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Chromatographic performance of large-pore versus small-pore columns in micellar liquid chromatography

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Abstract

Micellar liquid chromatography (MLC) is useful in bioanalysis because proteinaceous biofluids can be directly injected onto the column. The technique has been limited in part because of the apparently weak eluting power of micellar mobile phases. It has recently been shown [Anal. Chem. 72 (2000) 294] that this may be overcome by the use of large pore size stationary phases. In this work, large-pore (1000 Å) C_{18} stationary phases were evaluated relative to conventional small-pore (100 Å) C_{18} stationary phases for the direct sample injection of drugs in plasma. Furthermore, the difference between the large and small pore phases in gradient elution separations of mixtures of widely varying hydrophobicities was investigated. Large-pore stationary phases were found to be very effective for eluting moderately to highly hydrophobic compounds such as ibuprofen, crotamiton, propranolol, and dodecanophenone, which were highly retained on the small-pore stationary phases typically used in MLC. The advantages of direct introduction of biological samples (drugs in plasma) and rapid column re-equilibration after gradient elution in MLC were maintained with large-pore phases. Finally, recoveries, precision, linearity, and detection limits for the determination of quinidine and DPC 961 in spiked bovine plasma were somewhat better using MLC with wide pore phases.

Keywords: Micellar liquid chromatography; Large pore columns; Direct sample injection; Gradient re-equilibration; Highly retained compounds

1. Introduction

Direct sample injection (DSI) of drugs in biological fluids such as plasma is one of the more important applications of micellar liquid chromatography (MLC) [1–5]. Typically, DSI is conducted using conventional small (≈100 Å) pore-size stationary phases in which the plasma proteins are excluded from the stationary phase pores and elute in the void volume. This has been thought to be important for successful DSI since plasma contains very high concentrations of proteins (~60–80 mg/ml [6]) relative to typical drug concentrations (≤100 μg/ml).

We have recently reported that the use of wide-

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pore stationary phases is helpful in overcoming one of the important limitations in MLC, that of the apparently weak eluting power of micellar eluents [7]. In this paper, we describe an investigation of the use of large pore-size (1000 Å) 18 columns for DSI. With large pore-size stationary phases, it may be possible for plasma proteins to enter the pores of the large pore-size stationary phases and thus be retained and possibly interfere with the analytes. Therefore, the performance of large-pore columns in DSI was evaluated by comparison with the small-pore columns that have typically been used; various classes of drug compounds in bovine plasma were examined with sodium dodecyl sulfate (SDS) and polyoxyethylene (10) dodecanol (Brij-22) micellar mobile phases. In addition, recovery experiments were conducted, using moderately-to-highly hydrophobic and plasma bound drugs to verify that these compounds are satisfactorily released from plasma proteins on large-pore columns.

Gradient elution is important in the chromatography of mixtures of widely differing polarity, e.g., in the analysis of drugs and more polar metabolites. A major difficulty encountered in gradient elution MLC has been the inability to effectively elute highly retained species, such as nonpolar hydrophobic compounds, on long chain stationary phases (e.g., C_{18}) [8–13]. This problem has been attributed to the apparently weak solvent strength of micellar mobile phases [14]. The use of shorter chain length stationary phases has been recommended to reduce analysis times [14], but although this effectively reduces retention [8,10,12], resolution also decreases. We previously demonstrated that, with gradient elution using a SDS-based mobile phase on a large pore-size C_{18} stationary phase, hydrophobic solutes could be more easily eluted [7]. The reduction in retention relative to the small-pore column can only partly be explained by the difference in phase ratio between the columns; it is also due to the ability of the micelles to penetrate the larger pores. Here, results with other surfactant mobile phases in gradient MLC are described.

Another aspect of MLC that has proven to be important is rapid gradient re-equilibration. Generally it is believed that 15–20 column volumes of mobile phase are required to re-equilibrate a column in RPLC [15–17]. Often this re-equilibration time is as long as the sample elution time, a problem when trying to apply RPLC gradient elution to rapid analysis [15,16]. In MLC, gradient re-equilibration is rapid (it has been thought that this is because the micelles are restricted to the interparticle space) and this is one of its more attractive features [5,14,18,19]. However, in large-pore columns the micelles penetrate into the stationary phase pores, and thus a slower column re-equilibration time might be expected. Since previous studies of MLC gradient re-equilibration were conducted on conventional small pore-size stationary phases [9,20–23], MLC gradient re-equilibration was evaluated on large- versus small-pore size columns in order to verify that this important capability is applicable as well to large pore-size stationary phases.

2. Experimental

2.1. Apparatus

A Hewlett-Packard (Rockville, MD, USA) HP1090M Win Liquid Chromatography System equipped with a diode array UV detector was used for gradient MLC studies. For determination of quinidine recovery, a Hewlett-Packard HP 1050 HPLC system was used with an HP1046A fluorescence detector containing a 345-nm cutoff filter. A Waters (Milford, MA, USA) Alliance HPLC system with a Waters 2487 UV detector was used for the DPC 961 recovery studies. All of the HPLC systems were equipped with gradient pumps, temperature controlled column compartments, and variable volume autoinjectors. Nucleosil C_{18}, 150×4.6 mm, 7 μm columns, 100 and 1000 Å pore size, were from Alltech (Deerfield, IL, USA). The available physical characteristics of the stationary phases are given in Table 1. A Phenomenex (Torrance, CA, USA)

Table 1
Physical characteristics of Alltech Nucleosil C_{18} stationary phases

Pore size (Å)	Carbon load (%)	Surface area (m^2/g)	Bonded phase coverage ($\mu mol/m^2$)
100	14	350	2.06
1000	1	25	1.87

SecurityGuard C_{18} guard column cartridge (4.0 mm length×3.0 mm I.D.) was used in studies involving bovine plasma to protect the analytical column.

2.2. Materials

SDS, Brij-22, dodecyltrimethylammonium bromide (DTAB), lyophilized bovine plasma, and trifluoroacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA); HPLC grade methanol, *n*-propanol, and acetonitrile were obtained from EM Science (Gibbstown, NJ, USA); HPLC grade water was obtained from a Milli-Q water system (Millipore, Corp., Milford, MA, USA). Sodium phosphate monobasic, monohydrate and sodium phosphate dibasic, anhydrous were obtained from EM Science, and phosphoric acid was obtained from J.T. Baker (Phillipsburg, NJ, USA). The alkylphenones were obtained from the following sources: acetophenone (Fisher Scientific, Fair Lawn, NJ, USA); propiophenone and butyrophenone (Sigma); valerophenone, hexanophenone, heptanophenone, octanophenone, decanophenone, and dodecanophenone (Aldrich Chemical Company, Inc., Milwaukee, WI, USA); nonaphenone and undecanophenone (Acros Organics, New Jersey, USA). Drug compounds were obtained from the following sources: acetominophen, salicylic acid sodium salt, ibuprofen sodium salt, naproxen sodium salt, phenacetin, diethylstilbestrol (DES), dobutamine hydrochloride, and ephedrine hydrochloride (Sigma); crotamiton and propranolol hydrochloride (Aldrich); quinidine sulfate dihydrate (TCI America, Portland, OR, USA); DPC 961 (DuPont Pharmaceuticals Company, Wilmington, DE, USA). Structures of these drug compounds are shown in Fig. 1.

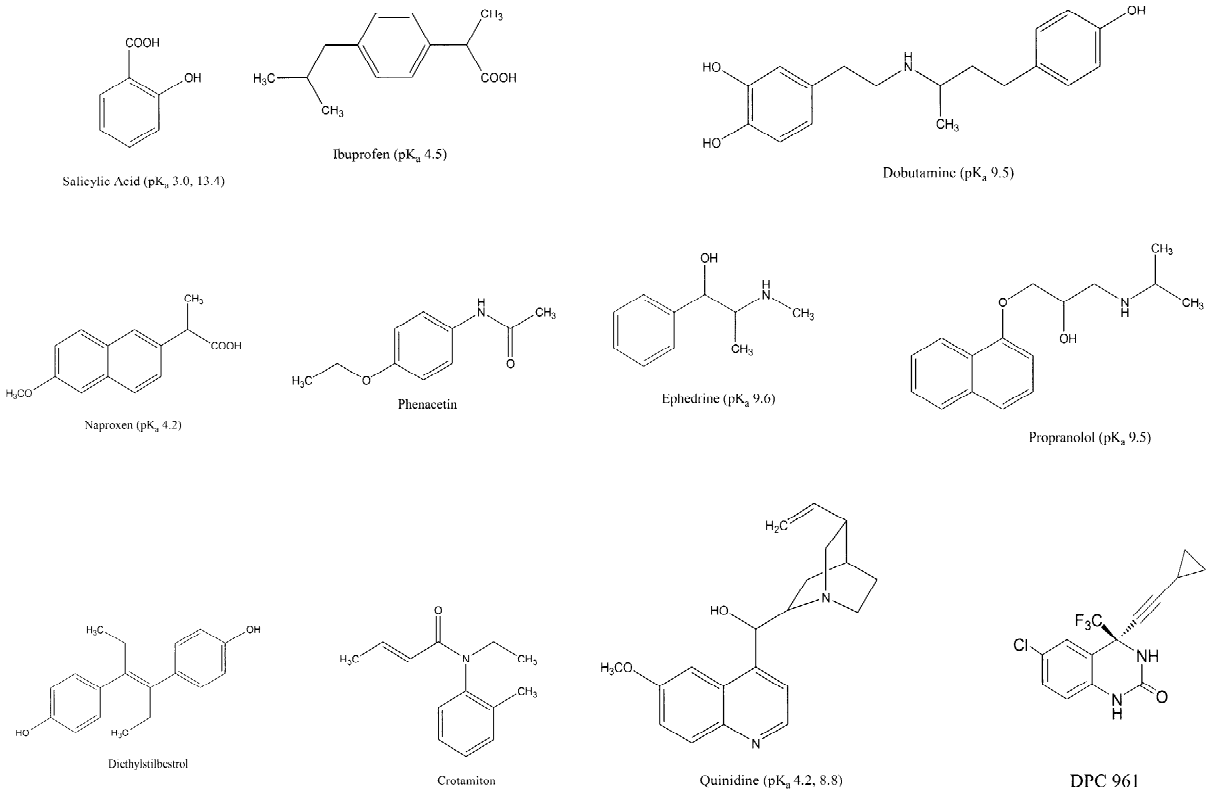
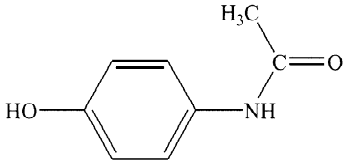
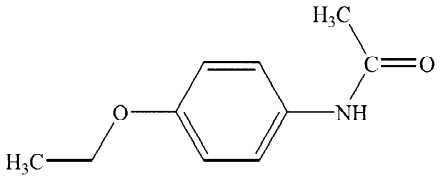


