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Review

Application of micellar mobile phases for the assay of drugs in biological fluids

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CONTENTS

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LIST OF ABBREVIATIONS

BRIJ® 35	Polyoxyethylene(23)dodecanol
CMC	Critical micelle concentration
CTAB	Hexadecyltrimethylammonium bromide
DSI	Direct serum injection
EMIT	Enzyme-multiplied immunoassay technique
GC	Gas chromatography
HPLC	High-performance liquid chromatography
ISRP	Internal-surface reversed-phase
k'	Capacity factor
MTX	Methotrexate
SDS	Sodium dodecyl sulfate
S/N	Signal-to-noise ratio
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography

1. INTRODUCTION

The assay of drugs in biological fluids presents many difficult analytical problems. In pharmacokinetic studies the drugs are typically present in the low ng/ml concentration range, while in therapeutic drug monitoring they are usually present in μg/ml concentrations. Frequently, the drugs are strongly bound to proteins and are present in a complex matrix. The analytical techniques used to overcome the above difficulties include immunoassays, enzyme-multiplied immunoassay (EMIT) and chromatographic methods, *i.e.*, thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC), coupled with both liquid–liquid and solid-liquid extraction [1–3].

The direct injection of biological fluids into a liquid chromatograph using conventional organic–water mobile phases is usually unfeasible due to the presence of endogenous compounds and high-molecular-mass proteins which tend to precipitate within the analytical column, leading to rapid loss of chromatographic efficiency and a large increase in column back-pressure. Thus, the drugs must first be extracted from their matrix and preconcentrated before an HPLC assay can be performed. Extraction and preconcentration steps are usually very labor-intensive and time-consuming, with each step introducing an additional source of error. While the use of robotics can allow complex sample preparation to be carried out with high precision and minimal labor costs, the equipment and devel-

opment time for such methods are only justified for cases where high sample throughput over an extended time period is expected. Previous workers who have attempted the direct injection of serum, using conventional acetonitrile or methanol–water mobile phases, reported an increase in column back-pressure, presumably due to the precipitation of proteins on the head of the column or in the injection port [4,5].

The problems associated with the direct injection of biological fluids onto an HPLC column, *i.e.* precipitation and column plugging, can be eliminated by the use of micellar mobile phases. Direct injection of biological fluids onto an HPLC column can be accomplished by the use of micellar mobile phases (instead of the traditional organic–water mobile phases). Micellar mobile phases are able to solubilize proteins that are present in the sample matrix and thus prevent their precipitation The major advantage to the use of micellar mobile phases for the analysis of biological fluids is the complete elimination of all sample preparation steps (and the errors associated with these steps), which results in a significant labor and time savings. Shihabi [6] and Westland [7] have discussed the analyses of drugs by direct serum injection (DSI). The scope of this paper is to discuss the use of micellar mobile phases for the analysis of biological fluids and not micelle theory, since it has been presented elsewhere.

2 SAMPLE PREPARATION TECHNIQUES

A major impetus to the development of techniques which use micellar mobile phases for the analysis of biological fluids was the elimination of sample preparation steps. It is desirable to reduce the complexity and amount of sample preparation because of the inherent problems associated with all sample preparation techniques (poor sample recovery and labor-intensive steps). Significant effort has been directed toward reducing the analysis time and number of steps required for the sample preparation, which has had the effect of increasing the efficiency and accuracy of the assays. Internal standards are frequently added to a sample before the sample preparation begins to account for the loss in analyte during the sample preparation steps and to increase the accuracy of the assay In order to more fully appreciate the advantages of using a technique which requires no sample preparation, some of the more common sample preparation techniques and their disadvantages are briefly discussed below.

