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Direct injection of untreated serum using non-ionic and ionic micellar liquid chromatography for determination of drugs

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Abstract: The chromatographic behaviour of nonionic micelles used as the mobile phase in liquid chromatography is similar to that of anionic and cationic micellar mobile phases, with reversal of retention order as the micelle concentration is varied. This behaviour follows the prediction of the model developed for anionic surfactants. Nonionic micelle chromatographic interactions are simplified by reducing the extent of electrostatic interactions with the solute and, as a result, their retention dependencies more closely follow the mathematical predictions. Drugs in blood serum samples are quantitatively determined by direct injection of the serum onto the chromatographic column, with no column clogging or pressure build-up. The results are similar to those found with anionic micellar mobile phases, but are in contrast to the protein precipitation observed with cationic micelles. Solute–micelle equilibrium constants and the critical micelle concentration of Brij-35, determined chromatographically, are reported. The potential usefulness of nonionic micelles for the determination of theophylline, paracetamol, phenobarbital, carbamazepine, quinine, quinidine, morphine, codeine and cocaine is demonstrated.

Keywords: *Therapeutic drug monitoring; micellar liquid chromatography; diastereomeric separation; cocaine; narcotics; chloramphenicol; analgesics; antiarrhythmic drugs.*

Introduction

Ionic surfactants at concentrations below the critical micelle concentration (CMC) have been used widely as mobile phase modifiers to enhance the separation of oppositely charged solutes [1–3]. A more complex mobile phase containing surfactants above their CMC, such as those formed by anionic sodium dodecyl sulfate (SDS), can interact with solutes by hydrophobic interactions with the hydrocarbon portion, and by coulombic interactions with the ionic head groups. These heterogeneous micellar properties produce unique solute–micelle association mechanisms which have been used to generate room temperature phosphorescence [4, 5], in reaction kinetics [6], for improvement of recovery of ubiquinone-10 from plasma [7], and as mobile phases in liquid chromatography (LC) [8–18]. Their powerful solubilizing properties allow direct injection of untreated serum and urine onto the chromatographic column for the determination of drugs without column clogging or pressure build-up [15, 16].

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Because micellar mobile phases provide both hydrophobic interaction sites which mimic aqueous/organic mobile phases, as well as electrostatic interaction sites, they can provide additional selectivity in separations. For example, retention order reversals occur as the surfactant concentration is varied [8–10]. For aqueous micellar solution, reduced mass transfer can diminish chromatographic efficiency in some separations. However, Dorsey and co-workers demonstrated efficiencies approaching those of hydro-organic mobile phases, when as little as 3% v/v propanol is added to the micellar mobile phase and the column temperature raised to 40°C [11]. Moreover, micellar concentration gradients speed the elution of mixtures of compounds without the lengthy re-equilibration step between samples [12]. Only one column system volume of the initial micelle concentration is necessary to return to initial system conditions.

Prediction of solute capacity factors as a function of surfactant concentration and pH can be made using equations that have been developed which also permit calculation of solute–micelle equilibrium constants [13, 14]. For neutral solutes, the capacity factor increases with increasing surfactant concentration to a maximum, then declines parabolically. This behaviour allows calculation of the micelle CMC value from chromatographic data. For example, Dorsey and co-workers determined the CMC of sodium dodecylsulphate (SDS) in 10% v/v aqueous propanol [11]. Since the separation process is controlled by both hydrophobic and electrostatic interactions, the nature of the interactions controlling the separation would be expected to be different depending on the hydrophobicity and charge type of the solute. For 2-naphathalenesulphonic acid anion, the retention time increases with SDS micelle concentration, while the opposite behaviour is observed for the more hydrophobic 1-pyrenesulphonic acid anion [14]. The equations fail to predict the chromatographic behaviour of charged species because no terms are included to describe the complex electrostatic interactions with the charged headgroups.

