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MODIFICATION OF REVERSED-PHASE SEPARATIONS OF SMALL MOLECULES USING NON-IONIC SURFACTANTS AND MIXED IONIC–NON-IONIC SURFACTANTS

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SUMMARY

The effects of non-ionic surfactants, Tween 20 and Tween 60, on reversed-phase separations of small molecules have been examined. Tween compounds were found to partition irreversibly into the ODS material used, markedly decreasing capacity factors for the compounds tested. Compounds which could hydrogen bond were less affected. Ion pairing using either anionic or cationic surfactants was possible in the presence of the non-ionic surfactants. While reversed-phase effects predominate under these conditions, secondary effects on retention order were observed and attributed to hydrogen bonding. Primary amines were retained longer than the corresponding secondary amine while catechols were retained longer than the corresponding methoxyphenols.

INTRODUCTION

The use of surfactants to modify reversed-phase separations of polar molecules is widespread[1–5]. This is generally accomplished by use of alkyl sulfates–sulfonates in the mobile phase to retain cationic species[2,7] and the use of a variety of tertiary and quarternary amines to retain anions[2,8]. The mechanisms involved are both ion-exchange and ion-pair reversed-phase[2,9]. As a result, separations obtained by ion-pair reversed-phase are generally quite similar to those obtained by older ion-exchange separations although the resolution and efficiency are generally much better with reversed-phase. This is particularly true for catecholamines, for example[3,7,10] where the order of elution in ion-pair reversed-phase or ion exchange is norepinephrine, epinephrine, dopamine, *n*-methyl dopamine. Similar observations can be made for acidic metabolites of these amines where O-methylated compounds, being less polar, always elute later than their dihydroxy precursors when ion suppression is used[2,10].

Non-ionic surfactants have found little utility in reversed-phase high-performance liquid chromatography (HPLC) separations. Chang[11] used a non-ionic surfactant with a diphenyl reversed-phase material to modify separation of proteins. In the present work, non-ionic surfactants were used in the hope of introducing some hy-

drophilic character to the stationary phase. This might be used to provide additional selectivity for separations of biogenic amines and/or acid metabolites.

EXPERIMENTAL

HPLC columns were 1.0–1.2 mm I.D., 5 or 10 cm in length, packed with 3-μm ODS material (Shandon Hypersil). Columns were packed as previously described[12] at a packing pressure of 10 000 p.s.i. for 45 min.

Non-ionic surfactants, polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monostearate (Tween 60) and Nonidet P-40 were obtained from Sigma (St. Louis, MO, U.S.A.), as were sodium dodecylsulfate and tetradecyltrimethylammonium bromide. Biogenic amines and metabolites were obtained from Sigma. All solvents were reagent grade. Water was distilled and deionized.

Detection was accomplished amperometrically at a glassy carbon electrode using an EC 230 amperometric LC detector (IBM Instruments, Danbury, CT, U.S.A.). Column dead volume was determined by using the first observed detector response following injection of sample. Since an amperometric detector was used, elution of ions caused a change in resistance and thus a detectable change in background current.

RESULTS AND DISCUSSION

Both Tween 20 and Tween 60 were used. Fig. 1 demonstrates the effect of Tween 20, 400 mg/l, on the retention of three organic acids, homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindoleacetic acid (5-HIAA). Equilibrium is reached in approximately 2 h and HVA is affected to a greater extent than 5-HIAA or DOPAC. The observed decrease in k' is irreversible for either Tween 20 or Tween 60. Use of larger quantities of either in the solvent has no additional effect. The effect of either surfactant was very similar.

Fig. 2 demonstrates the separation of a variety of indole compounds using Tween 20 to modify retention. This separation can be compared to previously published reversed-phase separations of these compounds[10]. Both the order of elution and relative capacity factors are altered, with compounds containing side chain hydroxy or carboxylic acid groups being affected to a lesser extent. The observed effect on melatonin is roughly equivalent to 20–30% methanol under normal reversed-phase conditions.

As the surfactant "loaded" column is irreversibly loaded, it might appear that this approach might find little utility. However, a number of other effects may be superimposed on the non-ionic surfactant effect.

Fig. 3 demonstrates the effect of addition of a cationic surfactant to the mobile phase. A variety of polar organic acids may be resolved under these conditions. Again, the order of elution is altered relative to reversed-phase separation of these compounds by ion suppression[2,10], with less polar O-methoxy analogues eluting before the dihydroxy analogues. The order is, however, similar to ion-pair reversed-phase separation of these same compounds using the same ion-pair agent, tetradecyltrimethylammonium bromide[13]. Effective ion-pairing requires higher concentration of the ionic surfactant, 150 mg/l when used with "Tween" loading, than with organic modifier, 50 mg/l.