2.1. Precipitating agents

A significant advantage of direct injection of biological fluids is the absence of a sample preparation step. Typical drug assays using HPLC require at least some sample preparation, with precipitation of the proteins in serum samples [with organics, sodium hydroxide, or trichloroacetic acid (TCA)] usually being the minimum prerequisite for HPLC analysis [8–12]. Without precipitation of the

proteinaceous material, the analytical column will quickly deteriorate with a concomitant increase in column back-pressure. More elaborate sample preparation techniques are frequently required, since drugs that are strongly bound to the proteins present in the sample may co-precipitate with the proteins. After precipitation of the serum proteins, the analyte is frequently extracted into an organic solvent

2.2. Ultrafiltration

Ultrafiltration has been used by Koenigbauer *et al* [13] for the determination of free (unbound) diazepam in serum and by Miller and Pinkerton [14] for the determination of free phenytoin in plasma Ultrafiltration removes the plasma or serum proteins in the sample, but it also removes any drug that is bound to the proteins, thus this technique is only applicable for the determination of the unbound drug in biological fluids.

2.3. Internal-surface reversed-phase columns

Internal-surface reversed-phase (ISRP) columns, first developed by Hagestam and Pinkerton [15,16], contain packing material whose particles have had their internal surfaces coated with a reversed-phase material, while their external surfaces are coated with a hydrophilic, non-protein-adsorbing material. This allows for the direct injection of biological samples (serum of plasma) without any sample preparation. Upon injection of biological samples, the proteins present in the sample pass through the column without adsorbing onto the packing material, since they are too large to enter the pores of the packing material. The analyte is able to enter the internal pores of the packing material and is preferentially retained by the reversed-phase surface. The disadvantages with this technique are the small sample volumes that must be used ($\leq$ 20 μl), the limited amount of organic solvent that may be present in the mobile phase ($\leq$ 25%), and the relatively high cost of these columns as compared to conventional C_{18} columns. Also, these columns tend to be less hydrophobic and less efficient (having only about one third to one half of the number of theoretical plates) than conventional C_{18} columns These restrictions result in detection limits in the μg/ml range for most compounds.

3 MICELLAR MOBILE PHASES

Surfactants are used to prepare micellar mobile phases and can be classified according to their charge type as either anionic, cationic, non-ionic, zwitterionic, or non-aqueous (reversed). They consist of a polar head, which can be charged or neutral, and a hydrocarbon tail Armstrong and Henery [17] first utilized surfactants in aqueous mobile phases, at concentrations above the critical micelle con-

centration (CMC), instead of the traditional organic–water mixtures or reversed-phase HPLC. In conventional chromatography, the primary equilibrium is established between the solute and the stationary phase, while in micellar chromatography, the solutes are subject to secondary equilibria as they enter and exit the micelle. This secondary equilibria can have a significant effect on the separation.

Since the introduction of micellar mobile phases, their usage has grown slowly but steadily. Subsequently, there have been several excellent papers and review articles written about surfactants and micellar mobile phases to which the reader is referred to for an in depth discussion of micelle characteristics and retention mechanism theory [18–23]

Only aqueous, anionic, and non-ionic surfactants have been sucessfully used as chromatographic mobile phases for the determination of drugs in biological fluids. Cationic surfactants, *i.e.* hexadecyltrimethylammonium bromide (CTAB), do not effectively solubilize serum proteins, but instead cause the serum proteins to precipitate and thus cannot be used for the preparation of micellar mobile phases for DSI [24,25]. Of the many types of aqueous surfactants (anionic, cationic, non-ionic, and zwitterionic), to date, only two have been used to prepare micellar mobile phases for the assay of drugs in biological fluids, *i.e.* sodium dodecyl sulfate (SDS), an anionic surfactant, and polyoxyethylene(23)dodecanol (BRIJ® 35), a non-ionic surfactant. SDS has by far been the most widely used surfactant for the assay of drugs in biological fluids.

Micellar mobile phases have hydrophobic and electrostatic interactions with the analytes and stationary phase, which can provide additional selectivity. If the surfactant concentration is varied, reversals in the retention order can occur Re-equilibration of the column after a gradient run is faster with micellar mobile phases than with conventional aqueous–organic mobile phases, since only one column volume is required to re-equilibrate the HPLC column [26–29].