Deviations from prediction for charged species can be reduced by the use of nonionic surfactants such as Brij-35, where electrostatic interactions are much less, allowing better control in studies of micelle–solute–stationary phase interactions. Previous studies showed that anionic surfactants effectively solubilized serum proteins permitting direct serum injection, but serum components precipitated when using cationic surfactants. This paper examines the ability of previously developed equations to predict the retention behaviour of solutes using the nonionic surfactant, Brij-35. The model accurately predicted the nonionic micelle liquid chromatographic behaviour of naphthalene, phenol, 2-naphthalenesulphonic acid and chloramphenicol over certain surfactant concentrations, and the experimental LC data were used to determine solute–nonionic micelle equilibrium constants and the CMC of the Brij-35. The potential usefulness of nonionic micelles, such as Brij-35, for the determination of drugs via direct injection of blood serum onto the chromatographic column, in particular, theophylline, acetaminophen, phenobarbital, carbaxazepine, quinine, quinidine, morphine, codeine and cocaine, is demonstrated.

Experimental

Apparatus

The high-performance liquid chromatography (HPLC) system consisted of a 'Fast LC' high pressure pump (Technicon, Inc., Tarrytown, NY), a LDC UV monitor detector (254 nm) (Laboratory Data Control, Rivera Beach, FL), and a Model FS970 LC

fluorometer (Kratos Instruments, Ramsey, NJ). The fluorometer settings are given in the figure captions. The columns were packed with: 5-μm Supelcosil CN (15 cm × 4.6 mm i.d.); 5-μm Supelcosil CN (25 cm × 4.6 mm i.d.); 5-μm Supelcosil C-18 (25 cm × 4.6 mm i.d.); and 10-μm μ-Bondapak C-18 (30 cm × 3.9 mm i.d.) (Waters Associates, Milford, MA). A precolumn (12.5 cm × 4.6 mm i.d.) (Whatman, Inc., Clifton, NJ) packed with silica gel (25–40 μm) (Whatman, Inc.) was located between the pump and the sample injector to saturate the mobile phase with silica to minimize dissolution of the analytical column packing. A Model 5000 Fisher strip chart recorder was used to record the chromatograms.

Reagents

Electrophoresis grade SDS obtained from Bio-rad, Inc. (Rockville Center, NY), and polyoxyethylene(23)dodecyl (Brij-35) from Sigma Chemical Co., St Louis, MO, were used as received. Serum blank samples were obtained from the General Diagnostic Division of Warner Lambert (Morris Plains, NJ). All solvents and sodium acetate were obtained from Fisher Scientific Co., Springfield, NJ, and the water was steam distilled. All solutes and other reagents were used as received.

Procedures

Micellar mobile phases were prepared by dissolving the appropriate amount of surfactant in distilled water or 0.05 M sodium acetate buffer containing the specified amount of organic modifier, followed by filtration through a 0.45-μm Nylon-66 membrane filter (Rainin Instruments, Woburn, MA), and were degassed under vacuum prior to use. Methanolic stock solutions of solutes were diluted to the desired concentrations with distilled water or dilute methanol. For direct serum injection, aliquots of the stock solutions were diluted with blank serum, and these serum standards were injected directly into the LC system. The specific conditions used for the chromatograms are given under the figure captions.

Results and Discussion

A quantitative equilibrium-based model that relates experimental chromatographic behaviour of neutral solutes to the concentration of SDS micelles in the mobile phase was developed recently [13]. The primary equation based on earlier work by Horváth and co-workers [2] is given below,

$$k' = \frac{\phi\,[L_s]K_1}{1 + K_2[M_m]} \tag{1}$$

where k' is the capacity factor of injected solute; ϕ is the ratio of the volume of the stationary phase, v_s, to the volume of the mobile phase, v_m, in the column; $[L_s]$ is the molar concentration of stationary phase sites; K_1 is the solute–stationary phase equilibrium constant; and K_2 is the equilibrium constant between the solute per monomer of surfactant in the micelle. The term, $[M_m]$, is the molar concentration of surfactant in the micelle in the mobile phase obtained from the relationship: $[M_m]$ = [surfactant] − CMC. Equation (1) predicts parabolic dependence of k' on $[M_m]$, and linear dependence of $1/k'$. The validity of this model was tested for nonionic micellar LC. Although it was not the purpose of this study, the pH dependence of k' must be

considered for ionizable solutes. Equations extending the one shown above have been developed to account for the pH of the mobile phase and the solute pK_a, and they predict sigmoidal dependence of k' with pH, as confirmed experimentally [14].