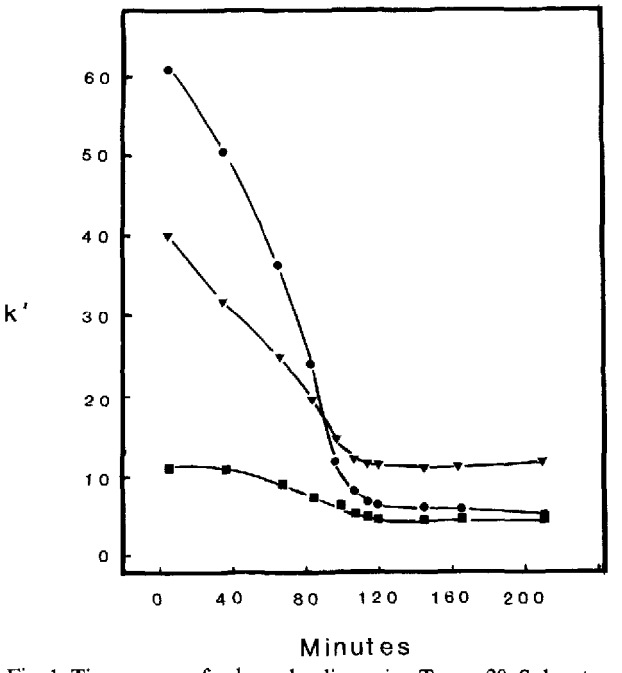

Fig. 1. Time course of column loading using Tween 20. Solvent was 0.1 *M* NaH_2PO_4 and 400 mg/l Tween 20, pH 4.5. The column was 10 cm × 1.0 mm I.D. packed with 3 μm ODS Hypersil. Flow-rate was 200 μl/min. ● = homovanillic acid, ■ = 3,4 dihydroxyphenylacetic acid, ▲ = 5-hydroxyindoleacetic acid. Detection was amperometric at a potential of 0.80 V *vs.* Ag/AgCl at a glassy carbon electrode.

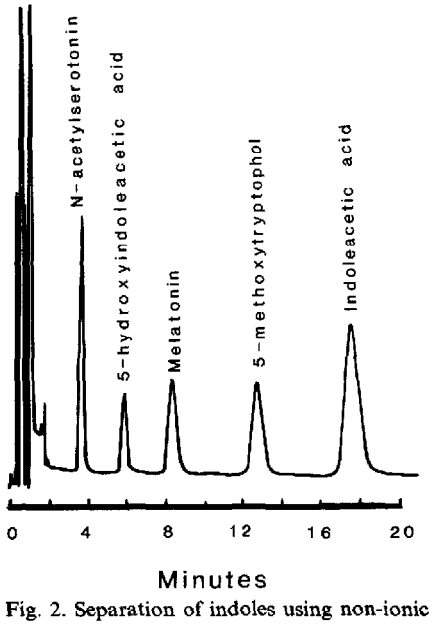

Fig. 2. Separation of indoles using non-ionic surfactant modified reversed-phase. Solvent and detection conditions are the same as for Fig. 1. The column was 10 cm × 1.0 mm I.D. packed with 3 μm ODS Hypersil. Flow-rate was maintained at 200 μl/min.

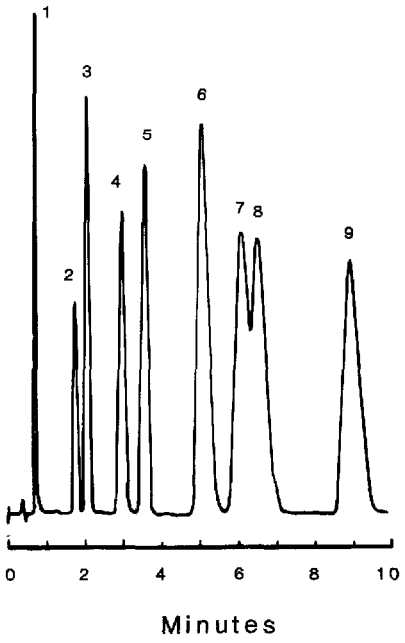

Fig. 3. Separation of phenolic acids using ion-pairing on a non-ionic surfactant modified reversed-phase column. Solvent was 150 mg/l tetradecyltrimethylammonium bromide, 400 mg/l Tween 20 and 0.1 *M* NaH_2PO_4, pH 4.5. Detector conditions were the same as in Fig. 1. The column was 10 cm × 1.0 mm I.D. packed with 3 μm ODS Hypersil. Flow-rate was maintained at 200 μl/min. Peak identities: 1 = ascorbic acid, 2 = vanillomandelic acid, 3 = 3,4-dihydroxymandelic acid, 4 = homovanillic acid, 5 = 3,4 dihydroxyphenylacetic acid, 6 = vanillic acid, 7 = 3,4-dihydroxybenzoic acid, 8 = 3,4 dihydroxycinnamic acid, 9 = 5-hydroxyindoleacetic acid.