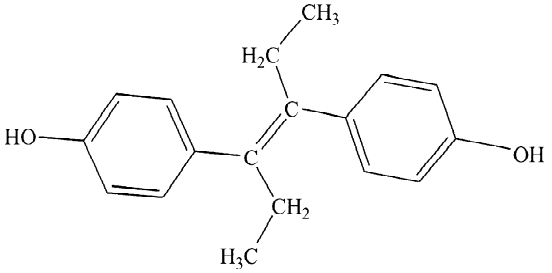
Fig. 1. Structures of test drug compounds used (note: pK_a data obtained from reference [33] for ibuprofen and reference [29] for all other compounds with reported values).



Acetaminophen



Phenacetin



Diethylstilbestrol

Fig. 1. (*continued*)

2.3. Procedure

2.3.1. Gradient elution of highly retained compounds

For gradient retention studies, an alkylphenone test mixture was prepared by combining the eleven alkylphenones together and dissolving them in *n*-propanol to give a stock solution containing 2% v/v or w/w of each alkylphenone. The stock solution was then diluted with methanol to give a final solution containing 0.01% (v/v or w/w) of each alkylphenone. The alkylphenone test mixture was run under both reversed-phase liquid chromatography (RPLC) and MLC gradient conditions on the Alltech C_{18} 100 and 1000 Å columns. The RPLC gradients were run on the columns first, for reference, followed by the MLC gradients. The columns were washed with methanol for 3 h when switching between micellar mobile phases composed of different surfactants. The RPLC gradient was a linear % B gradient from 10 to 100% methanol in 30 min. For MLC, both a linear % B gradient (i.e., a linear surfactant gradient) and a linear pM gradient (i.e., an exponential surfactant gradient) were run, where $pM = -\log[M]$ and $[M] = ([\text{surfactant}] - \text{cmc})/N$ with N being the surfactant aggregation number. With a linear pM gradient, the concentration of micelles in the mobile phase increases exponentially with time instead of linearly as in a % B gradient.

For all three micellar linear pM gradients, ΔpM was -0.3 and $\Delta pM/\Delta t$ was -0.06, where t is time in min. The surfactant concentrations in the micellar mobile phases and the gradient run times ranged as follows: 12.6–300 m*M* SDS in 31.7 min, 1.0–57.6 m*M* Brij-22 in 30 min, and 20–300 m*M* DTAB in 30.7 min. The gradient for each of the surfactants (SDS, Brij-22, and DTAB) is illustrated in Fig. 2. The final gradient conditions were held for 10 min for each gradient run (RPLC and MLC). Also, all micellar mobile phases contained 5% v/v *n*-propanol to improve efficiency [12]. The other HPLC conditions used in the RPLC and MLC gradient sepa-

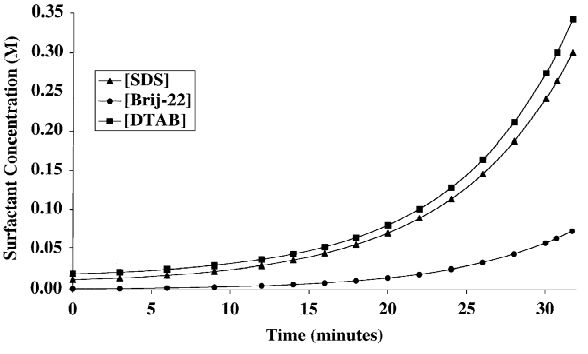


Fig. 2. Illustration of equivalent linear pM gradients for SDS, Brij-22, and DTAB ($\Delta pM = -0.3$ and $\Delta pM/\Delta t = -0.06$, see text). Surfactant concentration increases exponentially with time and is not the same for these surfactants due to differences in their critical micelle concentration and aggregation number.

rations of alkylphenones were: 1.0 ml/min flow-rate, 20 μl injection volume, 40 °C column temperature, and detection at 210 and 254 nm (235 and 254 nm for DTAB).

2.3.2. Gradient re-equilibration studies

These studies were conducted on the Alltech C_{18} 100 and 1000 Å columns using the procedures reported by Cole and Dorsey [15], and Madamba-Tan et al. [20]. Various low k (≤ 2) probe compounds were used with SDS, Brij-22, and DTAB micellar mobile phases. Acetaminophen was used as the probe compound for the 100 Å column re-equilibration studies with each surfactant type. Diethylstilbestrol was used for the 1000 Å column study with SDS, and phenacetin was used for the 1000 Å column studies with Brij-22 and DTAB. The probe compounds were all dissolved at a concentration of 1 mg/ml. DES was dissolved in methanol. Acetaminophen and phenacetin were dissolved in 50 m*M* SDS containing 5% (v/v) *n*-propanol, 1 m*M* Brij-22 containing 10% (v/v) methanol, or 50 m*M* DTAB containing 10% (v/v) methanol, respectively, for separations in SDS, Brij-22, and DTAB micellar mobile phases.

The columns were equilibrated with a high concentration of micellar mobile phase (300 m*M* for SDS and DTAB; 50 m*M* for Brij-22). After an injection with the higher concentration micellar mobile phase, the mobile phase was immediately switched (i.e., a step gradient) to a lower micellar concentration (50 m*M* for SDS and DTAB; 1 m*M* for Brij-22) and ten more injections were made. The run time for each injection was between 2 and 3 min. The following HPLC conditions were used for the gradient re-equilibration studies: 1.5 ml/min flow-rate, 2.0 μl injection volume, 40 °C column temperature, and detection at 210 and 254 nm (235 and 254 nm for DTAB). In addition, each of the micellar mobile phases contained 5% v/v *n*-propanol.

2.3.3. Direct sample injection

Various model drug compounds were utilized for DSI evaluations on the 100 and 1000 Å columns, representing acidic (salicylic acid, ibuprofen, naproxen), neutral (phenacetin, DES, crotamiton), and basic (dobutamine, ephedrine, propranolol) classifications. Acidic and basic drugs were dissolved in 50 m*M* SDS when using an SDS-containing mobile phase, and in 1 m*M* Brij-22 when using a Brij-22 mobile phase. Neutral compounds were dissolved in 50 m*M* SDS containing 10% methanol and 1 m*M* Brij-22 containing 10% methanol when using SDS and Brij-22 mobile phases, respectively. The only exceptions were dobutamine, a basic compound, which was dissolved in 50:50 acetonitrile: 0.1% trifluoroacetic acid, and DES, a neutral compound, which was dissolved in methanol.

After conducting a series of preliminary experiments using SDS and Brij-22 mobile phases, an isocratic micellar mobile phase composition was selected for each surfactant and compound class such that there was one compound in each class having a low (~1–10), mid (~10–20), and high (≥ 20) k value on the 100 Å column. The use of DTAB micellar mobile phase was avoided for DSI since it causes precipitation of plasma proteins [24,25], which may lead to column clogging. The final mobile phase surfactant concentration was 50 m*M* for both SDS and Brij-22 mobile phases except for basic solutes with SDS, which was used at a concentration of 125 m*M*. Neutral unbuffered micellar mobile phases were used for both the neutral and basic compounds, however for the acidic solutes the final micellar mobile phases were buffered at pH 2 with 50 m*M* phosphate buffer. All of the micellar mobile phases used contained 5% *n*-propanol to improve chromatographic efficiency.

Following the evaluation of the various drug compounds on the 100 Å column, the solutes were then spiked into bovine plasma and chromatographed on both the 100 and 1000 Å columns using the previously selected isocratic micellar mobile phases. Bovine plasma was prepared by reconstituting lyophilized bovine plasma with deionized (Milli-Q) water. When using SDS micellar mobile phases, the drug compounds were combined (by class) and spiked into bovine plasma, at concentrations of 0.1 mg/ml for the neutral compounds, 1.0 mg/ml for the basic compounds, and 0.5, 0.2 and 1.0 mg/ml for salicylic acid, naproxen, and ibuprofen, respectively. These were prepared by 1:10 dilution with bovine plasma from combination stock solutions at 1 mg/ml in methanol (neutral compounds), 10 mg/ml in 50:50 acetonitrile: 0.1% trifluoroacetic acid (basic com-

pounds), and 5, 2 and 10 mg/ml in 50 m*M* SDS for salicylic acid, naproxen, and ibuprofen, respectively.