3.1. Mobile phases with non-ionic surfactants

BRIJ 35, a non-ionic surfactant, has a CMC of 0.0001 *M* and an aggregation number of 40. BRIJ 35 is polymeric and has an average molecular formula of $CH_3(CH_2)_{11}(OCH_2CH_2)_{23}OH$ [19]. The cloud point, the temperature at which phase separation occurs, for aqueous solutions of BRIJ 35 in the 1–6% concentration range is approximately 100°C. Chromatography should be done below this temperature to prevent plugging or damage to the column [23]. The prediction of retention behavior is generally less complex with non-ionic micellar mobile phases as compared to ionic micellar mobile phases, since the retention mechanism is not complicated by charge effects of the surfactant.

3.1.1. Surfactant adsorption

The chromatographic retention mechanisms of ionic and non-ionic mobile

phases are generally similar, although there are some important differences in the way in which the surfactants and stationary phases interact. Ionic surfactants are strongly adsorbed onto and coat reversed-phase packing materials [26,30–34]. Langmuir adsorption isotherms accurately predict the adsorption behavior of ionic surfactants since there is little or no additional adsorption when the surfactant concentration is above the CMC in the mobile phase [26,30,31,35–37]. Non-ionic surfactants (BRIJ 35) also adsorb onto and coat reversed-phase packings, but they continue to adsorb onto the packing above the CMC. Borgerding and Hinze [23] have studied these differences and concluded that the major difference, chromatographically, between ionic and non-ionic surfactants is in the adsorption of the surfactant from the aqueous mobile phase onto the reversed-phase packing material. The continuous adsorption of surfactant from the mobile phase has the effect of further decreasing the chromatographic efficiency. This continuous decrease in efficiency may be a contributing reason for the general lack of use of BRIJ 35 mobile phases.

3.1.2. Efficiency

BRIJ 35 mobile phases suffer from significantly decreased chromatographic efficiency as compared to conventional organic–water mobile phases. The reasons for this reduced efficiency for non-ionic surfactants are poor wetting of the stationary phase by the micellar mobile phase [38] and restricted mass transfer between the various macroscopic phases [27] Attempts to improve the efficiency of non-ionic micellar mobile phases by the addition of organics have not been successful. Borgerding and Hinze [23] reported that the addition of 0–12% ethanol to a 6.0% BRIJ 35 mobile phase did not increase chromatographic efficiency. There has been an insufficient amount of work done to determine the effects of organic modifiers on the chromatographic efficiency of non-ionic micellar mobile phases.

3.1.3. Selectivity

Some of the parameters that are available to alter the chromatographic selectivity are column type (stationary phase), surfactant concentration in the mobile phase, and pH of the mobile phase. Changes in selectivity (peak order reversal) have been observed when the concentration of BRIJ 35 was changed from 0.04 to 0.08 *M* [24]. Since only a small percentage ($\leq$ 10%) of organic solvent (methanol, ethanol, 2-propanol, or acetonitrile) is allowed in the mobile phase without inducing precipitation, the added selectivity from the addition of different organic solvents is probably minimal. No studies have been reported which assessed the effect of organic solvents added to non-ionic micellar mobile phases for the analysis of drugs in biological fluids.

3.1.4. Direct serum injection

Cline Love *et al.* [24] have used non-ionic micellar mobile phases (BRIJ 35) for

the assay of drugs in biological fluids. They directly injected serum onto a C_{18} or CN column and determined the following drugs: acetaminophen, phenobarbital, carbamazepine, quinidine, morphine, codeine and cocaine. The (non-ionic) BRIJ 35 micellar mobile phases appear to be equally effective in solubilizing the serum proteins as (anionic) SDS micellar mobile phases, but they also suffer from the same problem of low chromatographic efficiency. Frequently, drugs are strongly bound to the serum proteins with as little as 10% of the drug existing in the unbound (free) state. When a serum sample is injected, the surfactant interacts with the serum proteins and displaces the bound drug. The displaced drug then can freely partition into the stationary phase. The protein components generally elute near or at the solvent front, while the drug elutes at a later time. The separation of the drug from the solvent front and serum proteins depends upon the hydrophobicity of the column and drug and the strength of the mobile phase (Fig. 1).

