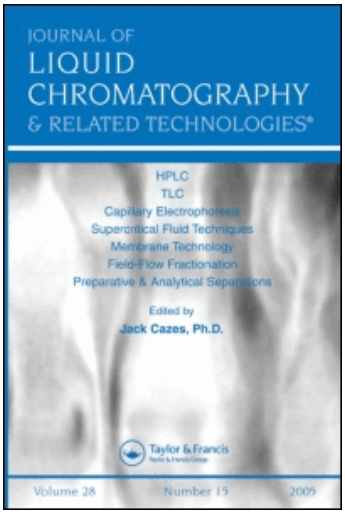

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Optimization of A Reverse Phase Ion-Pair Chromatographic Separation for Drugs of Forensic Interest Part I - Variables Effecting Capacity Factors

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OPTIMIZATION OF A REVERSE PHASE ION-PAIR CHROMATOGRAPHIC SEPARATION FOR DRUGS OF FORENSIC INTEREST PART I - VARIABLES EFFECTING CAPACITY FACTORS

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ABSTRACT

A study is presented employing approximately 50 drugs of forensic interest to determine the effect of stationary phase, water-methanol ratio, alkyl length and concentration of counter-ion and basicity of the compound chromatographed on capacity factors utilizing a reverse phase ion-pair separation. Microbondapak-C18, Microbendapak-Alkyl Phenyl and Microbondapak-CN are the columns examined. The mobile phases used contain water, methanol, acetic acid and an alkylsulfonate salt. Horvath's solvophobic theory is a useful model for explaining many of the chromatographic trends.

INTRODUCTION

Recently it was demonstrated that reverse phase ion-pair chromatography is a most versatile technique when applied to drugs of

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forensic interest[1]. This mode utilized a buffered aqueous-organic mobile phase containing a counter-ion which is available to form a lipophilic complex with the salt of a drug. This technique allows the simultaneous analysis of basic, acidic and neutral compounds. The methodology depicted by Lurie used a single isocratic system utilizing a Microbondapak-C18 column and a mobile phase consisting of 40% methanol, 59% water, 1% acetic acid and 0.005M heptanesulfonic acid at a pH of approximately 3.5. In general, by using this system, basic drugs are analyzed via ion pairing and acidic drugs by ion suppression.

This isocratic system is applicable to ergot alkaloids, phenethylamines, opium alkaloids, local anesthetics, barbiturates as well as other drugs of forensic interest. Although this technique approached the ideal situation of using a single HPLC system for a wide range of drugs of forensic interest, certain drawbacks existed. First of all the phenethylamines, amphetamine and methamphetamine were poorly resolved. In the case of opium alkaloids, heroin and acetylcodeine co-eluted. Although compounds related to cocaine were well resolved, their retention times were longer than optimum. LSD and Iso-LSD (a common component in LSD exhibits) had retention times that were fifteen and eighteen minutes respectively. PCP had a retention time of approximately seventeen minutes. Therefore, it was desired to optimize these and other various separations for resolution and speed. When dealing with semi-preparative reverse phase ion-pair chromatography, conditions must be optimized in order to obtain satisfactory resolution of the analytical separation[2].

With this goal in mind, a study was undertaken to determine the effect of column type, water-methanol ratio, counter-ion size, counter-ion concentration and basicity of drugs chromatographed on a reverse phase ion-pair chromatographic separation for drugs of forensic interest. This paper will discuss the effect of the above variables on the capacity factor for the various compounds chromatographed. The drugs include barbiturates, local anesthetics, phenethylamines, opium alkaloids, ergot alkaloids and other drugs of forensic interest. A subsequent paper will discuss the effect of the above parameters on selectivity factors[3].

EXPERIMENTAL

The liquid chromatograph consisted of the following components: Model 6000A pump (Waters Associates, Milford, MA); Model U6K injector (Waters); prepacked 3.9 mm x 30 cm stainless steel columns: Microbondapak-C18, Microbondapak-Alkyl Phenyl and Microbondapak-Cyanide (Waters); Model 770 variable UV detector at 254 nm (Schoeffel Instruments, Westwood, NJ) or Model 440 fixed UV detector at 254 nm (Waters); Systems IVB integrator (Spectra Physics, Santa Clara, CA).