When using Brij-22 micellar mobile phases, the drug compounds were individually spiked into bovine plasma since the resolution was not as good as it was with the SDS mobile phase. The final spiked drug concentrations in bovine plasma were 0.5, 0.2 and 1.0 mg/ml for salicylic acid, naproxen, and ibuprofen, respectively, and 1.0 mg/ml for each of the neutral compounds. These solutions were spiked using individual stock solutions of the drugs at 5, 2 and 10 mg/ml in 1 m*M* Brij-22 for salicylic acid, naproxen, and ibuprofen, respectively, and at 10 mg/ml in methanol for each of the neutral compounds. Separations were at 1.0 ml/min flow-rate, 40 °C column temperature, 20 μl injection volume, and detection at 210, 220 and 254 nm.

For the recovery studies, stock solutions of quinidine sulfate and DPC 961 were prepared at concentrations of 30 and 20 μg/ml, respectively, in water. The DPC 961 stock solution contained 20% methanol for solubility. The stock solutions were diluted 1:10 with bovine plasma in six replicates to prepare recovery samples spiked at 3 and 2 μg/ml with quinidine sulfate and DPC 961, respectively. Calibration curves were prepared by diluting aqueous (quinidine sulfate) or methanolic (DPC 961) 0.1 mg/ml stock solutions sequentially with water to obtain solutions containing approximately 1.0, 2.0, 3.0, 4.0 and 5.0 μg/ml of quinidine sulfate and 0.5, 1.0, 2.0, 3.0 and 4.0 μg/ml of DPC 961. Recovery of the drugs from the spiked bovine plasma samples was calculated via the calibration curves using the analyte's peak height response. Peak height was employed for quantitation rather than peak area since the sample concentrations were low and the analyte peaks were broad and tailing making it difficult to reproducibly integrate the peak areas. For this reason peak height is commonly used in bioanalytical work. One hundred and 300 ng/ml solutions of quinidine sulfate and DPC 961, respectively, prepared by aqueous dilution of 0.1 mg/ml calibration stock solutions, were utilized to ascertain the limit-of-detection (LOD) of the methods. The criterion used to determine the LOD was that amount where the signal-to-noise ratio equals three, with the noise measured as peak-to-peak noise.

The spiked bovine plasma recovery samples were analyzed in duplicate, with no further sample preparation, on the HP 1050 HPLC system with the fluorometric detector set at 277 nm excitation and 377 nm emission, 10 μl injection volume for quinidine sulfate, and on the Waters Alliance HPLC system with the UV detector set at 250 nm, 40 μl injection volume, for DPC 961. Three hundred m*M* SDS and 40 m*M* Brij-22 mobile phases containing 5% *n*-propanol were used with the 100 Å column for quinidine sulfate and DPC 961, respectively. With the 1000 Å column, 150 m*M* SDS and 20 m*M* Brij-22 mobile phases containing 5% *n*-propanol were used for quinidine sulfate and DPC 961, respectively. Flow rate was 1.0 ml/min, and the column temperature was 40 °C.

3. Results and discussion

3.1. Gradient elution of highly retained compounds

Linear-solvent-strength (LSS) gradients provide optimum separation in gradient elution chromatography [26]. Since hydro-organic mobile phases show an approximately linear relationship between log *k* and percent organic modifier, a linear gradient from solvent A to B is the preferred shape to give an LSS gradient in RPLC. However, with micellar mobile phases, linearity occurs between log *k* and log surfactant concentration [14]. Retention in MLC can be described as a secondary chemical equilibrium according to the following equation:

$$k = \left(\frac{[\mathrm{S}]}{[\mathrm{S}]+[\mathrm{SM}]}\right)k_s + \left(\frac{[\mathrm{SM}]}{[\mathrm{S}]+[\mathrm{SM}]}\right)k_{sm} \quad (1)$$

where k is the overall retention factor, k_s is the retention factor of the free solute, k_{sm} is the retention factor of the solute-micelle complex, [S] is the concentration of the solute and [SM] is the concentration of the solute-micelle complex. Eq. (1) can also be expressed as follows:

$$k = \left(\frac{1}{1+K_{sm}[\mathrm{M}]}\right)k_s + \left(\frac{K_{sm}[\mathrm{M}]}{1+K_{sm}[\mathrm{M}]}\right)k_{sm} \approx \left(\frac{1}{1+K_{sm}[\mathrm{M}]}\right)k_s \quad (2)$$

where K_{sm} is the solute-micelle binding constant. If $K_{sm}[M] \gg 1$, then:

$$k \approx \left(\frac{k_s}{K_{sm}[M]} \right) \quad (3)$$

and $\log k = \log k_s - \log [M] - \log K_{sm} = \log k_s + pM - \log K_{sm}$. Therefore, as pM decreases linearly, so does $\log k$. LSS gradients in MLC are therefore provided by a linear pM gradient in which the concentration of micelles (and total surfactant) in the mobile phase increases exponentially with time. Consequently, linear pM gradients should be the preferred shape for micellar mobile phase gradients, and they are analogous to the linear solvent gradients used in RPLC. However, the MLC gradient work that has been previously conducted has typically been linear surfactant gradients run on conventional small-pore stationary phase columns. Therefore, both linear % B and pM gradients were evaluated with the micellar mobile phases, for clarity they will be hereafter be referred to as linear and exponential surfactant gradients, respectively.

The chromatograms for the various gradient separations of the alkylphenone test mixture are shown in Figs. 3–6. Overall, retention times were lower on the 1000 Å column compared to the 100 Å, and more of the higher alkylphenone homologs were eluted, within the 30-min gradient window, on the 1000 Å column than on the 100 Å.

For comparative purposes, Fig. 3 shows the RPLC gradients for the 100 and 1000 Å columns. The difference in the retention times of the peaks reflects the difference in the stationary phase volume between the two columns. The efficiency of the 1000 Å column is lower than that of the 100 Å column, and in addition, the peak widths for both the 100 and 1000 Å columns decrease during the course of the RPLC gradient run (Fig. 3). In an ideal LSS gradient the average or effective value of k during migration is the same for every band, which results in equivalent resolution of every band and equal bandwidths for all components [26]. The capacity factor for each solute at the column inlet, k_i, decreases during gradient elution according to [27]:

$$\log k_i = \log k_0 - b(t/t_m) \quad (4)$$

where k_0 is the value of k_i determined isocratically in the starting solvent composition for the gradient, b is the gradient steepness parameter, t is the time after the start of the gradient, and t_m is the column holdup time. The gradient steepness parameter in RPLC is given by [27]:

$$b = \Delta\phi \, S t_m / t_g \quad (5a)$$

where $\Delta\phi$ is the change in the volume fraction of the stronger eluting solvent during the gradient, S is the change in $\log k$ for unit change in the volume fraction of stronger eluting solvent, t_g is the gradient time (i.e., the time from the start to the end of the gradient), and t_m is the column holdup time. Analogously, the gradient steepness parameter in MLC is given by:

$$b = \Delta pM(S') t_m / t_g \quad (5b)$$

ΔpM is the change in the logarithm of the concentration of micelles in the micellar mobile phase during the gradient and S' is the change in $\log k$ per unit change in the logarithm of the concentration of micelles in the micellar mobile phase.

Ideally, the gradient steepness parameter, b, should remain constant throughout the solvent program and hence have the same value for all sample components. However, the gradient steepness parameter will clearly vary with the value of S, which is only roughly constant for similar compounds. Systematic variations in S are generally observed among homologs, oligomers and samples comprising a parent skeleton with varying numbers of functional group substituents [27]. Because S increases systematically for higher homologs in RPLC, the focusing effect of the gradient is larger for higher homologs and thus their peak widths should be narrower. This would account for the decreasing peak widths that were observed during the RPLC gradient runs (Fig. 3). Resolution also decreased during the RPLC gradient runs since methylene selectivity decreases substantially in hydro-organic gradient elution as the percent organic modifier in the mobile phase increases.

Looking at the 1000 Å column gradient data (since more homologs were eluted), it can be seen that the peak widths for the micellar LSS gradients (Figs. 4D–6D) are larger than those obtained with the hydro-organic LSS gradient (Fig. 3). Also, the peak

