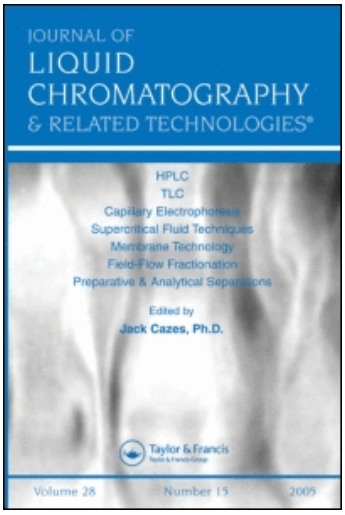

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Influence of Structural Differences of Dextromethorphan and its Three Metabolites on their Simultaneous Separation using Various Silica Columns with a Simple Aqueous Mobile Phase

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Abstract: The retention behaviors and efficiencies of the chromatographic separation of dextromethorphan (DM) and its metabolites (dextrorphan (DX), 3-methoxymorphinan (MM), and 3-hydroxymorphinan (HM)), on three different silica columns (Inertsil, μ-Porasil, and Lichrospher), were compared using a simple aqueous mobile phase consisting of an organic solvent (methanol or acetonitrile) and water at different volume ratios (1:9 to 9:1) containing triethylamine (TEA) and acetic acid (ACH). Results demonstrated that the retention capacities for basic compounds with the same ionization conditions of the silanol group were the largest for the Lichrospher column, followed by the μ-Porasil one, with the Inertsil column exhibiting the lowest level. Based on their physical characteristics, the larger retention capacities for DM and its three metabolites in the LiChrospher column compared to those of the Inertsil and μ-Porasil columns may have been because the LiChrospher column has the largest surface area for interaction. However, since the surface areas of the Inertsil and μ-Porasil columns are similar, the greater retention capacities for DM and its three metabolites in the μ-Porasil column can probably be attributed to the existence of a greater number of silanol groups than in the Inersil column. This also demonstrates that the

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interaction of tertiary amines with ionized silanol groups is greater than that of secondary amines resulting in the elution order being MM > DM and HM > DX. However, O-demethylation to expose metabolites with a phenolic hydroxy group seemed to decrease the retention capacity, thus yielding the elution order of DX > DM (tertiary amine) and HM > MM (secondary amine). This might be attributable to the negative charge repulsion between phenoxyl groups (PhO^-) and ionized silanol groups (SiO^-) decreasing the affinity of basic compounds.

Keywords: Silica column, Aqueous eluents, Dextromethorphan, Triethylamine, Acetic acid

INTRODUCTION

The separation of basic compounds by high performance liquid chromatography (HPLC), especially under reversed-phase conditions, is characterized by long variable retention times, poor efficiencies of separation, and excessive peak tailing. The chromatographic conditions reported by Jane using silica columns with high pH buffered eluents (ammonium nitrate) containing a high proportion of methanol resulted in one of most successful approaches for separating basic compounds.[1,2] Silica columns not only offer a powerful and versatile approach to analyzing a wide range of basic drugs and their metabolites with simple methanol-buffer eluents,[3] but also provide hydrophilic interactive chromatography for separating hydrophilic peptides[4,5] using a mixed acetonitrile-water solution containing 0.1% trifluoroacetic acid. However, they are often avoided because of several potential risks, including dissolution of the silica gel with the use of an aqueous mobile phase, particularly at pH values exceeding 8, and the consequent distortion of column efficiency. The application of silica gel columns to the assay of plasma concentrations was further modified by Lin et al.[6–8] with the use of low ammonium phosphate concentrations, the pH of which was adjusted to 7.0 by adding concentrated phosphoric acid.

Applications of silica columns were further simplified and improved for assaying basic drug concentrations in plasma[9] and pharmaceutical preparations,[10] with the use of methanol or acetonitrile with water containing a combination of less than 0.1% each of triethylamine (TEA) and acetic acid (ACH). In both cases, methods not only showed reduced times for analysis and simplified preparations of the mobile phase, and required less cleaning of the column and equipment tubing, but also achieved high precisions and accuracies with minimal interference and produced peaks with high symmetry. It has been proposed, that a combination of ionic exchange and interaction with siloxane and silanol groups over the entire range of concentrations of organic solvents is the underlying mechanism controlling retention[11] for the separation of basic compounds using (normal phase) silica columns. That study also revealed that various retention times on

silica columns were largely due to differences in ion exchange strengths of silanol groups and surface concentrations of siloxane bridges.