Role of Brij-35 mobile phase concentration

The capacity factors of four solutes measured at thirteen different concentrations of Brij-35 from 0 to 0.1 M are given in Table 1. Note that the CMC of this surfactant is 0.0001 M. The elution behaviour of 2-naphthalenesulphonic acid is considerably different compared to the other three compounds, and is illustrated graphically in Figure 1. The capacity factor increases with increasing Brij-35 concentration up to 0.02 M,

Table 1
Variation of capacity factors versus Brij-35 mobile phase concentration for four compounds on a Supelcosil CN column

[Brij-35]* (M)	Naphthalene	Chloramphenicol	Phenol	2-Naphthalenesulphonic acid
0	232.0	9.78	7.0	0.28
0.00005	189.0	10.00	7.58	0.50
0.00010	197.0	10.93	8.31	0.53
0.00025	205.0	11.23	8.67	0.57
0.0005	163.0	11.00	8.47	0.67
0.0010	126.0	10.71	8.36	0.98
0.0025	74.0	9.91	7.94	2.71
0.005	44.0	8.82	7.36	5.37
0.010	24.15	7.24	6.36	7.45
0.020	12.67	5.24	4.93	8.52
0.040	6.58	3.42	3.43	7.83
0.080	3.33	2.05	2.18	5.85
0.10	2.68	1.70	1.85	5.60

* The CMC and aggregation number of Brij-35 are 0.0001 M and 40, respectively [19].

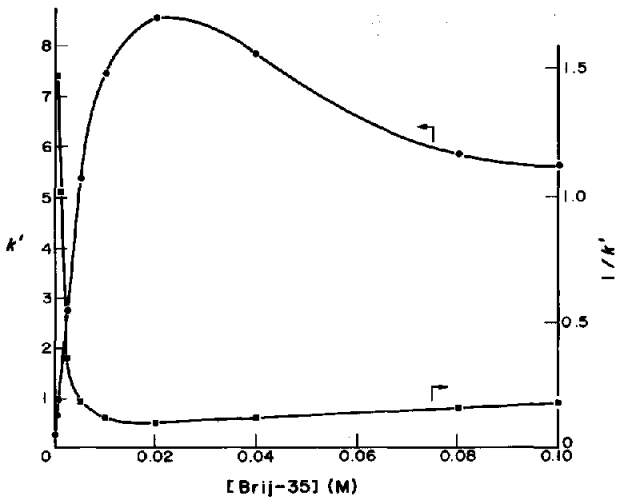

Figure 1
Dependence of k' and $1/k'$ on the concentration of Brij-35 for 2-naphthalenesulphonic acid: column, Supelcosil CN (15 cm); flow rate, 2.0 ml/min; mobile phase, aqueous Brij-35; UV detection at 254 nm.

followed by a gradual parabolic decline. At concentrations greater than the CMC, but less than 0.02 M, electrostatic repulsion effects dominate over hydrophobic attraction, causing an increase in capacity factor (expulsion of the anionic solute from the mobile phase). As the surfactant concentration approaches 0.02 M, the two opposing forces tend to balance one another, slowing the rate of increase in k' as 2-naphthalenesulphonic acid tends to solubilize into or onto the micellar assemblies. At Brij-35 concentration >0.02 M, the elution behaviour follows the prediction of equation (1) of parabolic dependence of k' on $[M_m]$. This behaviour is in contrast to that found for the same solute using anionic SDS micellar mobile phases, where k' increased over a wide SDS concentration range, when all concentrations studied gave increases in k' vs $[M_m]$ [14]. This resulted because of the much stronger electrostatic repulsion of the negative SDS headgroups with the anionic solute, preventing dominance of hydrophobic attraction to the micelle. The much less polar nonionic micelle exhibits less coulombic repulsion and can associate with negative solutes over certain micelle concentration ranges.