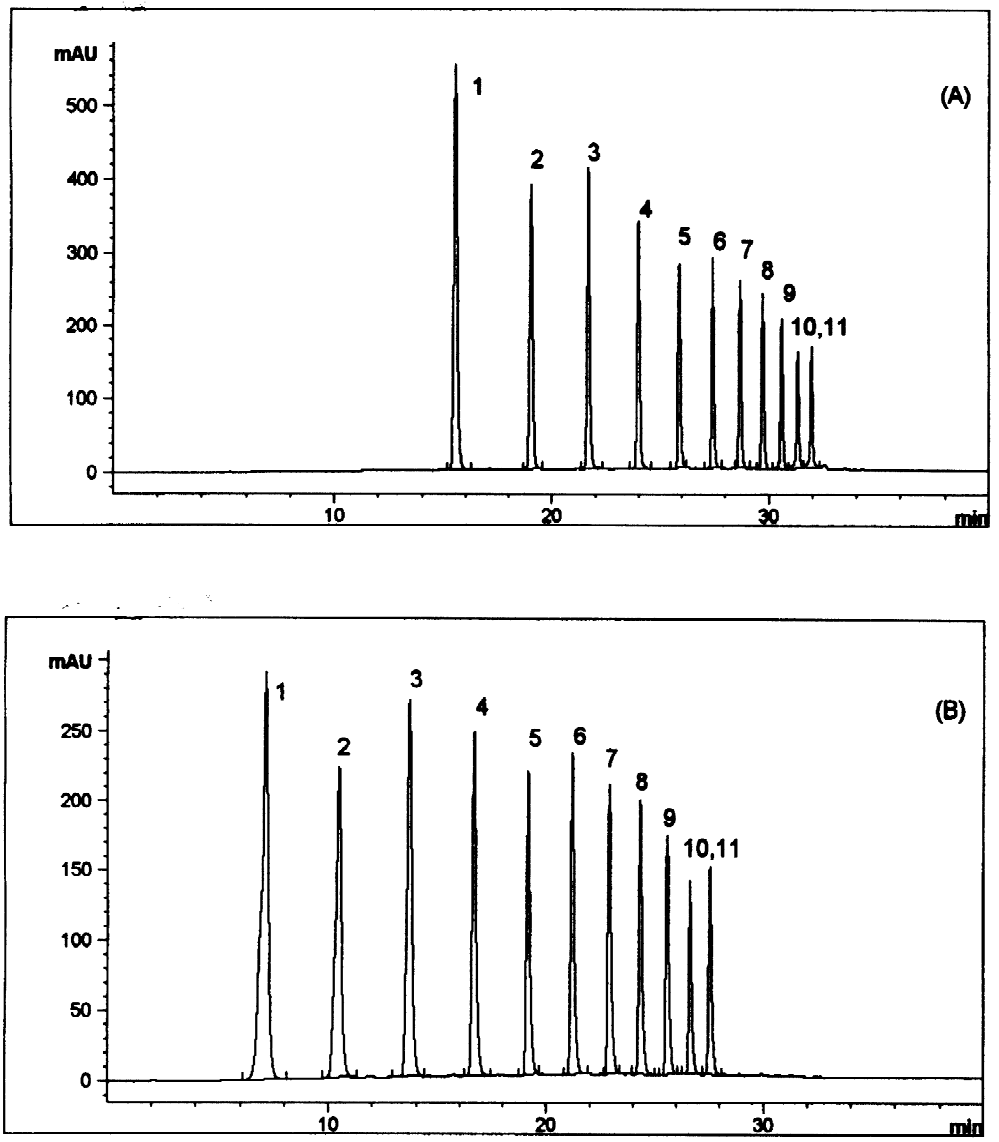


Fig. 3. RPLC separation of 0.01% alkylphenone mixture with linear solvent gradient (10–100% methanol in 30 min and hold 10 min) on Alltech C_{18} Columns; (A) 100 Å, (B) 1000 Å. Absorbance detection at 254 nm.

width tends to increase during the micellar LSS gradients as opposed to decreasing with the hydro-organic LSS gradients. This is due to the broadening effect of resistance to mass transfer (RTMT) which dominates over the focusing effect of the gradient in micellar gradient elution. RTMT dominates in MLC because of (i) the lower apparent diffusion coefficient of all homologs in MLC compared to RPLC and (ii) the more rapid decrease in the diffusion coefficient with increasing homolog number. The apparent (effective) diffusion coefficient of a solute in MLC is:

$$D_{\text{app}} = \left(\frac{1}{1 + K_{sm}[\text{M}]}\right) D_s + \left(\frac{K_{sm}[\text{M}]}{1 + K_{sm}[\text{M}]}\right) D_{sm} \quad (6)$$

where D_s and D_{sm} are the diffusion coefficients of

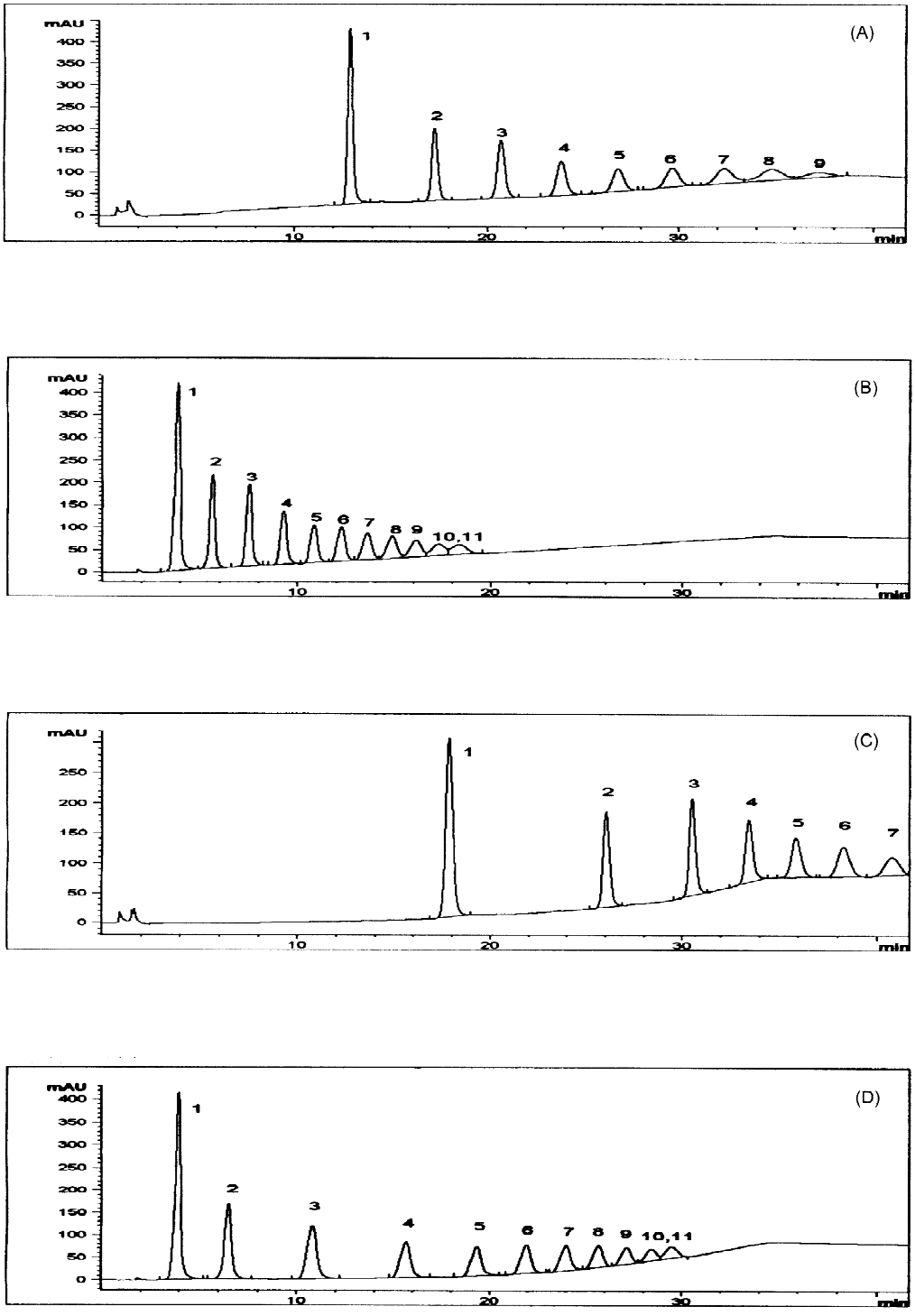


Fig. 4. MLC separation of 0.01% alkyiphenone mixture with linear SDS gradient (12.6–300 m*M* SDS containing 5% *n*-propanol in 31.7 min and hold 10 min) on (A) 100 Å, (B) 1000 Å columns. Separation of same mixture with exponential SDS gradient (12.6–300 m*M* SDS containing 5% *n*-propanol in 31.7 min and hold 10 min) on (C) 100 Å, (D) 1000 Å columns. Absorbance detection at 254 nm with a bandwidth of 4 nm. Gradient shape for (C) and (D) as in Fig. 2.