Materials

The following chemicals were used: methanesulfonic acid, butanesulfonic acid sodium salt, heptanesulfonic acid sodium salt (Eastman Chemicals, Rochester, NY); methanol, distilled in glass (Burdick and Jackson, Muskegon, MI); distilled water and other chemicals were reagent grade. Authentic drug standards of USP/NF quality were employed.

Procedures

The capacity factor k' was calculated according to the formula $k' = \frac{(tRi - tRo)}{tRo}$ where tRi is the retention time of component i and tRo is the retention time of a non-retarded component which in this instance was approximated to be methanol. The selectivity factor (α) is obtained from the formula $\alpha = k'j/k'i$, where k'j and k'i are the capacity factors of the jth and ith sample component.

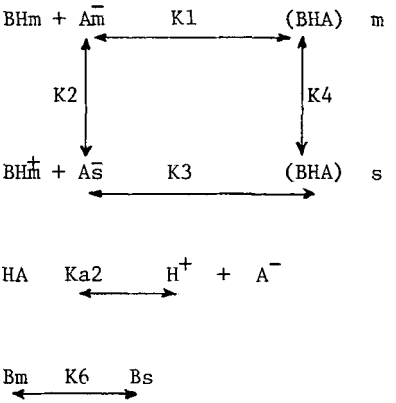
All mobile phases were prepared by dissolving an alkylsulfonic acid or alkylsulfonic acid salt in a solution consisting of glacial acetic acid, methanol and distilled water. After filtering and degassing the solution through a Millipore 0.50 micron filter (Millipore Corporation, Bedford, MA), the pH was adjusted to 3.5 with 2N NaOH.

All standards were dissolved in methanol. For the determination of capacity factors, co-injections consisting of 5 ml of drugs of interest and of methamphetamine (meth) were used. The k' values were then determined based on the above mentioned selectivity factor and the k' of methamphetamine in a given mobile phase based on the relationship $k'j = \alpha k'meth$ or $k'j = k'meth/\alpha$. The concentrations of these drugs were 0.5 mg/ml except for LSD, LAMPA and Iso-LSD.

THEORETICAL

The separations that take place can be described by the following equations:

$$BH^+ \xleftarrow{\text{Kal}} B + H^+$$



B and HA represent basic and acidic drugs respectively while A^- refers to a negative counter ion. The subscript m depicts mobile phase while s refers to the stationary phase. The mechanism of ion pairing is very much in dispute. The ion pair mechanism has been shown by Horvath et. al.[4] to proceed by equation Kal, Kl and K4 which represent ion pair formation in the mobile phase followed by adsorption of the ion pair on the stationary phase. Kissinger[5] and Scott and Kucera[6] believe the mechanism proceeds via equation Kal, K2 and K3 which depicts the counter ion being adsorbed onto the stationary phase and ion pairing occuring by an ion exchange mechanism.

At a low pH equation Ka2 is shifted to the left which would favor formation of the free acid. This situation represents ion suppression. Free base, if present in the mobile phase, can be adsorbed onto the stationary phase as represented by equation K6.

RESULTS AND DISCUSSION

Approximately 50 drugs of forensic interest were chromatographed using three different stationary phases, namely a Microbondapak-C18,

a Microbondapak-Alkyl Phenyl and a Microbondapak-Cyanide. For each column, mobile phases consisting of water, methanol, 1% acetic acid and a 0.005M alkylsulfonate counter ion at pH 3.5 were employed. For methanol concentrations of 40% and 30%, the counter ion was varied from heptanesulfonate to methanesulfonate in increments of three carbons. For 20% methanol only methanesulfonate was used because of excessive retention of many bases with butanesulfonate or heptanesulfonate counter ion. Retention data for the various drugs is presented elsewhere[3].

