin with the capacity factor increasing sharply below pH 5. Although the effect of buffer concentration on the elution of unconjugated bilirubin was not described, concentrations of ammonium acetate above 0.25 M increased the capacity factor of monoglucuronides of bilirubin indicating some ion-paired effect might be operating. These investigators used DMSO to increase the solubility of the compounds and acetonitrile to reduce the high back pressure produced by the DMSO. Manipulation of pH, buffer molarity, and organic solvent composition gave a flexible system, but the effect of these variations was only minimally discussed. Spivak and Yuey [6] utilized a gradient system of methanol and ammonium acetate buffered to pH 4.5 to analyze various mixtures of bilirubin and bilirubin conjugates.

A conventional ion-pair system was used by Onishi, Itoh, Kawade, Isobe, and Sugiyama [7] for the analysis of bilirubin and photochemical decomposition products utilizing a phosphate buffer pH 8 with 0.1 % (3.86 mM) tetrabutylammonium hydroxide and a linear gradient of acetonitrile from 10 to 40 %. Jansen and Tangerman [8] reported a similiar ion-paired system for the analysis of bilirubin and its conjugates. McCarthy, McClintock, and Purdy [9] utilized a mobile phase of acetonitrile, DMSO, and buffered tetrabutylammonium chloride with pH ranging from 6.7-8.5 to chromatograph bilirubin and related compounds. These authors

noted the marked dependence of elution time on organic/aqueous ratios and reported a 60/40 ratio resulted in no retention whereas a 44/56 ratio caused bilirubin to be retained indefinitely. The capacity factor of bilirubin rose sharply at "apparent" pH's below 8.5 and increased with ion-pair reagent concentrations from 0.2 to 5.8 mM. A rather unique ion-paired system [10],[11] consisting of 0.1 M di-n-octylamine acetate in methanol pH 7.6 was used as the mobile phase to separate photoisomerization products of bilirubin.

Goresky and Gordon [12] utilized a different ion paired system using solvent programing of an acetonitrile, DMSO, and aqueous system containing 1-pentanesulfonic acid at pH 4. The solvent programming system was somewhat complex. The retention time of bilirubin changed slightly in changing the ion-pairing reagent from 0.3125 mM to 12.5 mM, and changing the pH from 4.0 to 4.8 decreased the retention time of both conjugated and nonconjugated bilirubin isomers. The small change in retention time indicated that the effects are not due excusively to ion-pairing. Since bilirubin lacks groups of sufficient basicity to pair with an acid, this system would not be expected to behave as a typical ion-paired system.

The previous HPLC methods utilize either gradients or ternary solvent systems which tend to obscure simple direct relationships between retention time and mobile phase composition. This makes the development of new methods a complex and time consuming process. Previous studies often utilized too few data points to confidently define the curves relating retention time to mobile phase composition. We report the effects of pH, organic modifier, and ion-pair concentration on the capacity factor in a simple convenient HPLC system for the analysis of bilirubin. The results of a mathematical model are used to evaluate the effect of the mobile phase on the capacity factor.

MATERIALS AND METHODS

Apparatus

Liquid chromatography was performed on a system consisting of a Spectra-Physics SP8810 pump (Spectra-Physics, San Jose, CA), Rheodyne 7225 injector (Rheodyne Inc., Cotati, CA), a Waters 481 Lc spectrophotomer (Waters Assoc., Milford, MA), and a Hewlett-Packard 3396A Integrator (Hewlett-Packard Company,

Avondale, PA). The column was a Whatman Partisphere 5 C18 (110 x 4.7 mm, id) (Whatman, Inc., Clifton, NJ). The pH's were determined on a Corning model 125 pH meter (Corning Glass Works, Corning, NY)

Chemicals and Reagents

Bilirubin was obtained from Sigma Chemical and was used directly. Dibutylamine was obtained from Aldrich Chemical and was redistilled through a 10 cm column prior to use. Acetic acid was obtained from Dupont. The aqueous buffer was prepared using water passed through a Corning Mega-pure system. The concentrations of dibutylamine were calculated for the volume of solution produced on the addition of the organic component. The pH of the aqueous solution was adjusted using acetic acid prior to adding the organic modifier.