These results suggest that Equation (1), which accurately predicts the chromatographic behaviour of neutral solutes with charged surfactants but not that of charged solutes with the similarly-charged surfactant, can more accurately model behaviour as the electrostatic nature of the surfactant is reduced (nonionic surfactants). However, the CMC values of nonionic surfactants cannot be determined using charged species because they must be effectively associated with the micelle at surfactant concentrations near the CMC. The plot shown in Fig. 1 does not show a break in the slope at the CMC because the anionic solute is only weakly, if at all, associated with the micelle. Neutral solutes would allow determination of nonionic surfactants CMC values.

Similar changes were observed in the k' behaviour of naphthalene, phenol and chloramphenicol. The results for chloramphenicol shown in Fig. 2 are typical of neutral solutes. A sharp change in k' occurs at approximately 0.00025 M Brij-35, indicating that the surfactant is undergoing micellization. This is in fair agreement with the literature CMC value of 0.0001 M [19]. Assuming that the 0.00025 M value corresponds to the CMC, the solute–micelle equilibrium constants can be calculated from the slope/intercept of the plot of $1/k'$ versus $[M_m]$, as shown in Table 2 together with the associated statistical analysis.

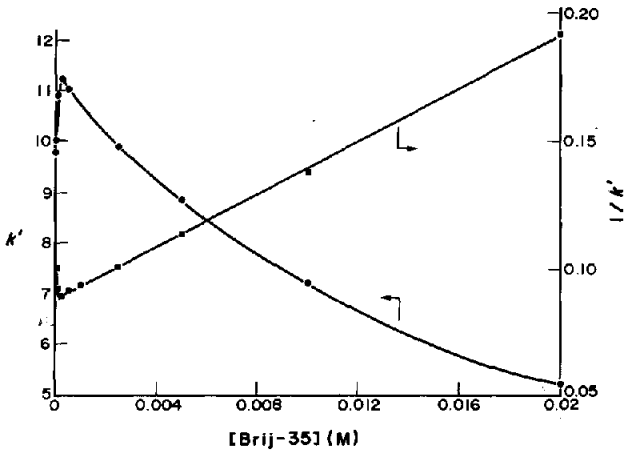

Figure 2
Dependence of k' and $1/k'$ on the concentration of Brij-35 for chloramphenicol: chromatographic conditions same as in Fig. 1.

Table 2
Solute–micelle equilibrium constants calculated from chromatographic retention data with associated statistical analysis

Compound	Slope	Intercept	% RSD		k_{eq} (l/mol)*
			Slope	Intercept	
Phenol	4.28	0.117	0.48	0.75	1.46×10^3
Chloramphenicol	5.00	0.0921	0.29	0.70	2.17×10^3
Naphthalene	3.69	0.00528	0.10	2.96	2.80×10^4

* K_{eq} is the solute bulk phase–micelle equilibrium constant per micelle obtained by multiplying the ratio of the slope/intercept from plots of $1/k'$ vs [Brij-35] by 40, its aggregation number [19].

Direct serum injection

HPLC has drawbacks for the routine analysis of drugs in biological fluids. These include lengthy analysis time and tedious sample preparation because the protein must be precipitated or the drug extracted to prevent column clogging. These additional steps also increase the error in the analysis results. Micellar HPLC provides a unique solution to these problems by solubilizing the protein components in the micellar medium, thus allowing direct injection of biological fluids on to HPLC columns with no column clogging or build-up of back-pressure. In addition, the surfactant monomers appear to displace the drug bound to the protein, releasing it to partition to the stationary phase. The protein components elute on or near the solvent front, and the separated drugs elute in the normal fashion.