Fig. 4A demonstrates the effect of addition of an anionic surfactant to a mobile phase containing Tween. Catecholamines are resolved under these conditions. As before, the order of elution is altered when compared to normal ion-pair reversed-phase separation of these compounds[7,10] or to cation-exchange separation of these compounds[14]. The order of elution using ion-pair reversed-phase or cation exchange is norepinephrine, epinephrine, dopamine, N-methyldopamine and α-methyldopamine. Fig. 4A shows that N-methylated catecholamines, epinephrine and N-methyldopamine, elute before their primary amine analogues, norepinephrine and dopamine, respectively. The advantages of this effect may be seen in Fig. 4B where an alumina extract of urinary catecholamines is shown. The use of the non-ionic surfactant causes rapid elution of most polar phenolic compounds which might interfere, yielding a relatively clean chromatogram with no late eluting peaks.

Non-ionic surfactants have found little utility in chromatography. In an earlier work, it was demonstrated that Tween compounds partition irreversibly into a diphenyl bonded phase and cause marked decreases in capacity factor for a series of proteins[11]. The elution order was related to log molecular weights and the column performance was similar to a reversed-phase column of low carbon number, C_1–C_3. No real advantage over conventional separations was observed.

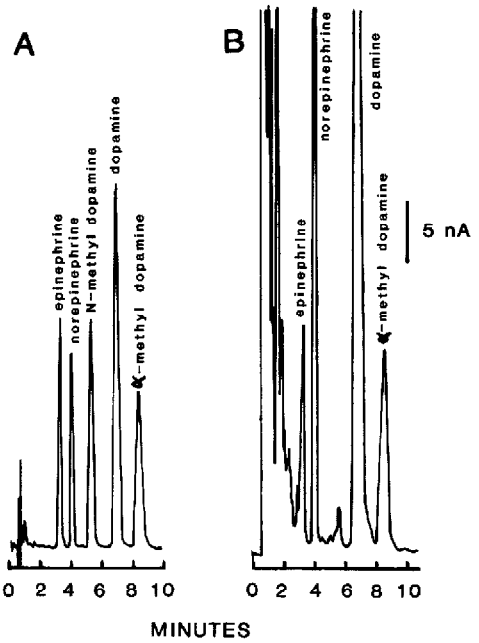

Fig. 4. (A) Separation of catecholamines by ion-pairing/non-ionic surfactant modified reversed phase. Solvent 0.1 *M* NaH_2PO_4, 400 mg/l sodium dodecylsulfate, 400 mg/l Tween 20. The column was 10 cm × 1.0 mm I.D. packed with 3 μm ODS Hypersil. Amperometric detection at +0.60 V *vs.* Ag/AgCl at a glassy carbon electrode. Flow-rate was 200 μl/min. (B) Separation of urinary catecholamines following alumina extraction. Conditions as in A. α-Methyldopamine was used as an internal standard.

In the present work, it is demonstrated that non-ionic surfactants can impart some novel properties to an ODS stationary phase. As in the earlier reported use of these surfactants, they appear to bind irreversibly to the stationary phase and are not removed by washing with acetonitrile–water (50:50) for up to 2 h. Separations appear to be governed by both reversed-phase (polarity) and hydrogen bonding interactions. For small molecules this leads to changes in the order of elution when compared to reversed-phase separations. This is demonstrated by the separation of a series of indole compounds (Fig. 2) whose structures vary by either phenol *vs.* methoxy or hydrogen in the 5 position or N-acetyl side chain *vs.* acetic acid or ethanol side chain. It is notable that side chain substituent has the greatest effect in altering relative retention. N-Acetylserotonin elutes before melatonin as is the case for the conventional reversed-phase separation. 5-Hydroxyindoleacetic acid elutes well before indoleacetic acid as is also the case with conventional reversed-phase separation[10] at this pH, ≈4.5. Unlike conventional reversed-phase, however, N-acetylated compounds, which are less polar, elute before their acetic acid or alcohol analogues. This would appear to be a hydrogen bonding effect. It is notable that this effect is more marked for indoleacetic acid analogues than phenylacetic acid analogues (see Fig. 1). Whether this is due to a difference in pK_a for the acid or the presence of the protonated ring nitrogen is at present unknown.