Chromatographic Procedure

The different mobile phases were prepared by varying the pH, the concentration of dibutylamine, or the per cent of methanol. The mobile phases were degassed by bubbling helium through the solution for 15 minutes. Bilirubin solutions contained 1 mg. per 100 ml. were injected using a 20 microliter sample loop on the Rheodyne injector. A flow rate of one ml. per minute was used for all runs. The effluent was monitored at 450 nm.

Data Processing

The retention times of bilirubin and the solvent front peaks were tabulated using Lotus 1,2,3 (Lotus Development Corporation, Cambridge, MA). The capacity factors were calculated and replicate runs were averaged using the spreadsheet. The averaged data were plotted using Grapher (Golden Software, Inc., Golden, CO) on a Zenith microcomputer (Zenith Data Systems, St. Joseph, MI) and a Hewlett Packard Laserjet printer (Hewlett-Packard, Boise Division, Boise, ID). The data were analyzed using the SAS statistical analysis system (SAS Institute Inc., Cary NC) on a Solbourne 5/802 computer system. First, either the capacity factor (K') or its logarithm were used as dependent variables and the methanol concentration, dibutylamine concentration, and hydrogen ion concentration, their squares, and their logarithms as independent variables were subjected to stepwise multiple linear regression analysis. From these results the best three variable equations were selected and analyzed using SAS's regression procedure with a thorough examination of all regression

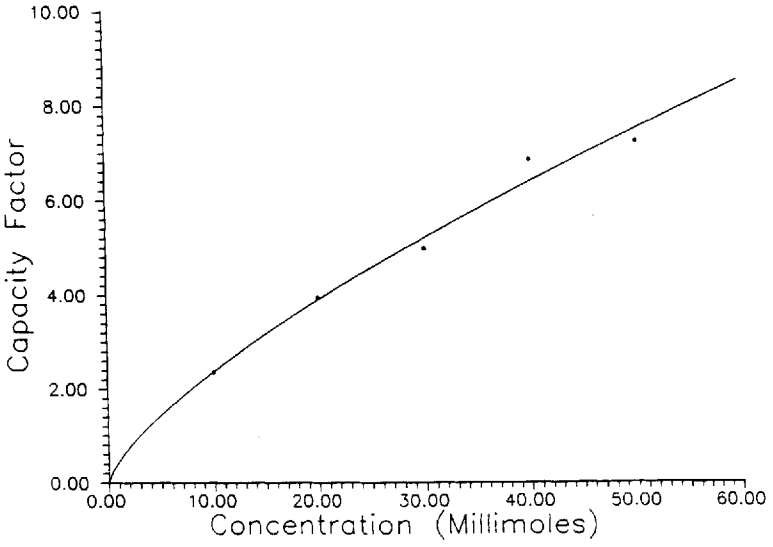

Figure 1. Effect of dibutylamine concentration on the capacity factor of bilirubin in a mobile phase containing 70% methanol and the aqueous phase at pH 7.

diagnostics for collinearity or influence. The results of the mathematical modeling were used to compute 3-D surface simulations, corrected for the contribution of the hydrogen ion concentration and the data plotted using Surfer, version 4 (Golden Software, Inc., Golden, CO).

RESULTS

Effect of Ion-Pair Reagent Concentration

The effect of dibutylamine concentration was studied by measuring retention time of bilirubin using mobile phases containing 70 % v/v methanol with a concentration of dibutylamine of 10, 20, 30, 40, and 50 mM while the pH of the aqueous solution was adjusted to 7.0. The results are shown in Figure 1 and show a nonlinear increase in capacity factor.