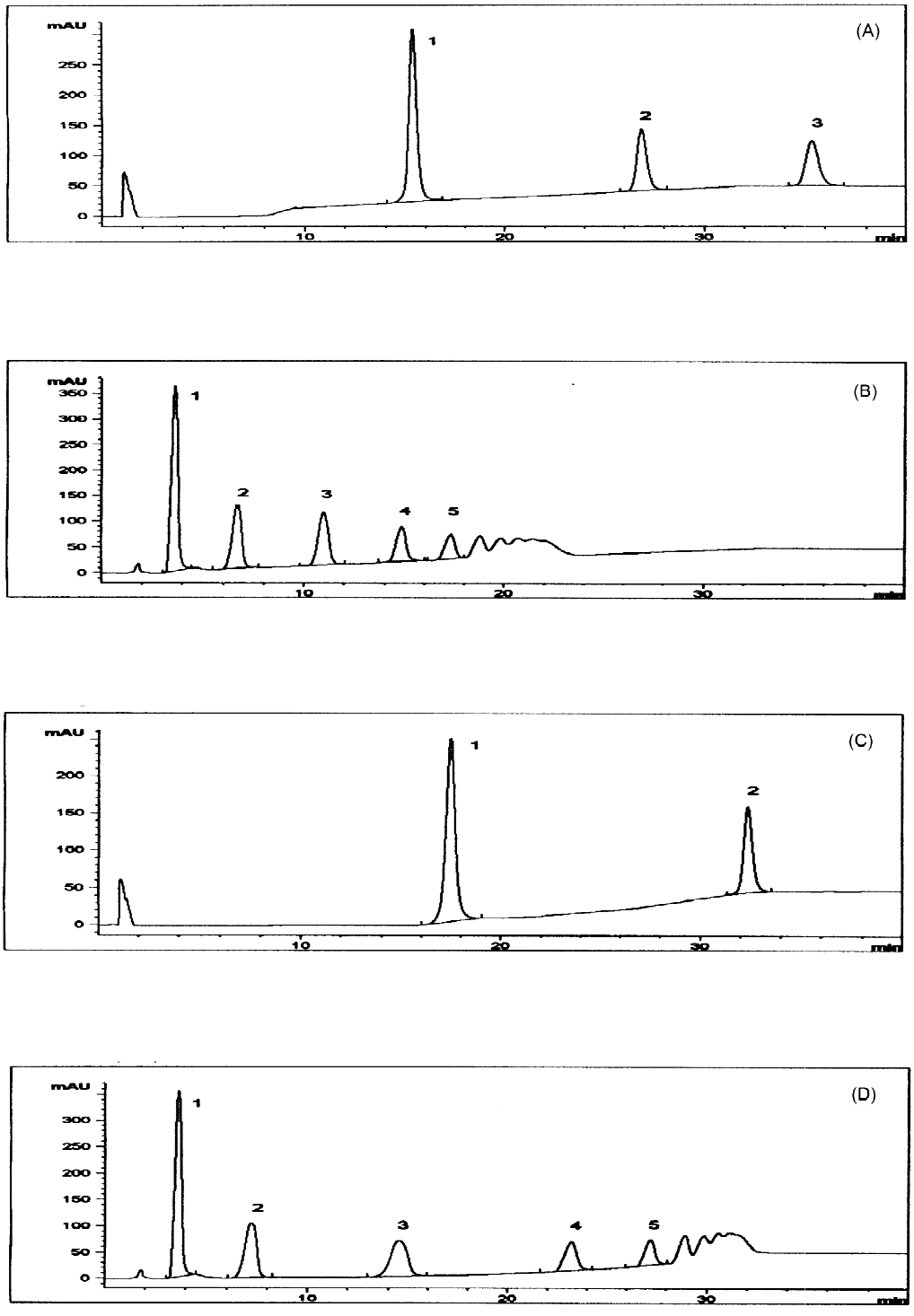


Fig. 5. MLC separation of 0.0 1% alkylphenone mixture with linear Brij-22 Gradient (1.0–57.6 m*M* Brij-22 containing 5% *n*-propanol in 30 min and hold 10 min) on (A) 100 Å, (B)1000 Å columns. Separation of same mixture with exponential Brij-22 gradient (1.0–57.6 m*M* Brij-22 containing 5% *n*-propanol in 30 min and hold 10 min) on (C) 100 Å, (D) 1000 Å columns. Detection as in Fig. 4; gradient shape for (C) and (D) as in Fig. 2.

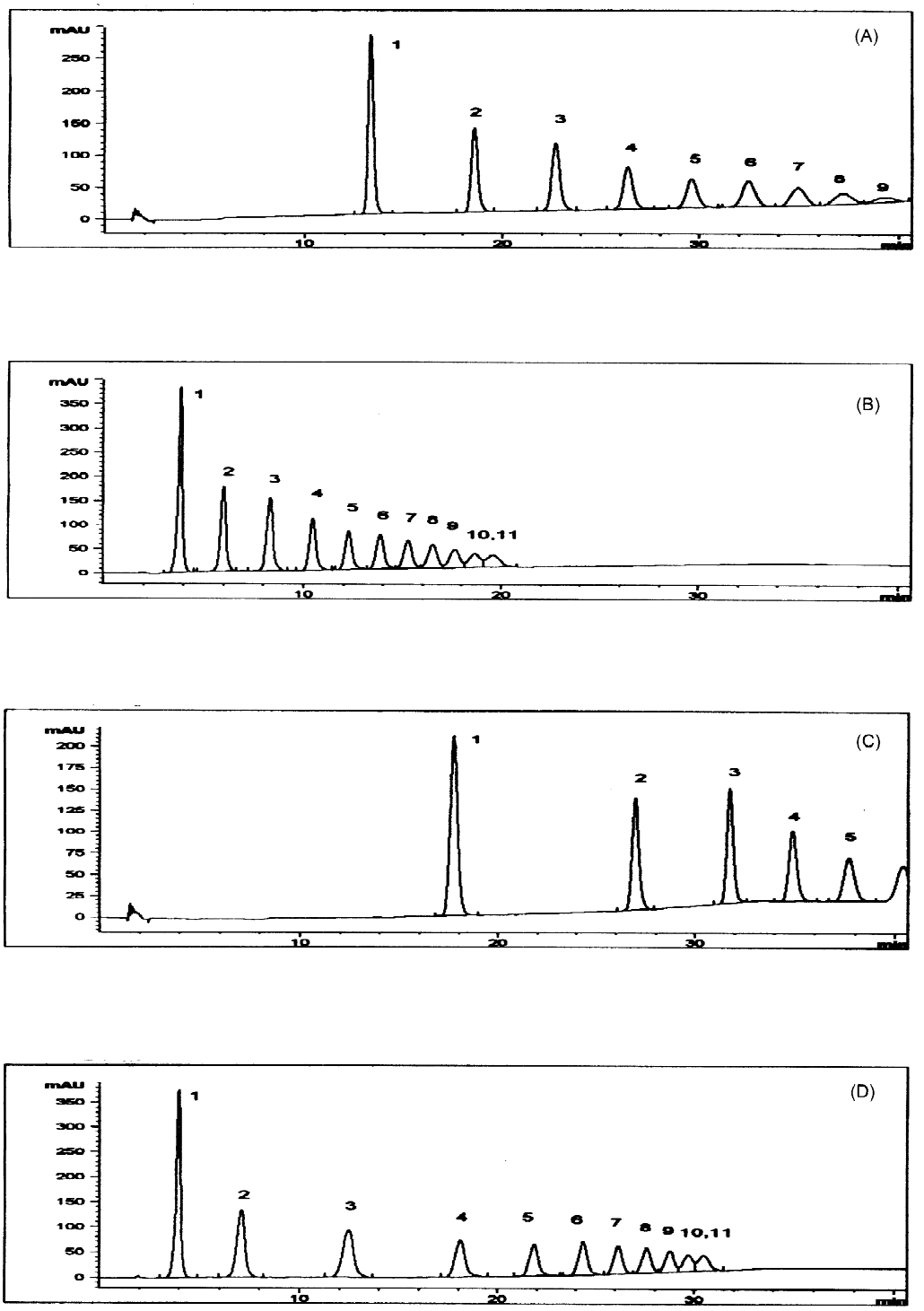


Fig. 6. MLC separation of 0.0 1% alkylphenone mixture with linear DTAB Gradient (20–300 m*M* DTAB containing 5% *n*-propanol in 30.7 min and hold 10 min) on (A) 100 Å, (B) 1000 Å columns. Separation of same mixture with exponential DTAB gradient (20–300 m*M* DTAB containing 5% *n*-propanol in 30.7 min and hold 10 min) on (C) 100 Å, (D) 1000 Å columns. Detection as in Fig. 4; gradient shape for (C) and (D) as in Fig. 2.

the free and micelle-bound solute, respectively. Given that $D_{sm} \ll D_s$, the apparent diffusion coefficient of a solute in MLC will be significantly less than in RPLC, i.e., $D_{app} \ll D_s$).

The peak widths for the linear surfactant (micellar) gradients (Figs. 4B–6B) are smaller for the earlier eluting peaks and larger for the later eluting peaks than with the exponential surfactant (micellar) gradients. This is the result of an asymptotic increase in eluent strength that occurs with a linear surfactant (micellar) gradient versus the linear increase in eluent strength with an exponential surfactant (micellar) gradient; the asymptotic increase in eluent strength results in a decreasing amount of focusing with increasing homolog number.

With SDS and DTAB micellar mobile phases, all eleven alkylphenone homologs could be eluted on the 1000 Å column, within the ~30-min gradient window, using both linear and exponential surfactant (micellar) gradients (Figs. 4B,D and 6B,D). On the 100 Å column, however, only partial elution of these homologs could be obtained (Figs. 4A,C and 6A,C). Both the linear and exponential gradients appeared to work well on the 1000 Å column for both SDS and DTAB, although elution was faster on the 1000 Å column with the linear surfactant (micellar) gradients. On the 100 Å column, more of the higher alkylphenone homologs were eluted with the linear surfactant (micellar) gradients run with SDS and DTAB than with the exponential surfactant (micellar) gradients.

Although all of the homologs were eluted on the 1000 Å column using a linear surfactant (micellar) gradient with Brij-22, only the first five homologs could be adequately separated (Fig. 5B). The last six homologs co-eluted as one large, broad underlying peak with some resolution of the individual peaks superimposed on top. The exponential surfactant (micellar) gradient with Brij-22 did not provide any better results (Fig. 5D). Only a few (2–3) peaks were obtained on the 100 Å column with the linear and exponential Brij-22 gradients (Fig. 5A,C)

The lower efficiency, selectivity, and resolution of the alkylphenones provided by the Brij-22 micellar mobile phase compared to the SDS and DTAB micellar mobile phases is probably due to the nature of the surfactant and the interactions of the solutes with the micelle and the surfactant modified stationary phase. Although the hydrophobic group is the same for all three surfactants, the hydrophilic group is larger on Brij-22 than on SDS and DTAB. Therefore, in order for the highly hydrophobic solutes (i.e., the higher alkylphenone homologs) to partition directly from the micellar core to the stationary phase they must traverse a larger hydrophilic region with Brij-22 micelles compared to SDS and DTAB micelles. With SDS and DTAB, even though there is electrostatic repulsion between the micelle and the surfactant modified stationary phase surface, the distance between the micellar core and the stationary phase may still be shorter than with Brij-22.

Figs. 3 and 4C,D appear to be visually similar to Fig. 3 in Ref. [7], however the data presented in this present manuscript were obtained with different base silica C_{18} columns. In Ref. [7], the columns were made with Macrosphere base silica material, whereas in this paper Nucleosil was used as the base silica in the manufacture of the columns. Given that this is only the second wide-pore MLC paper to be published, it is important to show the previous findings [7] apply to as many different RPLC columns as possible.

The results of these various micellar gradient elution studies using an alkylphenone test mixture demonstrate that large-pore stationary phases are indeed effective for the analysis of mixtures with widely varying properties, including highly hydrophobic solutes which are strongly retained on the conventional small-pore stationary phases typically used in MLC.