3.1.5. Recovery

No validation data have been reported in the literature on DSI using non-ionic surfactants, thus it is difficult to make a judgment on recovery. The drugs appear

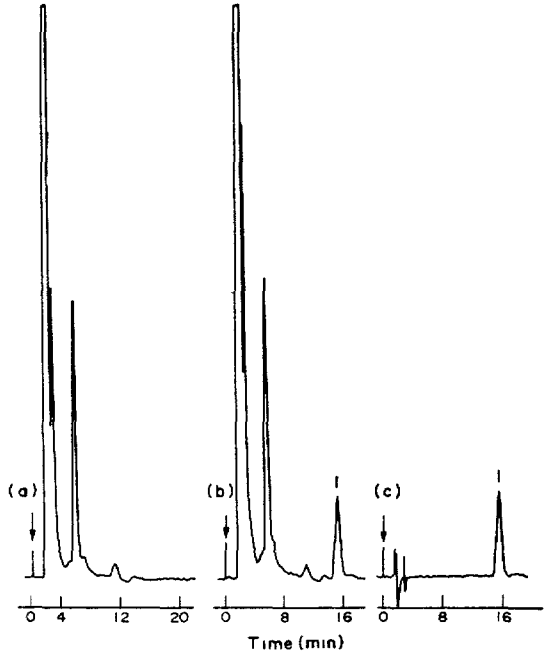

Fig 1 Chromatograms of (a) serum blank, (b) serum blank with 20 μg/ml chloramphenicol (1) added, and (c) 20 μg/ml chloramphenicol in water Column, Supelcosil CN (25 cm), mobile phase, 0 04 *M* aqueous BRIJ 35; flow rate, 1.0 ml/min, UV detector sensitivity, 0 08 a u f s. at 254 nm (Reproduced with permission from ref 24)

to be totally released by the surfactant and recovery should be near 100%, provided that the injection volume is not too large (<25 μl), there are no interferences, and the sample is in the concentration range of about 10–20 μg/ml. Injecting too large a sample will cause column deterioration and plugging. Frequently, the problems with assessing recoveries are due to the interferences from endogenous plasma peaks. Falsely high recoveries may occur if there are interferences from endogenous peaks and low recoveries may occur if the injection volume is too large, since the release of the drug from the serum proteins may be incomplete [39].

3.1.6. Detection

Both ultraviolet (UV) (254 nm) and fluorescence detection have been used with non-ionic micellar mobile phases and DSI [24]. The largest fluorescence responses were obtained using excitation at 215 nm for morphine, codeine, and cocaine and

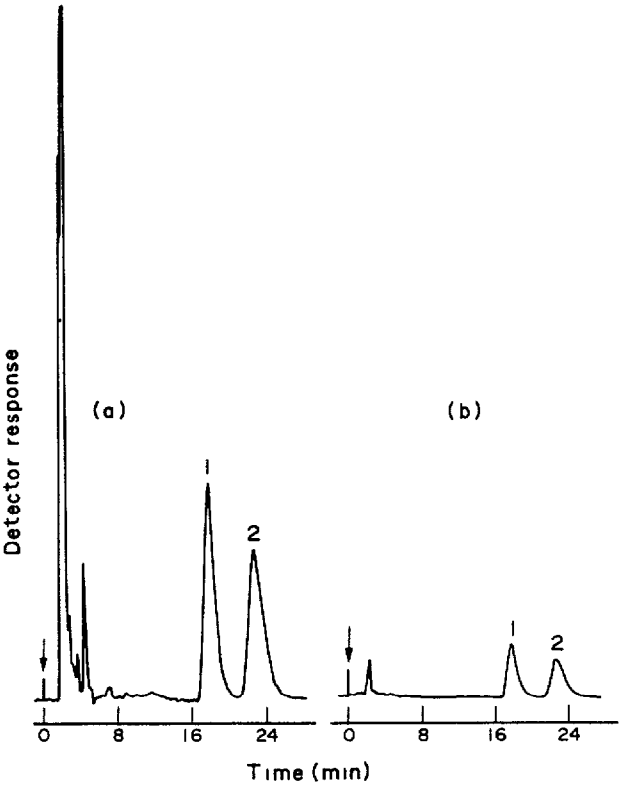

Fig 2. Chromatograms of serum blank with 2 μg/ml added quinidine (2) Column, Supelcosil CN (25 cm), mobile phase, 0 08 *M* aqueous BRIJ 35 with 10% propanol added, flow-rate, 1 0 ml/min, fluorescence detector voltage, 700 V, sensitivity range, 0 2 μA; excitation wavelength, 254 nm, emission cut-off filter, (a) 300 nm and (b) 470 nm (Reproduced with permission from ref 24)