Effect of Counter Ion Size on k'

The effect of varying the counter ion size from heptanesulfonate to methanesulfonate on the various classes of drugs is described below. For any given column and water-methanol ratio, the k' of barbiturates was independent of counter ion. This was expected since barbiturates are weak acids with pka values greater than 7[7]. This means these compounds would exist as the free acid at pH 3.5 and wouldn't be expected to ion pair.

For most bases studied at a constant water-methanol ratio, the k' increased with increasing size of counter ion with both the C18 and alkylphenyl columns. This effect has been well documented[4, 8]. Also, for most of the bases studied, the ratio of k's for any given set of counter ions is fairly constant. This relationship is demonstrated in Figure 1 & 2 by the constant slopes of the curves between any two data points. Since most of these compounds have the same charge, their ratios are independent of elute surface area. Quinine and quinidine in the majority of cases have ratios of k' values that

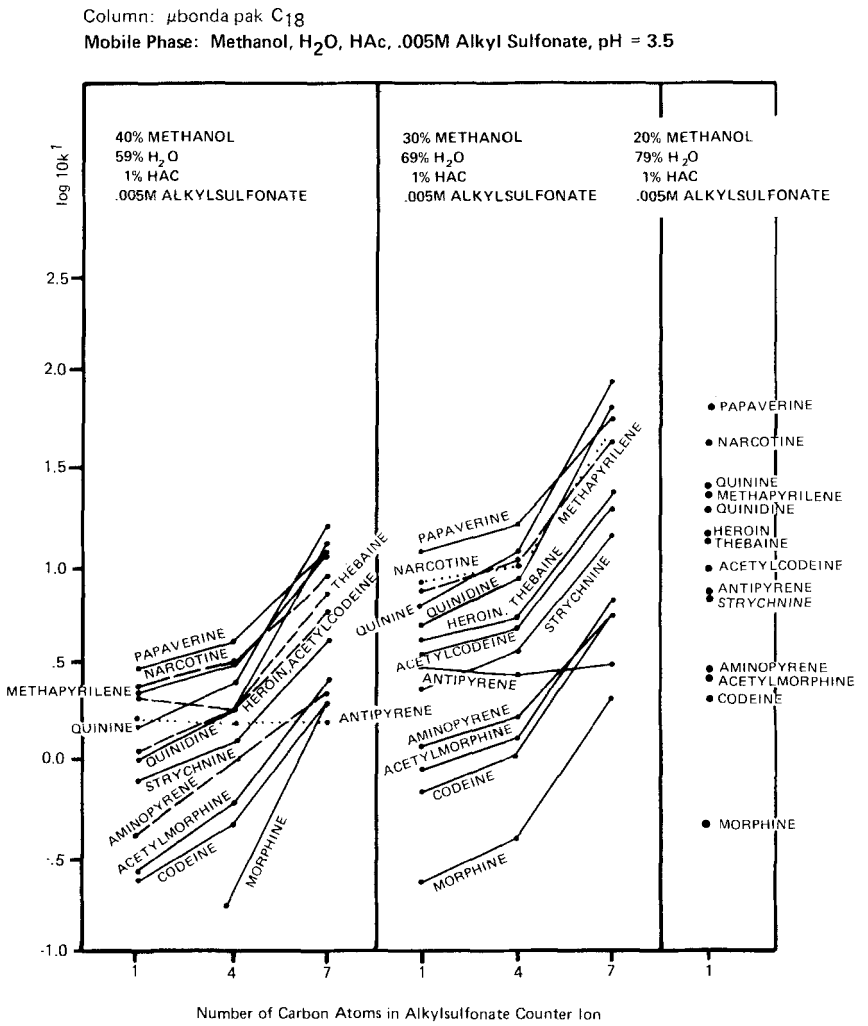


Figure 1 - Plot of log k' of opium alkaloids and related compounds versus number of carbon atoms in alkylsulfonate counter ion utilizing various methanol, water, 1% acetic acid, 0.005M alkylsulfonate mobile phases at pH 3.5; Column Microbondapak C-18.