Figure 3A shows the elution behaviour of serum blank, where most serum components elute at the solvent front. Figures 3B and 3C show the chromatograms using a Supelcosil CN column after injection of 20 μg/ml chloramphenicol dissolved in a serum matrix and in distilled water, respectively, both eluting at approximately 15 min. The resolution in the serum medium is sufficient to quantitatively determine chlorophenicol, and the sensitivity is adequate to monitor the normal therapeutic range in serum (10–20 μg/ml). The peak heights of the drug in Figs 3B and 3C are equal, indicating that the protein-bound drug is completely displaced by the surfactant monomers and/or micelles in the mobile phase.

Analogous results are obtained with C-18 columns. Figure 4 contains chromatograms of serum blank and serum containing 20 μg/ml chloramphenicol using a Supelcosil C-18 column and a Brij-35 aqueous mobile phase. The elution profiles are similar to those using a CN column. Figures 5 and 6 illustrate the typical chromatographic performance of Brij-35 mobile phases for selected drugs using Supelcosil CN and C-18 columns, respectively. Theophylline (peak 1) and acetaminophen (peak 2) elute rather quickly, and a lower surfactant concentration should be used for optimum separation. All of the drugs studied in Figs 5 and 6 have been quantitatively determined in serum using SDS micellar mobile phases [15]. Note that the retention orders obtained on the two different columns are different, and that the change in the order is column related and not due to surfactant concentration effects.

Effect of added organic modifiers to SDS mobile phases

The primary equilibria generally recognized to be important in micellar chromatography are those of the solute between the bulk water and the micelle aggregate, K_2, and of the solute between the bulk water and the stationary phase, K_1. Bulk phase water in

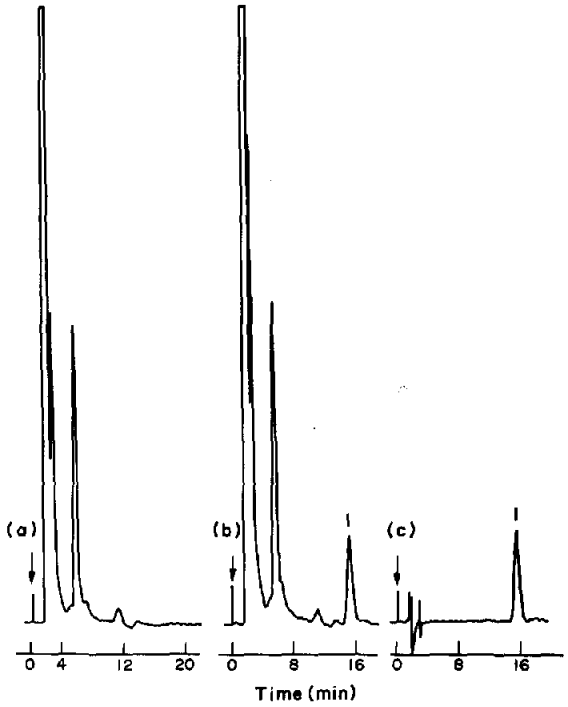

Figure 3
Chromatograms of (A) serum blank, (B) serum with 20 μg/ml chloramphenicol (1) added, and (C) 20 μg/ml chloramphenicol in water: column, Supelcosil CN (25 cm); mobile phase, 0.04 M aqueous Brij-35; flow rate, 1.0 ml/min; UV detector sensitivity, 0.08 a.u.f.s. at 254 nm.