excitation at 254 nm for quinidine. The use of fluorescence emission cut-off filters of 300 and 470 nm was demonstrated with quinidine. The 470-nm filter significantly reduced the endogenous interferences, but it also significantly reduced the signal (Fig. 2). It is difficult to determine if there was an improvement in the signal-to-noise ratio (S/N), since no numerical data were given [24].

3.2. Mobile phases with ionic surfactants

SDS has a CMC of 0.0081 *M*, an aggregation number of 62, and a molecular formula of $CH_3(CH_2)_{11}OSO_3^-Na^+$ [19]. SDS mobile phases have been used far more than any other type of micellar mobile phase, especially for the assay of drugs in biological fluids. Electrophoresis-grade SDS can be obtained in fairly high purity. It is sometimes recrystallized from methanol to increase its purity [39].

3.2.1. Surfactant adsorption

Unlike non-ionic surfactants, SDS does not continue to adsorb onto reversed-phase packing materials above its CMC. The surfactant initially adsorbs onto the stationary phase, but after 1–2 h of pumping a 0.01 *M* solution through the HPLC column an equilibrium is established

3.2.2. Efficiency

SDS mobile phases suffer from the same problem of decreased chromatographic efficiency as do BRIJ 35 mobile phases. The poor chromatographic efficiency has been predominately attributed to slow mass transfer in the stationary phase or poor wetting of the stationary phase by the mobile phase [40]. Dorsey *et al.* [38] have shown that increasing the temperature of the HPLC column to 40°C and the addition of 3% propanol to SDS mobile phases lowered the viscosity and increased the chromatographic efficiency to near that of conventional organic–water systems. The effect of added co-solvent on the CMC should be considered. The CMC of SDS has been shown to first decrease and then increase when small amounts of methanol are added to the mobile phase. In solutions of 0.27 mol fraction methanol, there is no micellar aggregation in SDS solutions [41]. Dorsey *et al.* [38] have stated "the use of shorter alkyl bonded phases or polar bonded phases should inherently yield better mass transfer, as the wetting problem will be less severe".

3.2.3. Selectivity

As with mobile phases prepared with non-ionic surfactants, the selectivity of mobile phases prepared with ionic surfactants is an important consideration and can be modified. A more detailed discussion of selectivity is presented in Section 4.1.2.

3.2.4. Direct serum and urine injection

The direct injection of serum or urine using ionic micellar mobile phases has been accomplished both with and without column-switching procedures. The major advantage to the column-switching procedures is their lower detection limits, but they are difficult to develop compared to procedures that do not use column switching. Both approaches are discussed in greater detail below.

4 TECHNIQUES

4.1. Procedures without column switching

SDS mobile phases for therapeutic drug monitoring using DSI was first introduced by Cline Love and co-workers [25,42,43]. SDS (anionic) micellar mobile phases solubilize the proteins in serum by binding with the hydrophobic sites, which releases any protein-bound drug. Grannaman and Sennello [44] have shown that antibiotics that are bound to proteins are released by the preferential binding of the proteins to the surfactant monomers.

SDS mobile phases were used for the analysis of morphine, codeine, propranolol, quinidine, and quinine in urine by direct injection [42]. The necessity of using SDS mobile phases for the analysis of urine samples is questionable since the protein content of urine is significantly lower than that of serum and protein precipitation is usually not a problem. The same assays probably could have been done with conventional mobile phases, which generally have greater chromatographic efficiencies [39]. A survey of drugs analyzed with micellar mobile phases is given in Table 1.