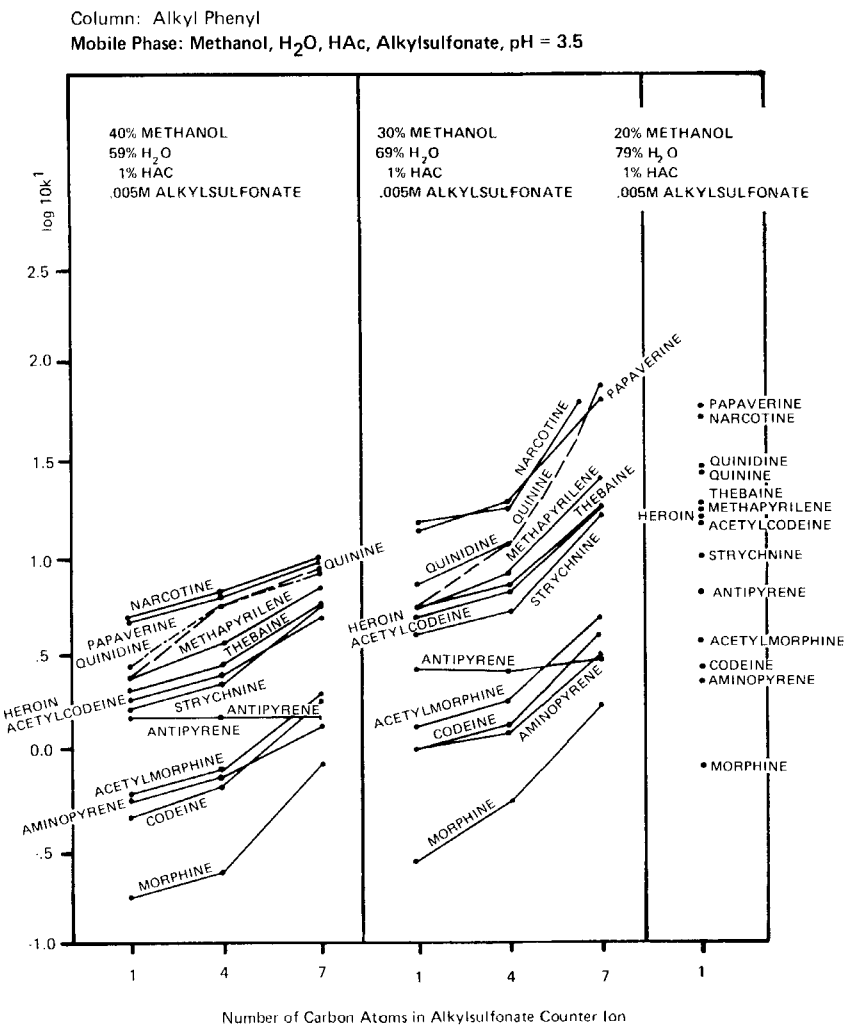


Figure 2 - Same as Figure 1 except column is a Microbondapak Alkyl Phenyl.

are approximately double that of the other bases. This is to be expected since both compounds have two basic pka values[7] representing two ionizable sites. We cannot explain why morphine and aminopyrene in certain instances exhibit anomalous behavior in relationship to their variation of k's with counter ion.

Tomlinson et. al.[9], who studied ion pairing between anionic solutes and alkylbenzyldimethylammonium chlorides, observed that a divalent solute exhibits twice the change in retention with increased size of counter ion than a monovalent solute. Tomlinson further points out that a 2:1 stoichiometric interaction between the counter ion and anionic solute would be sterochemically unfavorable if ion pairing occured by an ion exchange mechanism. Horvath et. al.[4] showed for cationic solutes with anionic counterions such as alkylsulfates and alkylsulfonates that the increase in retention with size of counterion is independent of the size of the solute but depends on its charge. Horvath's conclusions were based on working at counter ion concentrations where the solute's retention would be at a maximum. In our study, 0.005M does not represent for methanesulfonate, butanesulfonate and heptanesulfonate in most cases a counter ion concentration for which solute retention is at a maximum.