3.2. Gradient re-equilibration studies

Low k (≤ 2) probe compounds were used for these studies since failure to completely reequilibrate a column after a gradient will cause wide variation in the retention of early eluting peaks from one injection to the next [14–17,28]. Probe compounds were neutral, in order to eliminate electrostatic interactions with the mobile phase surfactants. Acetaminophen was found to be a suitable neutral low k probe for the 100 Å column, however it was virtually unretained on the 1000 Å column. Therefore, DES and phenacetin were used as the neutral low k probes

for the 1000 Å column since they provided slightly more retention. All three of these compounds are neutral in the pH range (6–7) of the mobile phases employed in this study.

The results of the gradient re-equilibration studies are given in Fig. 7. In order to compare the re-equilibration data for the 100 and 1000 Å columns, the volume of mobile phase eluted after the step gradient (V) was corrected for the delay volume (V_d) and normalized to the respective column void volume (V_m) using the formula $V_c = (V - V_d)/V_m$. Also, the k data for each injection was normalized to its respective equilibrium k value: $k_n = (k/k_{eq})$. The columns were considered re-equilibrated when $k_n = 1.00 \pm 0.01$.

The results of this study show that gradient re-equilibration in MLC is essentially as rapid on the 1000 Å column as it is on the 100 Å column using SDS, Brij-22, and DTAB micellar mobile phases. Although micellar exclusion occurs on the 100 Å columns, the similar results obtained on the narrow- and wide-pore phases are not unexpected since the rapid column reequilibration is based largely on the concept that the concentration of free surfactant monomer (whose concentration-dependent adsorption onto the stationary phase) does not change much during the micellar gradient. Re-equilibration occurs on both pore-size columns within approximately two injections after the step gradient from high to low micellar mobile phase concentration for each surfactant as shown in Fig. 7. This corresponds to 4.0, 6.7 and 2.6 column volumes required for re-equilibration on the 100 Å column with SDS, Brij-22, and DTAB mobile phases, respectively. For the 1000 Å column, 5.7, 3.7 and 3.6 column volumes were required for re-equilibration with SDS, Brij-22, and DTAB mobile phases, respectively. These column re-equilibration volumes are considerably lower than that necessary when using hydro-organic mobile phases, which typically require 15–20 column volumes for re-equilibration.

3.3. Evaluation of direct injection analyses of various classes of drug compounds in bovine plasma using SDS and Brij-22 micellar mobile phases

The results of the spiked bovine plasma studies for the 100 and 1000 Å columns using SDS micellar mobile phases are shown in Figs. 8–10, and selected chromatographic data are given in Table 2. The acidic, neutral and basic compounds were all adequately separated from the plasma peak on both the 100 and 1000 Å columns with the SDS micellar mobile phases. Interestingly, the salicylic acid peak was split into two peaks on the 1000 Å column when in the presence of bovine plasma, which did not occur when the bovine plasma was absent. This is apparently due to the peculiarities of the interactions between salicylic acid, SDS, and bovine plasma.

Retention of the drug compounds in bovine plasma decreased significantly between the 100 and 1000 Å columns with SDS mobile phase while selectivity (α) remained approximately the same (Table 2), which is particularly beneficial for the elution of mid- and high-k compounds. The efficiency of the 1000 Å column was less than the 100 Å column, although the peak widths were lower on the 1000 Å column (Table 2). In this case, peak width and efficiency are not redundant because of the large difference in retention times between the 100 and 1000 Å columns. Even though the efficiency is lower on the 1000 Å column, the narrower peak widths coupled with approximately the same selectivity as the 100 Å column may result in better resolution between sample components on the 1000 Å column compared to the 100 Å column. However, in some cases the lower efficiency of the 1000 Å column may be a problem if the analyte of interest has to be resolved from other similar compounds in the sample. The reasons for the lower efficiency of the wider-pore column are presently being investigated, but we believe that it is due at least partly to a greater resistance to mass transfer in the pores, i.e., the greater penetration of the micelles into the wider pores which lowers the effective diffusion rate of the analytes in the pores (Eq. (6)).

With the Brij-22 micellar mobile phases, the acidic and neutral compounds were all adequately separated from the plasma peak on both the 100 and 1000 Å columns. However, the basic compounds did not chromatograph well with Brij-22 micellar mobile phase and therefore they were not spiked into bovine plasma for the Brij-22 mobile phase studies. Similar trends in k, α and N were observed between the 100 and 1000 Å columns for the drug compounds in bovine plasma run with Brij-22 mobile phase as was noted above with SDS mobile phase.

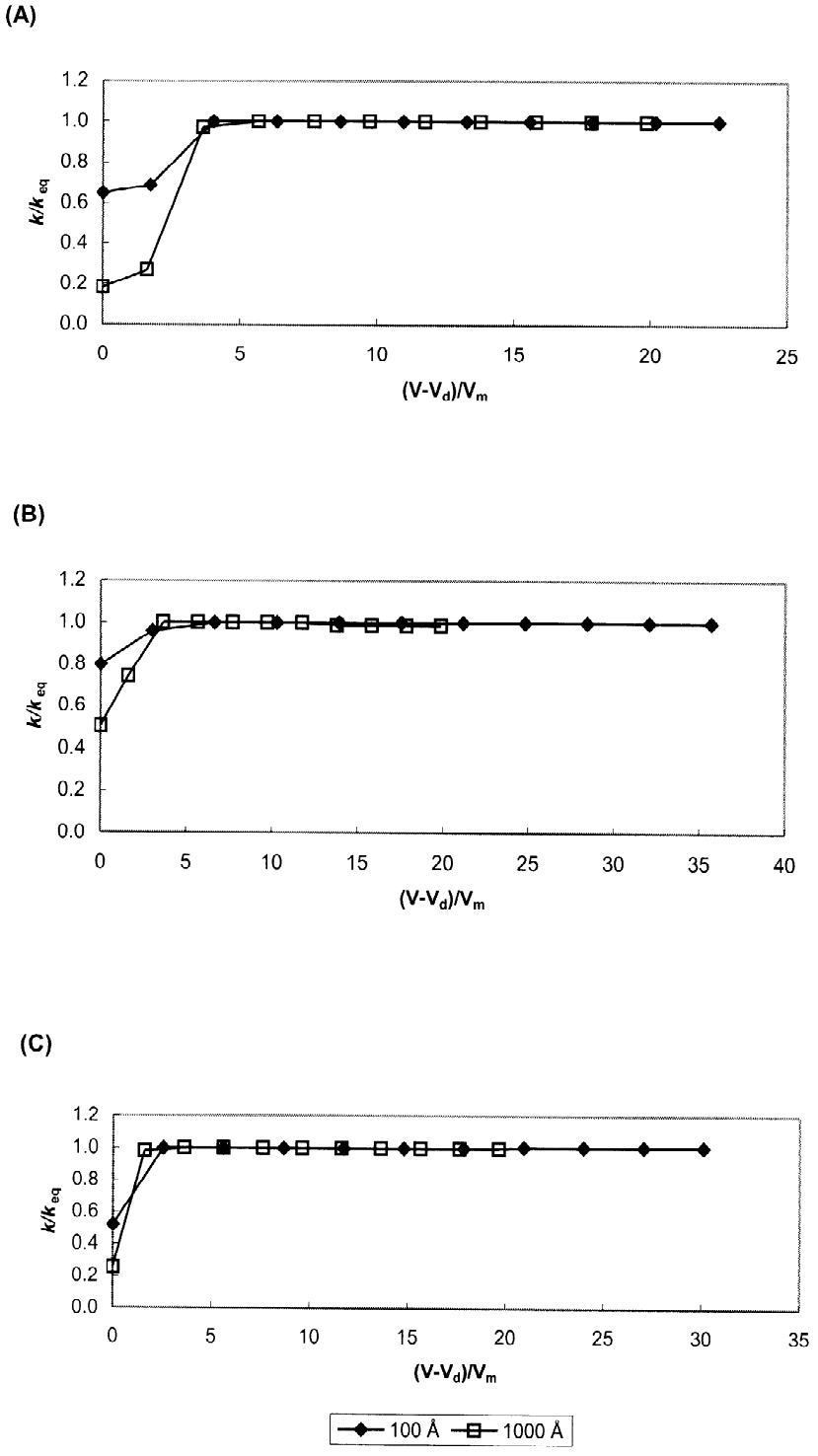


Fig. 7. MLC column re-equilibration plots for Alltech C_{18} 100 and 1000 Å pore-size columns using a micellar mobile phase of (A) SDS, (B) Brij-22, and (C) DTAB. The re-equilibration probe used for the 100 Å column was acetaminophen; for the 1000 Å column, diethylstilbestrol was employed with SDS and phenacetin was used with Brij-22 and DTAB.