4.1.1. Chromatographic apparatus

The equipment used for the assay of drugs in biological fluids using micellar mobile phases is that which is typically available in most analytical laboratories. Commercially available HPLC pumps have been used without modification. A saturation column packed with silica gel (25–40 μm) is placed between the pump and the injector to saturate the mobile phase with silicic acid. The injection volume is usually limited to 20 μl or less. Larger injection volumes can cause column plugging or incomplete release of protein-bound drugs. The columns that have been used have had either C_{18} or CN stationary phases, 5 or 10 μm particle size, lengths between 15 and 30 cm, and internal diameters of 0.46 cm. These columns have all been shown to be able to accomodate hundreds of injections of serum without any increase in back-pressure or decrease in column performance. After the analysis of a series of serum or urine samples, flushing the column overnight with mobile phase at 0.1 ml/min was found to remove the strongly retained serum components and increase column life.

TABLE 1

SURVEY OF DRUGS ANALYZED WITH MICELLAR MOBILE PHASES

Drug	Technique[a]	Limit of detection (μg/ml)	Detection[b]	Matrix	Reference
Acetaminophen	Direct	0 2	UV 254	Serum	25
Acetylsalicylic acid	Direct	2	UV 254	Serum	25
Carbamazepine	Direct	2	UV 254	Serum	25
	Switch	0 1	UV 287	Serum	50
Cefmenoxime	Direct	5	UV 260	Serum	45
Cefotiam	Direct	5	UV 260	Serum	45
Chloramphenicol	Direct	0 7	UV 254	Serum	25
	Switch	0 05	UV 278	Serum	50
	Switch	0 03[c]	UV 278	Serum	49
Codeine	Direct	0 3	F 215/300	Urine	42
Diazepam	Switch	0 03	UV 242	Serum	39
Diltiazem	Switch	0 002	UV 238	Serum	49
Furosemide	Switch	0 009	F 270/400	Serum	50
Methotrexate	Direct	0 04[c]	UV 305	Serum	46
Morphine	Direct	0 3	F 215/300	Urine	42
Phenobarbital	Direct	2	UV 254	Serum	25
	Switch	2	UV 208	Urine	39
Phenytoin	Direct	3	UV 254	Serum	25
Procainamide	Direct	1	UV 254	Serum	25
	Switch	0 006	UV 280	Serum	50
Propranolol	Switch	0 005	F 230/340	Serum	50
	Direct	0 01	F 215/300	Urine	42
Quinidine	Direct	0 3	F 366/400	Serum	25
	Switch	0 004	F 238/340	Serum	50
	Direct	0 03	F 215/300	Urine	42
Quinine	Direct	0 03	F 215/300	Urine	42
Theophylline	Direct	1	UV 254	Serum	25

[a] Direct injection or column switching

[b] UV (ultraviolet), detection wavelength given in nm, F (fluorescence), with excitation/emission wavelengths given in nm (in some cases an emission cut-off filter was used).

[c] Estimate.

4.1.2. Analytical method development

The types and proportions of organic modifiers that may be used with micellar mobile phases are more limited than with conventional water–buffer systems. This limits the degree to which the selectivity can be changed. The addition of organic modifiers to micellar mobile phases decrease micelle formation. If too great an amount of organic modifier is added no micelles will form and subsequent injections of serum will cause protein precipitation and plugging at the

head of the column. Propanol and acetonitrile (up to 10%) have been successfully used as organic modifiers without problems [25,42,43].

Surfactant concentration (which affects micelle formation) can have a profound effect on the capacity factor (k') and the retention of drugs. The number of micelles in the mobile phase may be increased by increasing the surfactant concentration or decreasing the concentration of organics in the mobile phase. Increasing the micelle concentration decreases the k' of neutral analytes. The k' for chloramphenicol in serum decreased from 9 to 2 when the SDS concentration was increased from 0.01 to 0.1 *M* [25].

The type of stationary phase can also affect the retention of the analytes. Highly hydrophobic analytes may be better separated on CN stationary phases than on C_{18} phases, since they will be less strongly retained by the CN phases. The proper selection of the stationary phase, organic modifier concentration, and SDS concentration should produce relatively good analyte peak shapes with retention times of 10 min or less.