In general, higher variations of k' with counter ion were observed on the C18 column than the alkylphenyl column as is shown in Figure 1 & 2. A possible explanation for this effect can be derived from Horvath's et. al. solvophobic theory for retention in reverse phase ion pair chromatography[4]. According to this theory, if we assume ion pairing occurs in the mobile phase followed by adsorption

of the ion pair onto the stationary phase, the equilibrium constant for the binding of the neutral ion pair is expressed as $\ln K = a - b + c \text{ delta } A$ where a, b and c are constants depending on solvent and column properties and delta A is the contact area which is the difference between the molecular surface area of the ion pair stationary phase complex and the surface areas of the stationary phase ligand and the ion pair. This contact area is proportional to the molecular surface area of the complex formed between the ion pair and hydrocarbonous ligand. Similarly, the C18 column which consists of 18 carbon bonded groups has more hydrocarbon character than the Alkyl Phenyl column which consists of ethylbenzene groups and thus a greater contact area with the ion pair. Thus the greater increase in retention with size of counter ion on the C18 column would be expected.

In addition, the variation of k' with counter ion on the C18 and alkyl phenyl columns appear to be fairly independent of water-methanol ratio (Figures 1-2). On both columns, k' increases exponentially with the carbon number of the counter ion. Although antipyrene, benzocaine, caffeine, diazapam, mecloqualone, methaqualone and theophylline all have basic functional groups, they exhibit no significant variation of k' with counter ion size as is illustrated in Figure 1 for antipyrene. For antipyrene, benzocaine, caffeine and methaqualone who have basic pka values of 1.4, 2.8, 0.6 and 2.5 respectively[7], no appreciable protonation of these bases would be expected at the mobile phase pH of 3.5. Equation Kal shows that this protonation would be required for ion pair formation Diazapam has a basic pka value of 3.4 while theophylline has a basic pka of

3.5 and an acid pka of 8.6[7]. At a pH of 3.5, some protonation of the basic functional group would be expected. The unionized compound, of which a significant amount would exist at pH 3.5 could be adsorbed by the stationary phase. This chromatographic process could be represented by the equation K5. The non-variation of k' with counter ion for diazapam and theophylline could be explained by domination of process K5 over K1 and K4 which represent ion pairing. Although we could not ascertain for sure, it is probable that these pKa values were determined in water. Pka values for bases tend to be lower when alcohol greater than 20% is present in the mobile phases[9]. A lower pKa value would mean that less ionized base would be present and could account for the above behavior of diazapam and theophylline. No pKa data was available for mecloqualone. Glutethimide, which is a weak acid with a pka of 4.52[7], as expected, exhibits no appreciable variation of k' with counter ion size.

No significant variation of k' with counter ion size was observed for any drugs on the cyanide column as is shown in Figure 3. This is probably related to the small aliphatic character of this column. As stated earlier, retention is proportional to the molecular surface area of the complex formed between the ion pair and the hydrocarbonous stationary phase.

Effect of Stationary Phase on k'

For any mobile phase the retention order of barbiturates on a given stationary phase was C18 ≈ alkylphenyl > cyanide (Figure 4). This is consistent with the work of Scott and Kucera[6] and Hennion et. al.[10] who show that a constant surface coverage of the parent