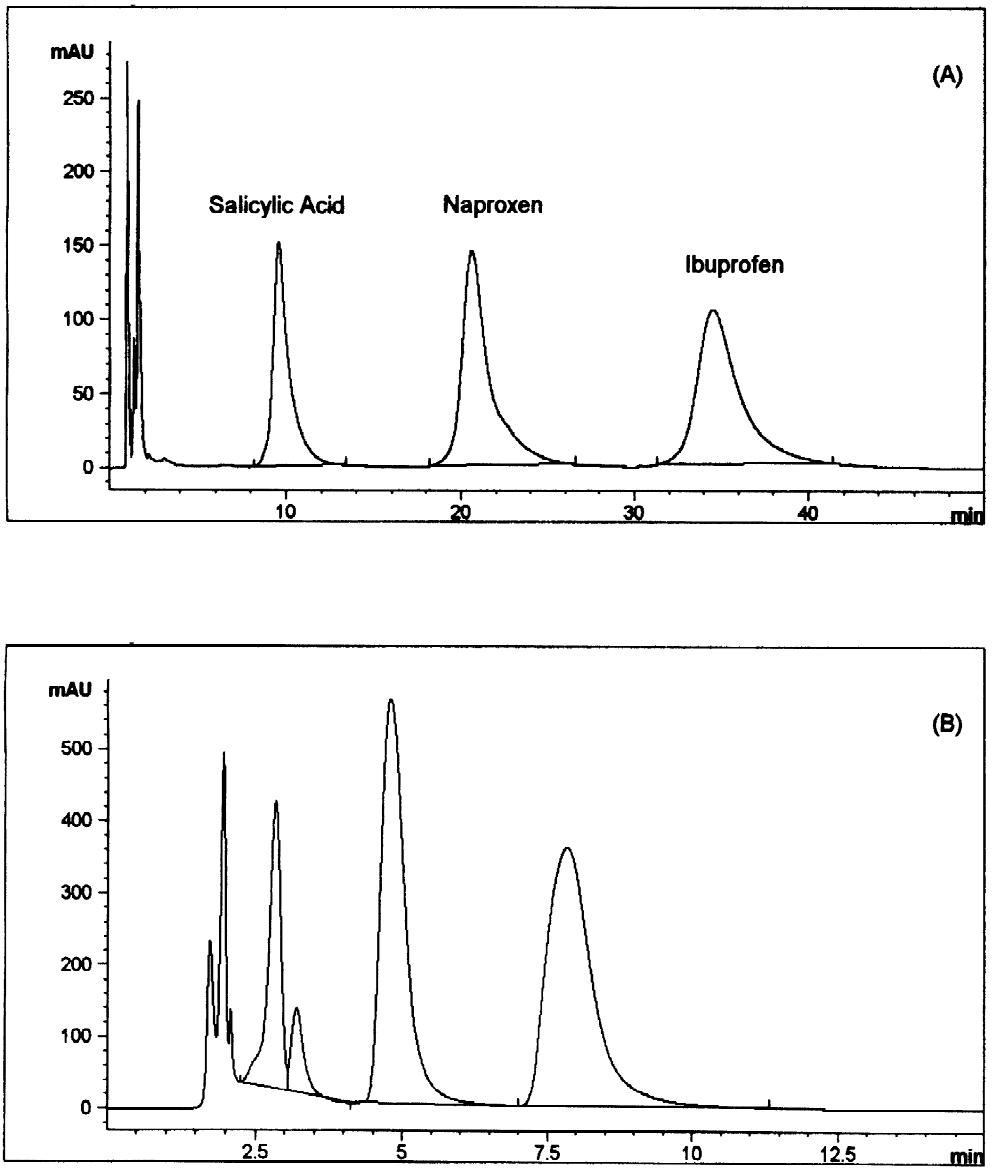


Fig. 8. Separation of a mixture of acidic drugs in bovine plasma (0.5/0.2/1.0 mg/ml salicylic acid/naproxen/ibuprofen) with 50 m*M*, pH 2 SDS mobile phase (containing 5% *n*-propanol) on Ailtech C_{18} columns: (A) 100 Å, (B) 1000 Å. Absorbance detection at 220 nm with a bandwidth of 4 nm.

Overall, the results of the spiked bovine plasma studies showed that plasma proteins do in fact elute with the void volume on the 1000 Å column as they do on the 100 Å column with both SDS and Brij-22 micellar mobile phases. Moreover, the drug compounds that exhibited excessive retention ($k>10$) on the 100 Å column could be eluted within a more optimum k range ($k<10$) on the 1000 Å column while still being adequately separated from the plasma proteins.

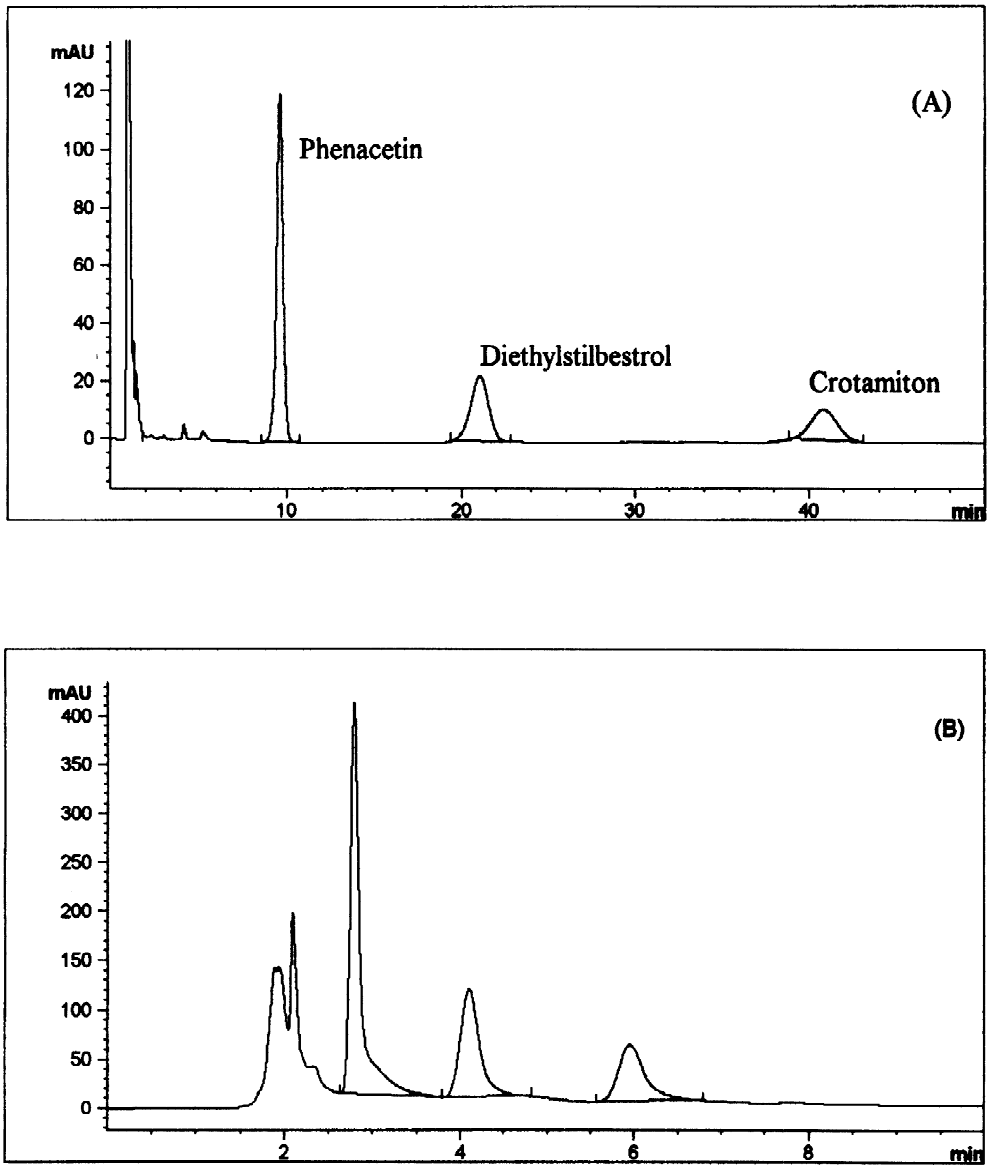


Fig. 9. Separation of a mixture of neutral drugs in bovine plasma (0.1 mg/ml each phenacetin/DES/crotamiton) with 50 m*M* SDS mobile phase (containing 5% *n*-propanol) on Alltech C_{18} Columns: (A) 100 Å, (B) 1000 Å. Detection as in Fig. 4.

3.4. Recovery of highly retained drug compounds from bovine plasma

Two drug compounds, quinidine sulfate and DPC 961, were selected for recovery studies with bovine plasma. Quinidine sulfate, a basic (pK_a 4.2, 8.8) antiarrhythmic drug [29–31], is moderately hydrophobic (Log $D = -0.07$ at pH 5.0 [32]) and moderately plasma bound (90%). Also, it was reported to be highly retained in MLC using SDS mobile phase and a C_{18} column [2]. Therefore, it was chosen for recovery studies using SDS mobile phase since basic compounds were previously shown to chromatograph well with neutral unbuffered SDS mobile phase. DPC 961, a non-nucleoside reverse transcriptase inhibitor for the treatment of AIDS which was under