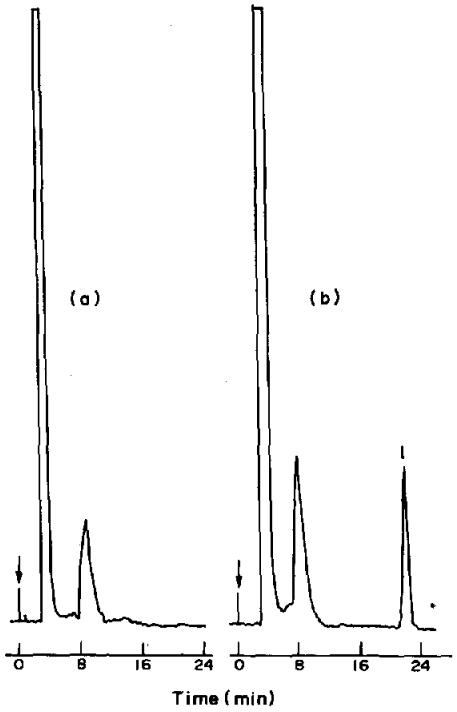

Figure 4
Chromatograms of (A) serum blank, (B) serum blank with 20 μg/ml chloramphenicol: column, Supelcosil C-18; mobile phase, 0.08 M aqueous Brij-35; flow rate, 0.6 ml/min; UV detector sensitivity, 0.08 a.u.f.s. at 254 nm.

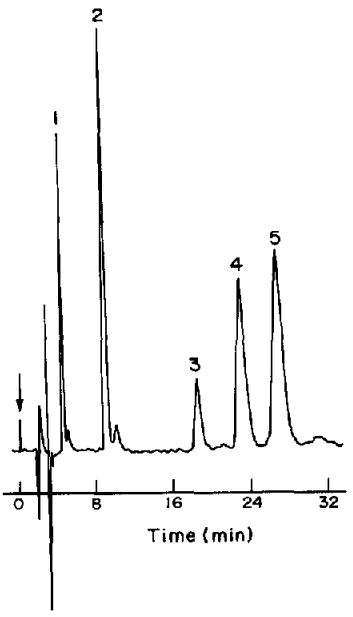

Figure 5
Chromatograms of (1) 10 μg/ml theophylline, (2) 10 μg/ml acetaminophen, (3) 40 μg/ml phenobarbital, (4) 40 μg/ml chloramphenicol, and (5) 20 μg/ml carbamazepine: column, Supelcosil CN (25 cm); mobile phase, 0.04 M aqueous Brij-35; flow rate, 1.0 ml/min; UV detector sensitivity, 0.08 a.u.f.s. at 254 nm.

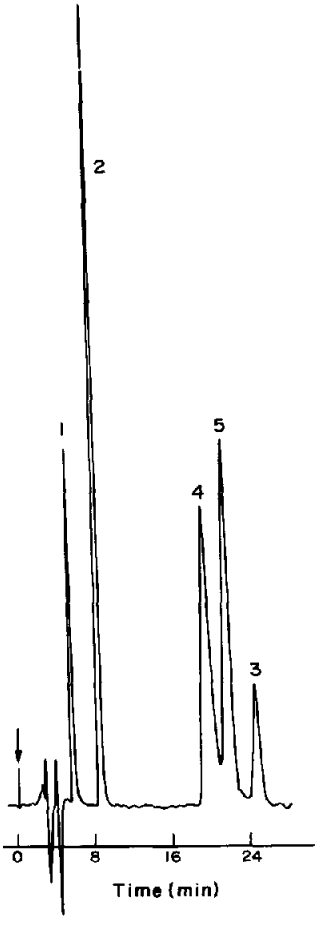

Figure 6
Chromatograms of the same sample shown in Fig. 5: column, Supelcosil C-18 (25 cm); mobile phase, 0.08 M aqueous Brij-35; flow rate, 0.6 ml/min; UV detector sensitivity, 0.08 a.u.f.s. at 254 nm.

these systems acts as a barrier through which the solute must pass, and it slows down mass transfer between the micelles and the stationary phase. The key to improving the efficiency of separation is to reduce the water barrier by addition of organic modifiers, or to increase the mass transfer rate constants by other means such as elevated temperature, reduced flow rate, and/or reduced micelle concentration [17].

Organic modifiers, such as propanol, present in the micellar mobile phase increase chromatographic efficiency by wetting the hydrophobic stationary phase surface and thus promoting transfer from the aqueous phase [11]. They also blend with the bulk water phase, making it less polar, allowing nonpolar solutes to more rapidly exit the micellar assembly and desorb from the stationary phase, thereby improving efficiency [17]. Organic modifiers must not adversely alter the micelle characteristics which provide the distinct advantage of selectivity afforded by micellar chromatography. If they only alter kinetic and thermodynamic processes, and do not damage the integrity of the micellar aggregate, then improved efficiency can be achieved with no loss in selectivity. Selectivity for certain compounds can also be improved by using detection schemes such as room temperature phosphorescence or sensitized room temperature biacetyl phosphorescence [18, 20].