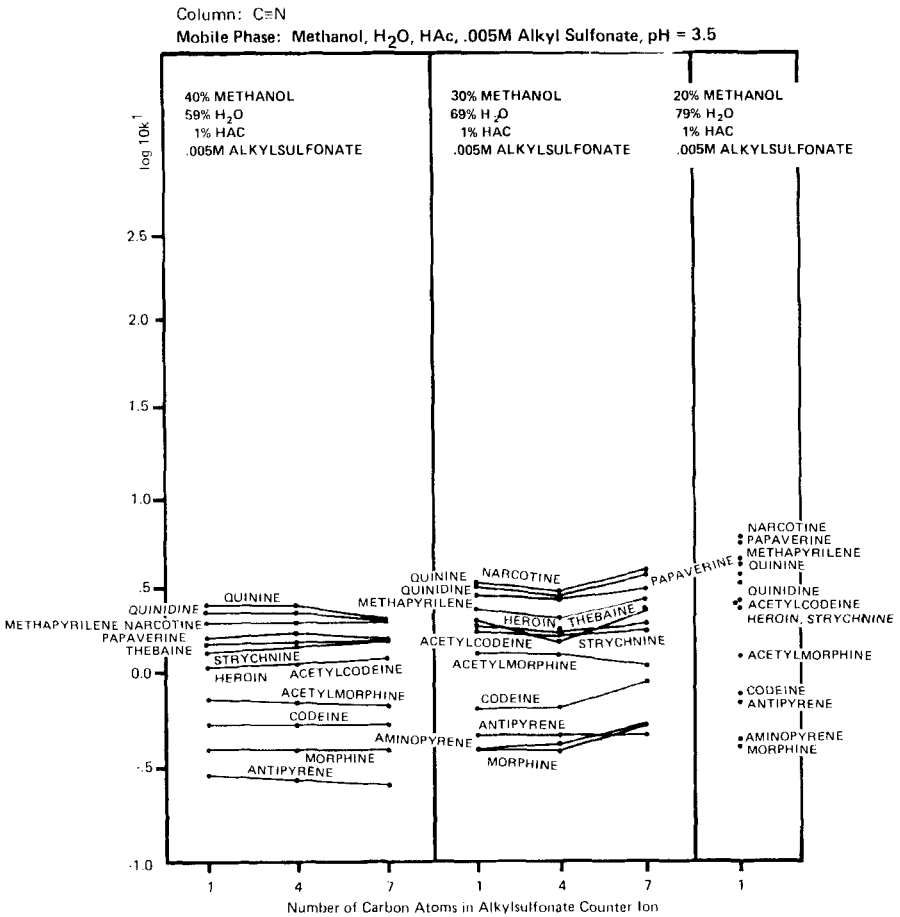


Figure 3 - Same as Figure 1 except column is Microbondapak CN.

silica increases retention with the carbon chain length of the bonded group. In general, the Microbondapak-C18, Microbondapak-Alkyl Phenyl and Microbondapak-Cyanide have constant surface coverage[11]. The carbon chain lengths of the C-18, alkylphenyl and cyanide columns are