Simultaneous analysis of dextromethorphan (DM) and its three metabolites, dextrorphan (DX), 3-methoxymorphinan (MM), and 3-hydroxymorphinan (HM), to determine their concentration time profiles for the metabolic characterization and as a biomarker of CYP3A4 and CYP2D6 activity was developed by employing a variety of techniques, including LC-MS/MS[12–15] and high performance liquid chromatography (HPLC) with fluorescence detection.[16–18] Most such systems use Phenyl and Cyano stationary phases. Since DM and its three metabolites are basic compounds with some structural differences, the influence of these structural differences on the retention behaviors and efficiencies of these compounds with a simple aqueous mobile phase on various silica columns were evaluated in this study.

EXPERIMENTAL

Chemicals

Dextromethorphan HBr (DM), dextrorphan tartrate (DX), and 3-methoxymorphinan HCl (MM) were purchased from RBI (USA). (±)-3-Hydroxymorphinan HBr (HM) and levallorphan (LP) (used as the internal standard) were supplied by Sigma-Aldrich Chemie (Germany). Methanol and acetonitrile were LC grade and obtained from Lab-Scan (Ireland). Triethylamine (TEA, $d_4^{25} = 0.7255$, $pK_a = 11.01$) and acetic acid (ACH, $d_{25}^{25} = 1.049$, $pK_a = 4.74$) were both purchased from Merck (Germany). All other reagents used were of reagent grade or better.

Apparatus

The HPLC system was equipped with a pump (Jasco PU-980 intelligent HPLC pump, Japan), a fluorescence detector (Thermo Separation Products, Spectra-SYSTEM FL3000, USA), and an autosampler (Jasco, AS-1555-10). LiChrospher Si-60 (4 × 250 mm, Merck), μ-Porasil (3.9 × 300 mm, Waters), and Inertsil (4.6 × 250 mm, GL Science, Japan) columns were used after preconditioning and with no other treatments. Detailed descriptions of the physical characteristics of these silica columns are given in Table 1. Silica columns were initially preserved in hexane. Before the analysis, serial preconditioning was conducted by elution with solvents of gradually increasing polarity from ethyl acetate, dichloromethane, acetonitrile, methanol, and then finally water (the volume for each solvent was approximately 100 mL). Thereafter, the silica column could be used with aqueous eluents.

Table 1. Physical characteristics of the three different silica columns

Column	ID × length (mm)	Particle shape and size	Pore size (Å)	Pore volume (mL/g)	Surface area (m^2/g)
Metasil	4.6 × 250	spherical 5 μm	80	0.50	220
LiChrospher	4.0 × 250	spherical 5 μm	60	0.85	700
Inertsil	4.6 × 250	spherical 5 μm	150	1.15	320
μ-Porasil	3.9 × 300	irregular 10 μm	125	1.00	330

Chromatographic Measurements

The mobile phase consisted of methanol (or acetonitrile) and water in varying proportions by adding different percentage ratios of TEA and ACH (v/v). The flow rate was set to 1.0 mL/min. The eluent was detected with fluorescence (excitation at 230 nm and emission at 330 nm). The capacity factor (k') as defined in the following equation was calculated for these basic compounds on these silica columns as described above:

$$k' = \frac{t_R - t_0}{t_0}$$

where t_0 and t_R are the respective retention times of the solvent peak and corresponding compound.