While the effect of the non-ionic surfactant in decreasing k' and introducing

some hydrophilic character to the stationary phase offers some possibilities for novel separations, polar amines and phenolic acids are eluted in or very near the solvent front. As a result, columns treated with these surfactants might have limited utility. Superimposing ion-pairing with ionic surfactants offers the possibility of resolving these polar amines and acids. This was accomplished using long chain alkyl ammonium and alkyl sulfates. The longer side chain has been shown to lead to higher loading of reversed-phase columns with the ion-pairing agent[2]. For this reason, C_{12} and C_{14} compounds were used. For separation of amines, sodium dodecyl sulfate (SDS) was used. The amount of SDS required to effect separation was much greater than necessary using reversed-phase with methanol as the organic modifier. The separation achieved, however, is in some respects preferable to the conventional ion-pair separation of these compounds. First, k' values for all the catecholamines are relatively similar, but even the earliest eluting is well removed from solvent. Under conventional ion-pair reversed-phase conditions, this would not be the case as dopamine and α-methyldopamine would have very large k' relative to epinephrine and norepinephrine[7,10]. As was the case without ion-pairing, hydrogen bonding effects appear to be involved in the separation of amines. N-Methylated secondary amines, epinephrine and N-methyldopamine elute before their primary amine analogues. In addition to this secondary effect, the separation is generally governed by polarity as the least polar primary amines have the greatest k', norepinephrine < dihydroxybenzylamine < dopamine < α-methyldopamine. This also appears to be the case for secondary amines with k' for epinephrine less than for epinine (N-methyldopamine).

Ion-pairing with a cationic surfactant can also be superimposed upon the effects of the non-ionic surfactant. Phenolic acids which elute in or near the void can be resolved using tetradecyltrimethylammonium as the ion-pairing reagent. As was observed with the separation of catecholamines, a higher concentration of ion-pairing agent is required when the non-ionic surfactant is used in lieu of methanol. Approximately twice as much is required to achieve the equivalent retention of the phenolic acids (Fig. 3) as is required when 10% acetone is used in the same phosphate buffer instead of Tween (personal observation); however, the use of Tween allows more discrimination between dihydroxy and O-methoxy analogues. Hydrogen bonding effects appear to play a small role in resolving dihydroxy and O-methoxy acids while polarity appears to be the predominant mechanism for this separation. Only 3,4-dihydroxycinnamic acid elutes differently, eluting prior to 5-hydroxyindoleacetic acid instead of after, under conventional ion-pair reversed-phase conditions.

Columns treated with the Tween non-ionic surfactants may be considered "derivatized" as the material appears to bind irreversibly to the reversed-phase material. This is true of both Tween 20 and Tween 60 is likely true of other polyoxyethylene sorbitan surfactants. The orientation of the surfactant appears to place the lipophilic alkyl group into the alkyl surface, exposing the polar polyoxyethylene sorbitan containing hydroxy groups to the aqueous mobile phase. No notable difference was observed between the use of Tween 20 and Tween 60. The concentration present in the solvent affects only the length of time necessary to load the column. A low concentration, 0.01% or so, is maintained in the mobile phase after column loading to protect against any possible leaching from the column. A non-ionic phenolic surfactant, Nonidet P-40, was used to modify retention on Tween derivatized columns. Only minimal effect was observed in further decreasing k' and this effect was reversible[11].

CONCLUSIONS

Although not routinely used to modify retention of small molecules in reversed-phase HPLC, non-ionic surfactants such as Tween may offer some novel effects which may be of value in separations. The introduction of apparent hydrophilic effects offers an additional mechanism which can selectively alter the order of elution for compounds which can hydrogen bond. When coupled with ion-pairing, this hydrophilic interaction leads to novel separations governed by hydrophobic, hydrophilic and electrostatic interactions.

The present work demonstrates the possible utility of non-ionic Tween surfactants in the separation of a variety of small electroactive compounds. These separations are accomplished without organics such as methanol, acetonitrile and acetone. This should be useful with amperometric detection where both background current and noise are negatively affected by high solvent organic content[6,10]. Although untested, the greatest utility of combined ionic–non-ionic surfactant modification of reversed-phase chromatography may be in the separation of proteins. Separations should reflect charge, size and a mixture of hydrophilic and hydrophobic interactions. In addition, gradient elution using ionic strength could be used to overcome ionic effects allowing separation without denaturation while taking advantage of the high efficiency of reversed-phase materials.

The variety of surfactants available is quite large such that a large number of novel separations might be possible using this approach.

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