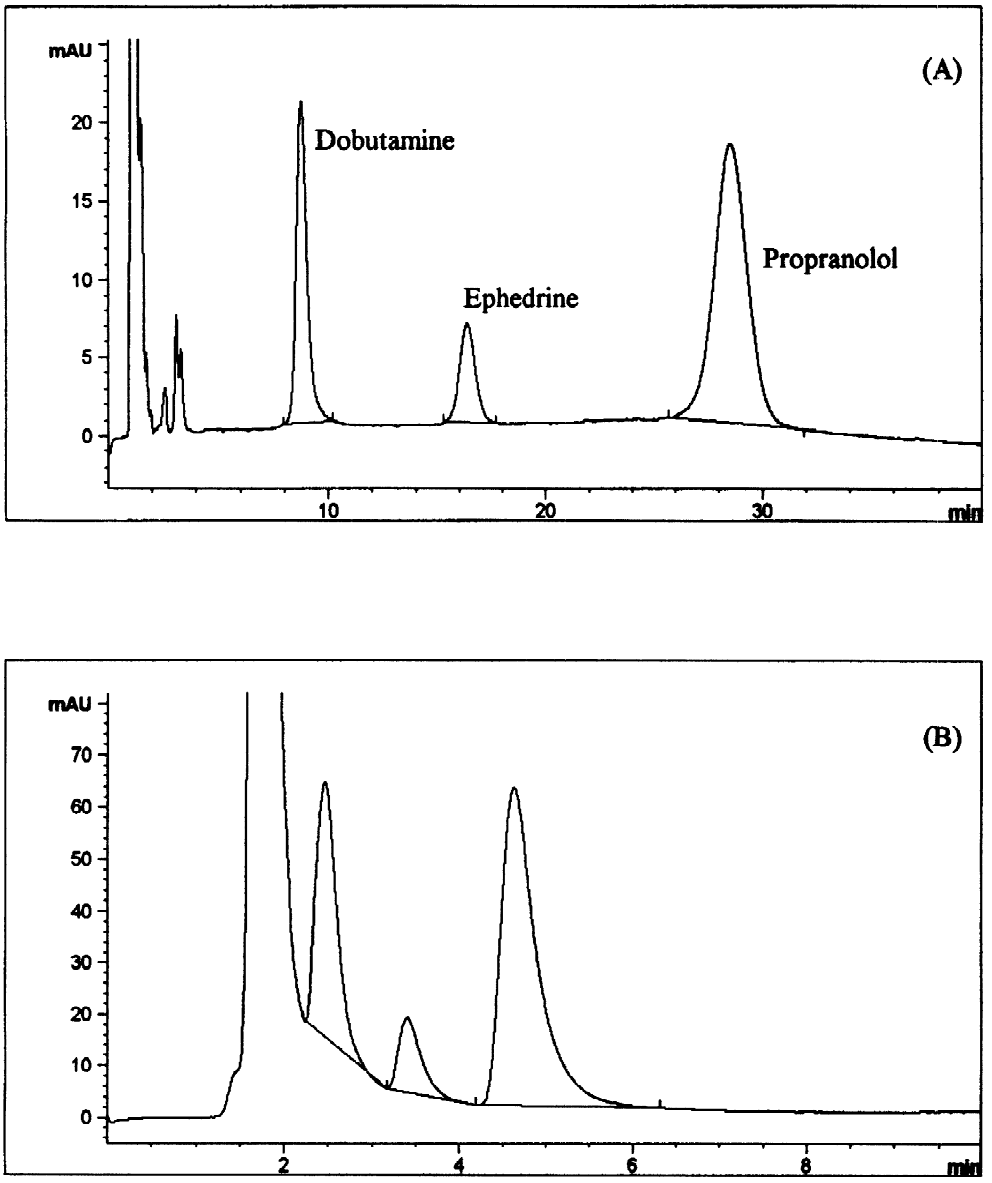


Fig. 10. Separation of a mixture of basic drugs in bovine plasma (1.0 mg/ml each dobutamine/ephedrine/propranolol) with 125 m*M* SDS mobile phase (containing 5% *n*-propanol) on Alltech C_{18} Columns: (A) 100 Å, (B) 1000 Å. Detection as in Fig. 4.

development at the DuPont Pharmaceuticals Company, is a neutral, poorly water soluble (15 μg/ml), highly hydrophobic (Log $P=3.2$), highly plasma bound (98.5%) compound. It was chosen for recovery studies using Brij-22 mobile phase since previous experiments showed that only neutral compounds chromatographed well with neutral unbuffered Brij-22 mobile phase.

Quinidine sulfate and DPC 961 were spiked into bovine plasma at concentrations of 3 and 2 μg/ml, respectively, since their therapeutic blood levels are 2–5 and 0.53 μg/ml, respectively. Recovery of quinidine sulfate from bovine plasma (Table 3) was

Table 2
Chromatographic data for acidic, neutral, and basic drug compounds in bovine plasma run on the Alltech C_{18} 100 and 1000 Å columns

Moblie phase[a]	Compound	Pore size (Å)	Retention time (min)	Peak width (min)[b]	k'	α	N^c
50 m*M* ph 2 SDS	Salicylic acid	100	9.55	0.780	5.77	2.36	830
		1000[d]	–	–	–	–	–
	Naproxen	100	20.60	1.323	13.61	1.72	1343
		1000	4.80	0.459	1.46	2.07	606
	Ibuprofen	100	34.51	2.040	23.48	n/a	1585
		1000	7.84	0.874	3.02	n/a	446
50 m*M* SDS	Phenacetin	100	9.61	0.392	5.82	2.39	3330
		1000	2.79	0.121	0.62	2.22	2945
	DES	100	21.03	1.113	13.91	2.01	1978
		1000	4.10	0.237	1.38	1.78	1658
	Crotamiton	100	40.82	1.516	27.95	n/a	4017
		1000	5.95	0.326	2.46	n/a	1845
125 m*M* SDS	Dobutamine	100	8.75	0.498	5.21	2.04	1710
		1000	2.46	0.264	0.39	2.36	481
	Ephedrine	100	16.35	0.744	10.60	1.81	2675
		1000	3.40	0.274	0.92	1.75	853
	Propranolol	100	28.51	1.508	19.22	n/a	1980
		1000	4.63	0.433	1.62	n/a	633

[a] All mobile phases contained 5% *n*-propanol. drugs given in Figs. 8–10, respectively.
[b] Peak width at half height.
[c] Efficiency calculation based on peak width at half height.
[d] Salicylic acid peak split into two peaks on the 1000 Å column.

essentially equivalent between the 100 and 1000 Å columns. The results were also in agreement with previously reported results of 100%±3% RSD for DSI analysis of quinidine spiked at 3 μg/ml [3]. DPC 961 recovery from bovine plasma (Table 3) was poor with the 100 Å column, whereas satisfactory recovery was obtained with the 1000 Å column. Also, the DPC 961 peak shape became progressively worse with the 100 Å colunm as the number of sample injections increased. The peaks became broader and more distorted with increased tailing. Conversely, the peak shape of DPC 961 remained the same throughout the chromatographic run with the 1000 Å column.

A linear response with varying analyte concentration was obtained ($r^2 > 0.99$) over the specified

Table 3
Percent recovery of quinidine and DPC 961 from spiked bovine plasma uing the 100 and 1000 Å columns

	100 Å column		1000 Å column	
	Quinidine[a]	DPC961[b]	Quinidine[a]	DPC961[b]
Mean ($n=6$):	97.7	81.7	99.1	103.8
% RSD	4.9	16.3	1.0	2.4
Coefficient of Determination (r^2)[c]	0.996	0.997	0.999	0.998
LOD (ng, at $S/N=3$)[d]	0.83	12.0	0.65	2.4

[a] Quinidine spiked at 3 μg/ml.
[b] DPC 961 spiked at 2 μg/ml.
[c] Quinidine linearity curve range: 1–5 μg/ml ($n=5$); DPC 961 linearity curve range: 0.5–4 μg/ml ($n=5$).
[d] Noise measured as peak-to-peak noise.

concentration ranges for quinidine sulfate and DPC 961 on both the 100 and 1000 Å columns (Table 3). LODs were lower on the 1000 Å column for both quinidine sulfate and DPC 961 (Table 3), which was due to the lower retention on the 1000 Å column resulting in narrower peaks and thus greater peak height. The LODs for quinidine sulfate were also much lower than the reported LOD of 0.3 μg/ml using fluorescence detection [3]. As expected, retention was significantly lower on the 1000 Å column for both compounds. In contrast, no trend was observed for the efficiency of these compounds on the 100 and 1000 Å columns.

Overall, the spiked bovine plasma recovery experiments demonstrated that large pore size columns perform equivalent to or better than small-pore columns in DSI-MLC. In addition, retention was drastically reduced, ~3–4 times, on the large-pore column at half the surfactant concentration (mobile phase strength) and with a lower LOD. Highly hydrophobic and plasma bound drug compounds such as DPC 961 can be rapidly and successfully analyzed in plasma using DSI-MLC and large pore size C_{18} stationary phases. This may provide a useful alternative to some time-consuming RPLC methods, which typically require a number of sample preparation steps in order to remove the plasma proteins from the sample.

4. Conclusion

The effectiveness of large-pore stationary phases for the analysis of hydrophobic, highly retained solutes in micellar liquid chromatography (MLC) was demonstrated using gradient elution and an alkylphenone test mixture. The inability to elute and separate all 11 alkylphenones on the 100 Å column using linear and exponentional micellar gradients is most likely the result of micellar exclusion effects occurring on the small-pore column. However, since exclusion effects are overcome on large-pore columns, all 11 alkylphenones were separated on the 1000 Å column with micellar gradients. Gradient elution in MLC is therefore more effective with large pore-size columns, which, in practice, should aid chromatographers who wish to employ MLC to separate highly hydrophobic compounds. Although exponential surfactant gradients (linear pM gradients) provide greater resolution for both small- and large-pore phases, linear surfactant gradients are more practical small-pore phases because of the lower retention times.

Gradient re-equilibration studies with micellar mobile phases have shown that column reequilibration after a micellar mobile phase gradient is just as rapid on large-pore columns as it is on small-pore columns. The number of column volumes required for re-equilibration on both the large and small pore-size columns was considerably lower with the micellar mobile phases than that typically required with hydro-organic mobile phases. Thus, the important capability of rapid gradient re-equilibration in MLC has been demonstrated to be applicable to large pore-size stationary phases.

The results of the direct sample injection (DSI) studies demonstrate the advantage of using large pore-size stationary phases for the DSI of drugs in biological fluids using MLC. It was shown that plasma proteins elute in the void volume on large-pore columns with both SDS and Brij-22 micellar mobile phases. Also, highly retained drug compounds were eluted much more rapidly off of the large-pore column while still being adequately separated from the plasma proteins. Recovery of moderately-to-highly hydrophobic and protein bound drugs from bovine plasma was found to be equivalent or better with the large-pore column compared to the small-pore column. In addition to drastically reduced retention, only half the surfactant concentration was needed in the micellar mobile phases with the large-pore columns; detection limits were also somewhat lower.

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