RESULTS AND DISCUSSION

A large number of different phases in various proportions in the stationary phase of silica columns are in existence as a result of different manufacturing procedures and silica used, leading to large differences in ion exchange capacities for separating basic compounds. Figure 1 shows changes in k' values for DM and its three metabolites (DX, MM, and HM) eluted with the mobile phases containing different ratios of methanol/water, at the same volume ratio of TEA/ACH (0.02%/0.02%) in Inertsil (Fig. 1A) and μ-Porasil columns (Fig. 1B). Both columns demonstrated similar elution orders of HM > DX > MM > DM with respect to the low volume percent of methanol, and as HM > MM > DX > DM with respect to the high volume percent of methanol. This also illustrates, that the retention capacities of DM and its three metabolites in both columns decreased with an increasing volume percent of methanol in the mobile phase, with a smaller k' value of the corresponding compound being observed in the Inertsil column than in the μ-Porasil column. Furthermore, the retention capacities of DM and DX in the Inertsil column were found to have increased when the volume percent of methanol was increased to 90%, and those for four compounds in the

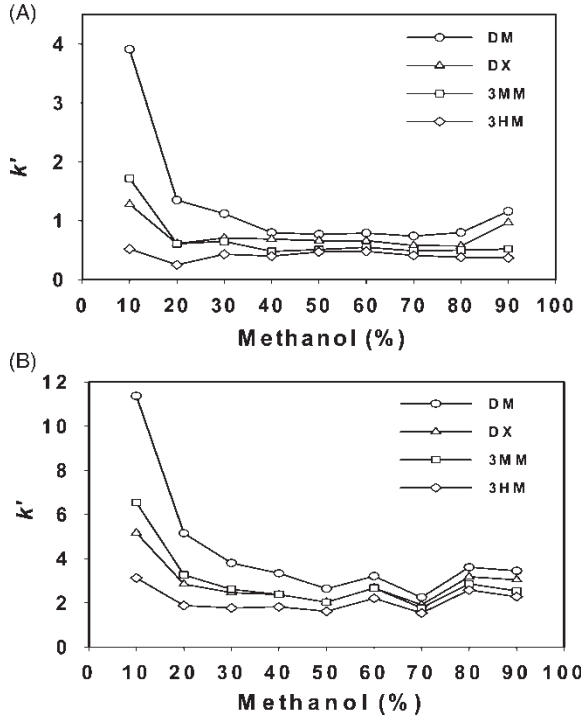

Figure 1. Changes in the k' values eluted with different ratios of methanol/water as the mobile phase at the same volume ratio of triethylamine (TEA)/acetic acid (ACH) (0.02%/0.02% v/v) in an Inertsil column (A) and a μ-Porasil column (B).

μ-Porasil column increased when the volume percent of methanol was increased to higher than 80%.

Due to the presence of acidic silanol (SiOH) functions on the surface of the silica gel, the retention behaviors of basic compounds on silica columns are thought to primarily depend on ion exchange mechanisms and to predominately be controlled by the pH value, and the nature and concentration of the organic modifier.[19] Therefore, ionization of acidic silanol (SiOH) to form negative ions can only function as a cation exchanger; the higher the pH of the eluent is, the higher the ion exchange capacity of this material will be. Likewise, silica exhibits cation exchange properties at all pH values, even down to pH 2, but the ion exchange capacity falls sharply with decreasing pH. Since an organic solvent is expected to decrease the dissociation of a weak electrolyte as a result of reducing the polarity of the mobile phase, the retention capacity of those basic compounds was expected to decrease with an increase in the percent volume of the organic solvent in the mobile phase, due to a decrease in the number of ionized acidic silanol (SiOH) groups. However, since the pH gradually increased with an increase in the

added methanol, reaching a value of 6.4 at 75% (data not shown), the silica surface is increasingly ionized at higher methanol concentrations. This can probably explain why increasing the methanol concentration to higher than 80% reversibly increased the capacity factors of DM and its three metabolites.

Since the retention behaviors of basic compounds on silica columns are thought to primarily depend on ion exchange mechanisms, their retention capacities should decrease with a decrease in the pK_b value of basic compounds. This can be explained by the elution order of compounds with a secondary amine being faster than those with a tertiary amine (MM > DM and HM > DX). However, O-demethylation to expose metabolites with a phenolic hydroxy group seemed to decrease the retention capacity, and yielded an elution order of DX > DM (tertiary amine) and HM > MM (secondary amine). This can be attributed to the negative charge repulsion between the phenoxyl group (PhO^-) and the ionized silanol group (SiO^-), which decreases the affinity of basic compounds having the same tertiary or secondary amine with a stationary silica phase. Furthermore, this repulsion effect was more profound when the mobile phase had a lower percent volume of organic solvent than that with a higher percent volume of organic solvent, thus leading to an elution order of DX > HM with the former mobile phase and of HM > DX with the latter mobile phase.