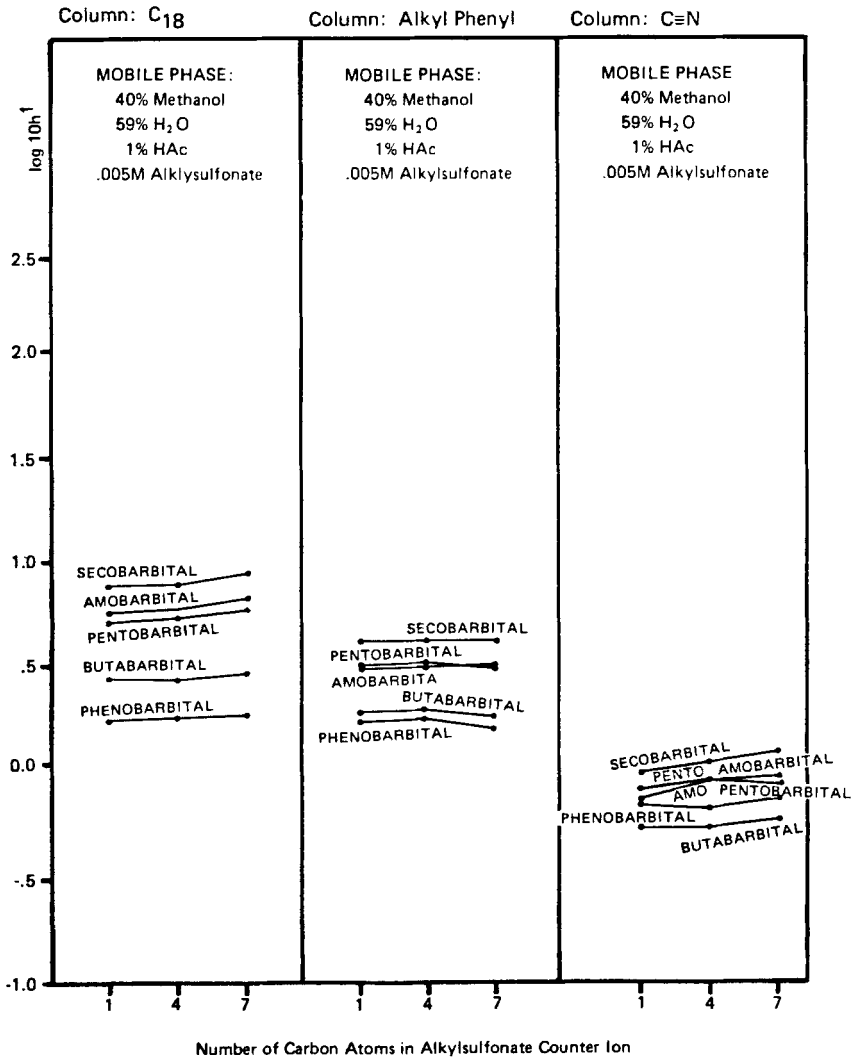


Figure 4 - Plot of log k' of barbiturates versus number of carbon atoms in alkylsulfonate counter ion utilizing mobile phase of 40% methanol, 59% water, 1% acetic acid, 0.005M alkylsulfonate at pH 3.5.

18, 8 and 4 respectively. The other workers were refering to linear chains. The alkylphenyl column contains a benzene ring with an attached ethyl group bonded to the silica while the cyanide column contains a cyanide group attached to propyl group. Phenobarbital, which is the only barbiturate studied to contain a benzene ring, has the greatest retention on the alkylphenyl column relative to the C-18 column. Both dipole[12] and pi orbital interactions[13] have been hypothesized to occur between the alkylphenyl column and solutes. This could explain the behavior of phenobarbital. Retention for phenobarbital on the cyanide column is greatest relative to its retention on the C-18 and alkylphenyl columns. This effect could also be attributed to dipole and pi orbital interactions.

In comparing retention of basic drugs, whose k' increases with counter ion size, some interesting trends were observed. When heptanesulfonate was used as a counter ion, an appreciable variation of k' with stationary phase was observed. The order of retention was C-18 > alkyl phenyl > cyanide. Archari and Jacob[14] obtained a similar result with a mobile phase consisting of 49% methanol, 50% water, 1% acetic acid and 0.005M heptanesulfonic acid at a pH of 4.0 in a study involving seventeen bases, seven of which are studied in this report. However, when butanesulfonate or methanesulfonate were used as counter ions a much smaller variation of k' with stationary phase was observed. In many instances retention was actually greater on the cyanide or alkylphenyl column than on the C-18 column. Horvath et. al.[4] has shown that when assuming ion pairing in the mobile phase mechanism, retention is proportional to the difference in the molecular

surface area of a complex formed between the non-polar hydrocarbonous ligand of the stationary phase and the non-polar moiety of the ion pair. This interaction is hydrophobic in nature, meaning it is based on repulsion between the ion pair and ligand with water causing the ion pair and ligand to associate. Thus a smaller hydrocarbonous ligand on a stationary phase would retain an ion pair less. Based on the size of their hydrocarbonous ligands, retention of the ion pairs on the three columns studied would be expected to be C-18 > alkylphenyl > cyanide with an appreciable difference of magnitude. It appears that when using a larger counter ion the above relationship was observed for the bases studied. The lesser variation of k' with stationary phase that was observed for bases when smaller counter ions were used could be explained by the decreased hydrophobic interactions between the ion pair and the ligand. These smaller interactions could favor the dipole and pi orbital interactions that occur on the cyanide and alkylphenyl columns. All of the bases whose retention times vary with counter ion have one or more benzene rings or multiple sites of unsaturation.