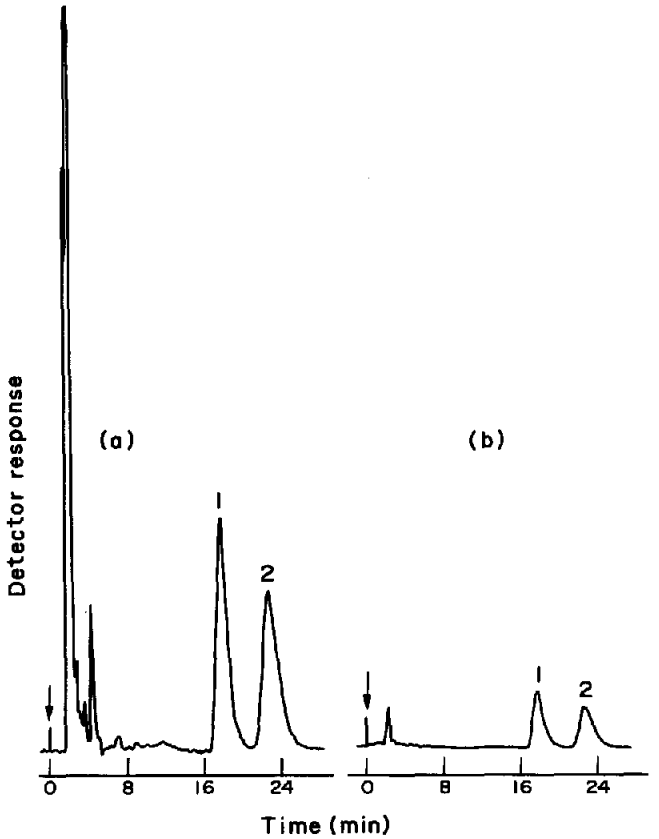

Figure 7
Chromatograms of serum blank with 2 μg/ml added quinine (1) and 2 μg/ml added quinidine (2): column, Supelcosil CN (25 cm); mobile phase, 0.08 M aqueous Brij-35 with 10% propanol added; flow rate, 1.0 ml/min; fluorescence detector voltage, 700 V; sensitivity range, 0.2 μA; excitation wavelength, 254 nm; emission cutoff filter (A) 300 nm and (B) 470 nm.

The effect of 10% propanol in a 0.08 M SDS mobile phase is shown in Fig. 7 for the separation of quinine and quinidine alkaloids in directly injected blood serum. The two solutes are diastereoisomers and elute at the same time at all concentrations of aqueous SDS mobile phase employed. However, the addition of 10% propanol to the SDS mobile phase results in good baseline separation. This indicates that propanol can not only enhance sensitivity by improving efficiency, but can also enhance selectivity. Generally, other organic modifiers such as acetonitrile, 2-propanol and methanol can be used with similar results, although propanol gave better selectivity and methanol gave the worst selectivity. The longer hydrocarbon chain of propanol may be incorporated into the micelle to form co-micelles, and it can more effectively reduce the polarity of the bulk water phase. Both of these factors should facilitate mass transfer.

Choice of excitation and emission filter

Improvement in signal-to-noise (S/N) ratio and therefore sensitivity is also illustrated in Fig. 7A and 7B. The difference in chromatographic conditions in the two traces only concerns the emission cutoff filters used in the fluorometric detector. Figures 7A and 7B were obtained using 300 and 470 nm filters, respectively, and the results show complete elimination of the serum background signal simply by using an appropriate emission cutoff filter.