Retention of basic compounds on the stationary phase of silica particles should occur on the exposed surface of particles including the outer surfaces of the particles and inner surfaces of the pores. By assuming that similar amounts of silanol groups and siloxane bridges are contained per unit surface area and that the pore size is large enough to be reached by the solute, the retention capacity of the solute should be related to the surface area of the silica column. However, the physical characteristics, including pore size, pore volume, and surface area, of the Inertsil and μ-Porasil columns (Table 1) only slight differed even though the shape and diameter of the silica particles of these two columns significantly differed. It was inconclusive whether this slight difference in the surface area could have such a large influence on the retention capacity. A reasonable assumption is that a higher amount of silanol groups existed on the stationary phase in the μ-Porasil column than in the Inertsil column. Another possible reason is that the extent of ionization of silanol groups caused by the impurities, which existed on the stationary phase, might be different for those columns with the same amount of silanol groups.

Figure 2 demonstrates the effect of changing the total percentages of TEA and ACH (both at the same ratio) in the mobile phase on the capacity factors of DM and its three metabolites, with the mobile phase consisting of the same volume ratio (50:50) of methanol to water (Fig. 2A), and the influence of using acetonitrile as the organic solvent (Fig. 2B). Apparently, their capacity factors gradually decrease with increasing total percentages of TEA and ACH. The pH values of these four compositions of the mobile phase (without an organic solvent) were found to be within the range of

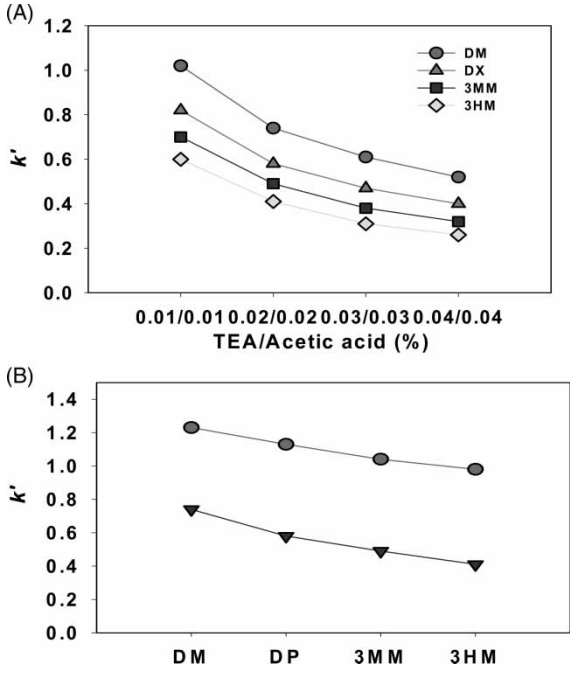

Figure 2. Changes in the k' values eluted with different total triethylamine (TEA) and acetic acid (ACH) percent volumes at the same ratio (A) and different organic solvents (acetonitrile or methanol) (B) as the mobile phase in an Inertsil column.

4.63–4.52. With similar ionization conditions for silanol groups on the silica surface, the retention of these basic compounds would, as expected, be dependent on the concentration of the competing ions, TEA in this case. Therefore, the influence of an increasing TEA concentration in the mobile phase on the capacity factors of basic compounds complies with that predicted by the ion exchange theory as previously revealed.[9] On the other hand, the influence of acetonitrile on increasing the capacity factors of these four compounds was greater than that of methanol. The same mechanism as discussed above, of the effect of the change in methanol concentration on the capacity factors, is applicable to explain this similar observation. The difference in the retention capacities between acetonitrile and methanol can be ascribed to the difference in the capacities for inducing ionization of silanol groups on the silica surface.