The pH of the micellar mobile phase can be an important variable in the analysis of ionizable drugs The recovery and retention times can be significantly affected. Acetylsalicylic acid ($pK_a = 3.50$) elutes in 3 5 min on a C_{18} column with a mobile phase of 0.08 *M* SDS (pH 3.0). If the pH of the mobile phase is increased to 6.5 an acetylsalicylic acid peak is not seen, since the drug probably elutes with the serum proteins. When the drug is in its anionic form ($pH > 6.5$) it is weakly retained by C_{18} columns, but at pH 3.0 the drug is in its neutral form and is well retained by C_{18} columns [25].

Haginaka *et al.* [45] observed split peaks when cefmenoxime in serum was chromatographed with an eluent of 0.80 *M* SDS at pH 3.3 and 3.1, but not at pH 2.9. Acidified serum samples also produced only one peak at all of the above eluent pHs. The split peaks have been attributed to protein-bound and unbound species. A similar phenomenon was reported by Palmissano *et al.* [46] for the determination of methotrexate (MTX) in body fluids using a mobile phase of 0.1 *M* SDS and a C_{18} column. The pH of the mobile phase was maintained at 5 7 and 5.2 by a phosphate buffer for the serum and urine assays, respectively. They concluded that the peaks at short and long retention times were due to protein-bound and unbound drug. Doubled peaks were not observed if the ionic strength was sufficiently high.

For most analyses, the concentration of SDS in the mobile phase is generally 0.02–0.10 *M* and the pH is maintained between 3 and 7. The proper pH and SDS concentration in the mobile phase depends upon the hydrophobicity and pK_a of the analytes. The addition of organic modifiers to SDS mobile phases for the determination of drugs in serum has usually helped improve chromatographic efficiency and peak shape. Although, Palmissano *et al.* [46] stated that for the assay of MTX in serum the addition of 1-propanol or 2-propanol (up to 6%) increased the efficiency by 40%, but also increased the peak asymmetry and decreased the retention. They concluded that the addition of organics to SDS

mobile phases has little value, since the elution profile of the unretained serum components changed. This change required the k' of MTX to be increased (so it would elute in an area free of interferences), which negated any gain in sensitivity.

4.1.3. Quantitation and validation

Fig. 3 shows a chromatogram of acetaminophen, phenobarbital, and chloramphenicol in serum using a CN stationary phase. The typical chromatographic profile for blank serum is characterized by a large peak due to the serum proteins, which occupies the first 3 min of the chromatogram. The signal then gradually returns to the baseline. The appearance of several smaller peaks in the chromatogram can cause problems with quantitation, depending upon the level of quantitation required. The sloping baseline can also cause problems when quantitation is done by peak area, since drawing an accurate baseline is difficult. For this reason, most quantitation has been done by peak-height instead of peak-area