Careful consideration of the optimum excitation wavelength must be done when using fluorescence detection of chromatographic eluates. It was found that an excitation wavelength of 215 and a 300 nm emission cutoff filter provided optimum results for the determination of drugs in serum and urine using SDS mobile phases [16]. In that study, the serum background signal was found to be the limiting factor in improvements in limits of detection (LOD). In the present study, use of a 254 nm excitation wavelength produced a considerable reduction in the serum background signal. Thus, for optimum LOD values, the chromatograms should be obtained at various excitation wavelength intervals. Figures 8A and 8B illustrate the changes in peak height for several drugs excited at 254 and 215 nm, respectively. The greatest fluorescence intensity was obtained for morphine, codeine and cocaine using 215 nm excitation, and for quinine and quinidine using 254 nm excitation. Unfortunately, for the analysis of mixtures, a compromise wavelength capable of being absorbed by all components must be chosen using both absorbance or fluorescence detection modes.

Conclusions

Both nonionic and anionic micellar mobile phases provide remarkably reproducible, sensitive and rapid results for the analysis of drugs in body fluids such as blood serum and urine. Addition of organic modifiers not only improves chromatographic efficiency and sensitivity, but can also improve selectivity. The background response level of unretained proteins can be completely eliminated in many systems by use of fluorescence detection with an emission filter whose wavelength is longer than that of the protein fluorescence. Further studies in progress involve the use of room temperature phosphorescence and sensitized room temperature biacetyl phosphorescence for the determination of other drugs such as naproxen and cocaine in body fluids. These phosphorescence-based detection schemes have the decided advantage of producing considerably red-shifted spectra, well away from the protein fluorescence, allowing improved S/N ratios and limits of detection.

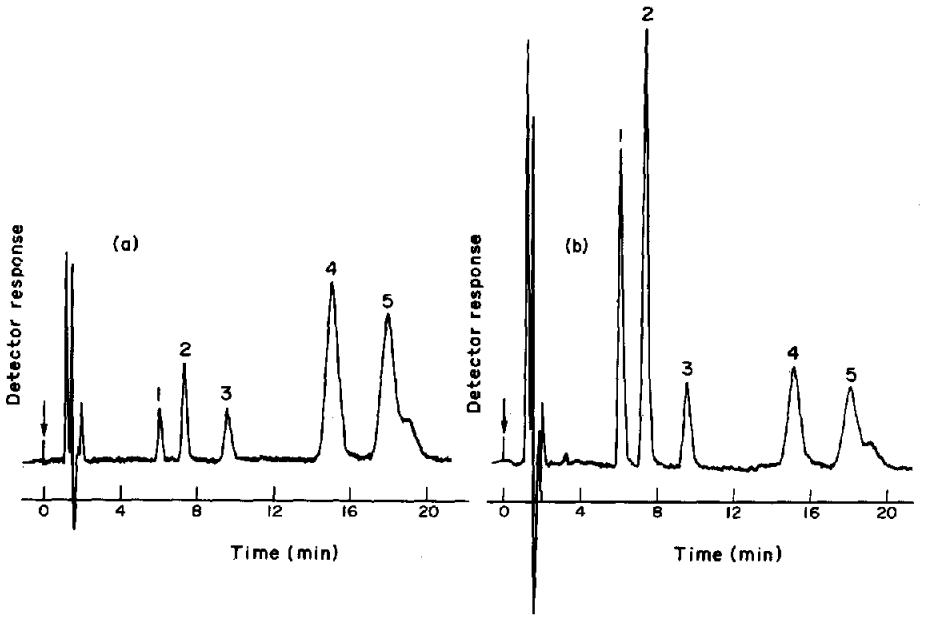

Figure 8
Characteristic separation pattern of (1) 20 μg/ml morphine, (2) 20 μg/ml codeine, (3) 200 μg/ml cocaine, (4) 0.5 μg/ml quinine, and (5) 0.5 μg/ml quinidine: column, Supelcosil CN (25 cm); mobile phase, 0.04 M SDS with 10% propanol and 0.05 M acetate buffer (pH 4.6) added; flow rate, 2.0 ml/min; fluorescence detector voltage, 700 V; sensitivity range, 0.1 μA; excitation wavelength, (A) 254 nm and (B) 215 nm; emission cutoff filter, 300 nm.

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