Figure 3 shows changes in the capacity factors for the chromatographic separation of DM and its three metabolites on a LiChrospher column, with the mobile phase consisting of water and acetonitrile at various volume ratios (90/10, 80/20, 70/30, and 60/40) containing TEA and ACH both at 0.01% v/v (Fig. 3A), with varying concentrations of TEA in the mobile phase composed of water and acetonitrile at a volume ratio of 80/20

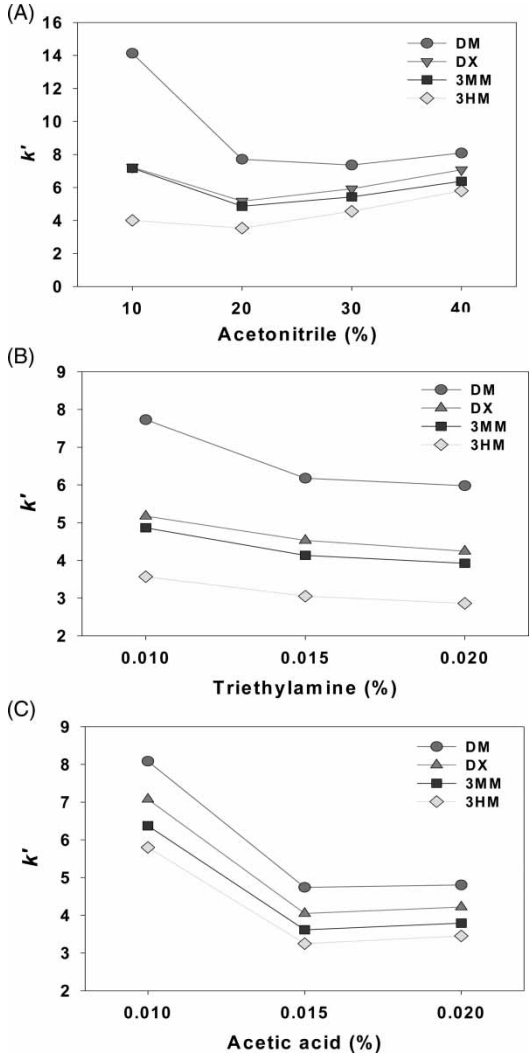

Figure 3. Changes in the k' values eluted with different percentages of acetonitrile (A), triethylamine (B), and acetic acid (C) as the mobile phase in a Lichrospher Si 60 column.

(Fig. 3B), and with varying ACH concentrations in the mobile phase composed of water and acetonitrile in a volume ratio of 60/40 (Fig. 3C). Figure 3A indicates that the elution order was HM > MM ≅ DX > DM, which was the same for the four different ratios of water to acetonitrile. However, the capacity factor for those compounds decreased and then increased, and the elution order gradually changed to HM > MM > DX > DM with an increase in the volume ratio of acetonitrile in the mobile phase

of 10% to 40%. The increase in the TEA concentration in the mobile phase decreased the capacity factors for all four compounds with no change in the elution order. Similarly, the increase in the ACH concentration in the mobile phase decreased the capacity factors of the four compounds but the elution order did not change.

As discussed above, the retention of basic compounds on the stationary phase of silica particles should occur on the exposed surface of particles, including the outer surfaces of particles and inner surfaces of pores. By assuming that similar amounts of silanol groups and siloxane bridges are contained per unit surface area and that the pore size is large enough to be reached by the solute, the retention capacity of the solute should be related to the surface area of the silica column. As revealed in Table 1, the surface area for the LiChrospher column was about two fold larger than that for the Inertsil and μ-Porasil columns. Since the effects of TEA and ACH on the capacity factor can be explained by the similar ion exchange mechanisms, it is reasonable to conclude that the longer retention in the LiChrospher column is due to its larger surface area.

CONCLUSIONS

In conclusion, the principal mechanism responsible for the separation of the basic compounds of DM and its metabolites, using various silica columns eluted with a simple mobile phase containing an organic solvent and water, with the addition of only trace concentrations of TEA and ACH, still complies with the ionic exchange theory. Based on this theory, the retention capacity of basic compounds is dependent on their interactions with silanol groups on the surface of silica particles. Therefore, increasing the surface area of silica particles in a silica column should increase the retention capacity by assuming a similar amount of silanol groups per unit surface area. The elution order of basic compounds with a secondary amine was found to be faster than those with a tertiary amine. However, the substituent groups on the structure of basic compounds that ionize to produce negative charges could further influence the interactions between ionized silanol groups and basic functional groups.

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