Effect of Water-Methanol Ratio on Retention

Increasing the ratio of water to methanol increases retention on all three columns for all the barbiturates studied. This is typical of retention in reversed phase systems where chromatography is based on hydrophobic interactions[15], [16]. For all three columns studied, the increase in retention with the water-methanol ratio was linear. A similar result was reported by Tjaden et. al.[17] for a methyl silica column. This increase is independent of barbiturate and column

type for the C-18 and Alkyl Phenyl column. This result is consistent with the findings of Karch et. al[18] using alcohols and phenols on C-18 and C-4 straight chain bonded columns. The increase in retention with water was independent of barbiturate on the CN column.

On the C-18 and alkylphenyl column all bases exhibited an increase in k' with an increase in water-methanol ratio as is illustrated in Figures 1 & 2 for opium alkaloids. Achari and Jacob[14] reported similar results using a Microbondapak C-18 column. According to Horvath et. al.[4], adsorption of the ion pair onto the stationary phase increases with increased surface tension of the mobile phase. The surface tension of a mobile phase increases by adding water.

The increase in retention with increased water concentration for all compounds studied was considerably less on the cyanide column. In certain instances there was no increase in retention. This result is consistent with Horvath et. al.'s[15] theory of hydrophobic interactions which depends on a complex being formed between a hydrocarbonous ligand and the non-polar substituents on an eluite. This interaction is proportional to the contact area between the ligand of the stationary phase and the solute. Since the cyanide column has a small hydrocarbonous ligand, the hydrophobic effect would be expected to be smaller than on the C-18 and alkylphenyl columns.

Effect of Counter Ion Concentration on Retention

Using eleven drugs of the original fifty, a limited study of the effect of varying counter ion concentration on retention was conducted. On both the C-18 and Alkyl Phenyl columns, the counter ion concentrations of 0.005M and 0.02M methanesulfonate, butanesulfo-

nate and heptanesulfonate were employed. The drugs included butabarbital, methamphetamine, procaine, lidocaine, cocaine, ephedrine, codeine, heroin, quinidine, LSD, and PCP. On the C-18 column the k' for the various solutes did not vary with the change in methanesulfonate concentration. The k's of methamphetamine, lidocaine, and ephedrine increased by approximately 10% with a four fold increase of butanesulfonate concentration while the retention of the other solutes did not change. All basic solutes, except quinidine, increased by a factor of approximately 1.5 with an increase in heptanesulfonate concentration from 0.005M to 0.02M. The k' of quinidine increased by a factor of approximately 2.2. Butabarbital, since it does not ion pair, exhibited no change of k' with counter ion concentration.

On the Alkyl Phenyl column, the k' of the various solutes (except for butabarbital) increased approximately 12% with increased concentration of methanesulfonate. The k' of most solutes increased by approximately 1.45 with a four fold increase of butanesulfonate or heptanesulfonate concentration. For quinidine and PCP k' increased by a factor of approximately 1.15 with an increase in butanesulfonate concentration. When quadroupling the heptanesulfonate concentration, the k' of quinidine increased by a factor of 1.7. Butabarbital did not vary with butanesulfonate or heptanesulfonate concentration. Both parabolic and hyperbolic relationships have been observed for the increase of k' with counter ion concentration for bases[6]. According to the solutes studied by Horvath et. al.[6] on the Microbondapak-C-18 column, 0.02M butanesulfonate and heptanesulfonate is near the concentration that k' does not vary with counter ion concentration. The

greater increase in k' for quinidine with heptanesulfonate concentration is consistent with having two ionizable sites available for ion pairing. Why this effect for quinidine was not observed for butanesulfonate is not apparent at this time.

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