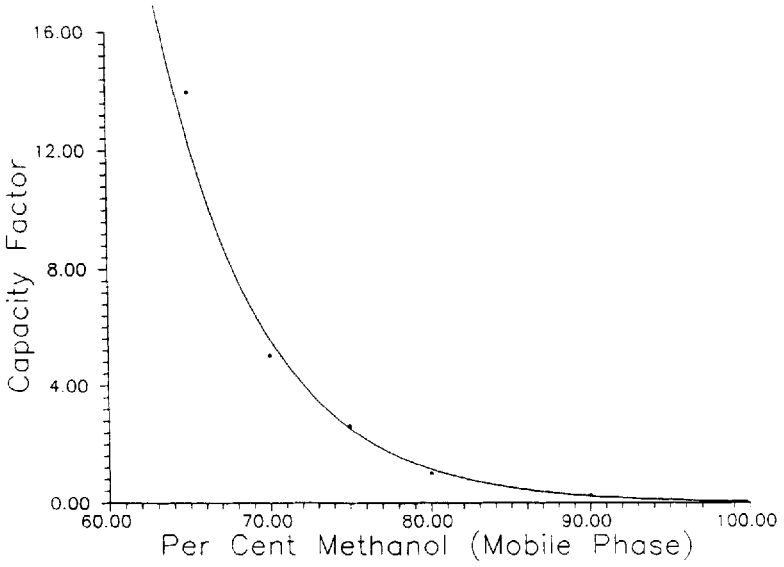

Figure 2. Effect of methanol concentration on the capacity factor of bilirubin in a mobile phase containing 30 mM dibutylamine and the aqueous phase at 7.0

Effect of Methanol Concentration

Mobile phases were prepared containing methanol concentrations of 90, 80, 75, 70, and 65 per cent with a dibutylamine concentration of 30 mM, and an aqueous pH of 7.0. Figure 2 shows the results and demonstrates the rather rapid increase in capacity factor occurring at methanol concentrations less than 70 per cent.

Effect of pH

The pH of the aqueous component was adjusted to 7.0, 6.0, 5.5, 5.0, and 4.5 by titration with acetic acid. The mobile phase was 30 mM dibutylamine and 70 % v/v methanol. The results are shown in Figure 3. A very sharp increase in the capacity factor occurs at about pH 5.

Modeling of Chromatographic Behavior

A careful statistical analysis of the data resulted in the selection of the following equation to describe the effect of all the variables on the capacity factor of bilirubin.

$$\log(K') = 10.08 + 0.432*[\log(\text{dibutylamine}) - 0.141*\text{methanol} + 42372*(H^+)]$$

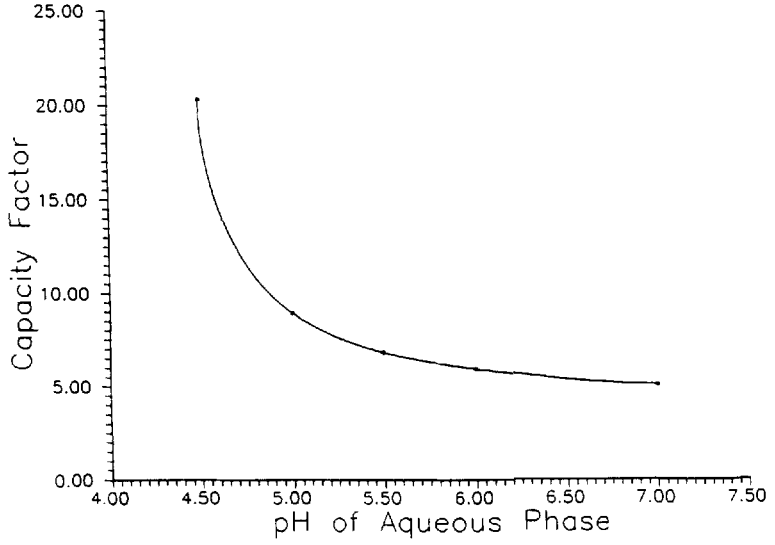

Figure 3. Effect of pH on the capacity factor of bilirubin in a mobile phase containing 30 mM dibutylamine and 70 % methanol.

The fit was satisfactory with R squared of 0.981 and a root mean square error of 0.202 with the dependent variable varying from -1.33 to 3.01. Figure 4 shows the variation in capacity factor with changes in dibutylamine, methanol, and hydrogen ion concentration. Figure 5 shows the agreement between observed capacity factors and those calculated from the above equation.

DISCUSSION

The use of dibutylamine as an ion-pairing agent has proven to produce useful predictable variations in capacity factor of bilirubin with changes in mobile phase composition. Changes in dibutylamine concentration produced moderately nonlinear changes in the retention of bilirubin. Methanol concentrations produce a very drastic increase in the capacity factor at approximately 70 % (pH 7 and 30 mM dibutylamine). This phenomenon of a change from non-retained to non-eluted over roughly a 20 per cent change in organic/aqueous ratio was previously observed by McCarthy and

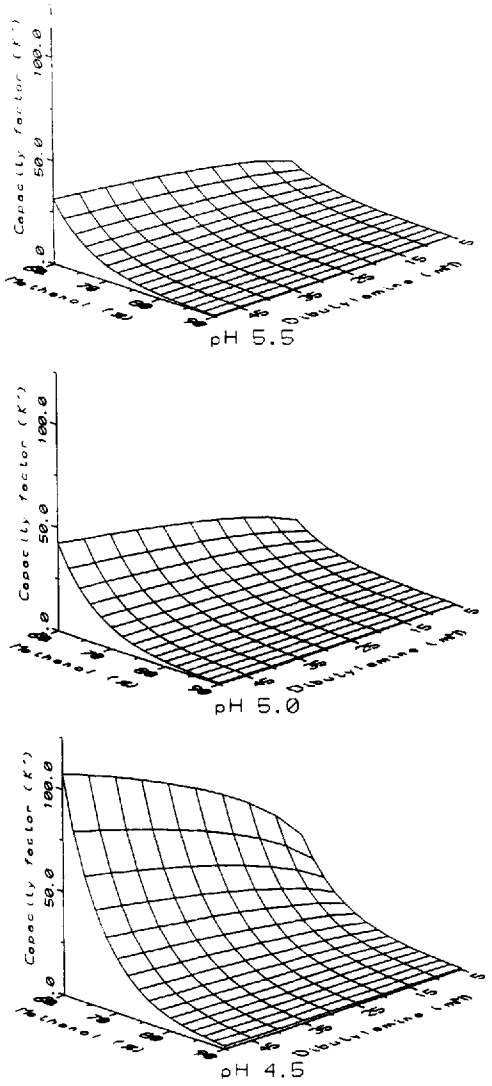

Figure 4. The variation in Capacity factor produced by changes in methanol, dibutylamine and pH predicted by the mathematical model. (see text for the equation)

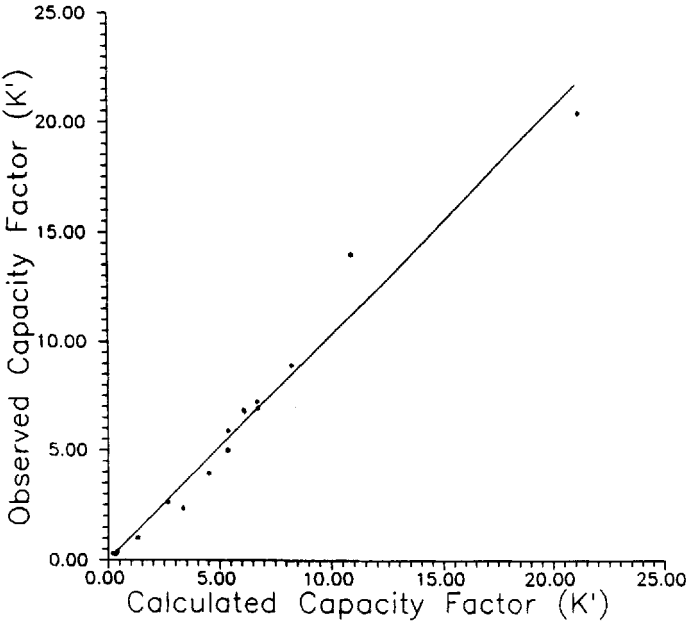

Figure 5. Comparison of Calculated and Observed Capacity Factors

coworkers. A similar phenomenon was observed in our system with very little change in retention time when the pH was changed from 7.0 to 5.0 but a pronounced increase in retention time at pH 4.5.

In plotting the curves of capacity factor versus mobile phase composition the smoothness of the data indicated that a mathematical equation might be found to model the data. Although other workers have modeled one or two variables [13], [14], very few workers have modeled all the common variables which affect retention time in a chromatographic system. Our model works relatively well although it is not based on theory and would not be expected to be adequate thoughout all the possible ranges of mobile phase. The model serves well as an empirical guide within the range of mobile phase conditions used in our experiments. The behavior of the system is clearly shown in Figure 4, where the non-linear relationship between the capacity factor and all of the mobile phase components is clearly indicated. Figure 5 shows good

agreement between the observed capacity factors and those calculated by the mathematical model.

The ion-pair system using dibutylamine as the ion-pair reagent produces an analytical system which is useful for bilirubin and in which the retention time can be controlled by modifying any one of three fundamental parameters. The rapid change of retention time at certain critical points of methanol concentration and pH need to be noted in the development of new procedures for the separation of bilirubin from related compounds.

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