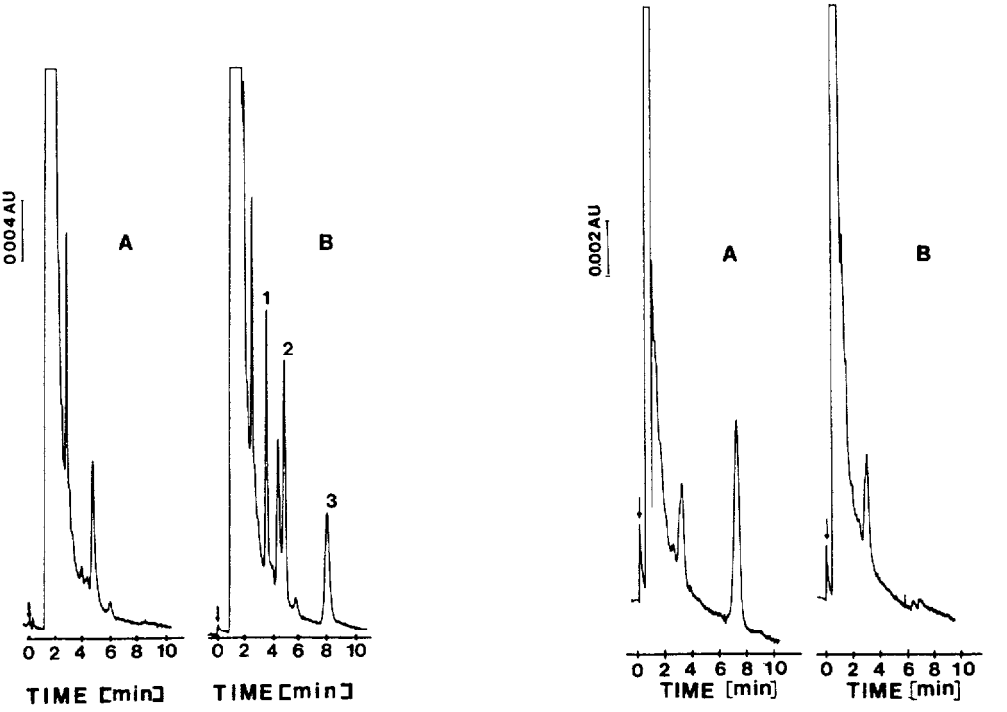

Fig 3 Chromatograms of (A) serum blank, (B) serum with 2 5 μg/ml acetaminophen (1), 15 μg/ml phenobarbital (2), and 10 μg/ml chloramphenicol (3). Chromatographic conditions were as follows column, Supelcosil CN (15 cm), mobile phase, 0 02 *M* SDS adjusted to pH 7 0 with phosphate buffer, flow-rate, 1 0 ml/min, UV detection at 254 nm (Reproduced with permission from ref 25)

Fig 4 Chromatograms of (A) serum with 20 μg/ml chloramphenicol (retention time 7 5 min) and (B) serum blank sample Column, Supelcosil C_{18} (15 cm), mobile phase, 0 03 *M* SDS, flow-rate, 2 0 ml/min, UV detection at 254 nm (Reproduced with permission from ref 25)

measurements. Similar serum profiles were obtained for the analysis of chloramphenicol using a C_{18} column (Fig. 4).

The results obtained with DSI have been compared with EMIT for serum samples containing known drug concentrations. The values obtained from the two techniques were generally in good agreement, although the precision from EMIT was usually about 25% better than that of DSI [25]. When the results from DSI assays of MTX in serum were compared with results from EMIT the regression line $[MTX]_{DSI}$ *versus* $[MTX]_{EMIT}$ had a correlation coefficient of 0.994, a slope of 0.945, and an intercept of -0.086. The slope and intercept were not significantly different from 1 and 0, respectively, as determined from a *t*-test at the 95% confidence limit [46].

4.1.4. Detection

The detection limit for drugs in serum ranges between 0.2 and 5.0 μg/ml using UV detection, which is suitable for the therapeutic monitoring of most drugs. The detection limit for both quinidine and quinine in urine was 0.03 μg/ml. A detection limit of 0.3 μg/ml was obtained for quinidine in serum using fluorescence detection. The higher detection limit in serum was caused by a greater level of interfering endogenous peaks that are present in serum [25,42].

4.2. Procedures involving column switching

The major shortcomings of DSI without column switching are the interference from endogenous plasma peaks, the limited sample injection volume, and the limited selectivity because the full range of organic modifiers cannot be used. This generally limits its utility to therapeutic studies in the μg/ml range Column switching was first used in an attempt to increase the sensitivity of micellar chromatographic techniques down to the low ng/ml range by improving resolution and sample injection volumes. This would allow the direct injection of physiological fluids for pharmacokinetic studies. Koenigbauer and Curtis [47] first used micellar mobile phases (0.01 *M* SDS) and microbore column switching for the determination of diazepam in serum. Untreated serum samples (200 μl) were injected onto a 15 mm × 3.2 mm I D. C_{18} pre-column using a 0.01 *M* SDS mobile phase. The analytes were trapped on the hydrophobic pre-column while the serum proteins were flushed to waste. The pre-column was then backflushed with a methanol–water (65:35, v/v) mobile phase onto a 25 mm × 1 mm I.D. C_{18} analytical column. Recovery was linear and quantitative (89.4%) over the range 30–3000 ng/ml for diazepam. The method was also specific against the three major metabolites of diazepam: oxazepam, nordiazepam, and temazepam (Fig. 5). The direct injection of urine (10 μl) for the analysis of phenobarbital was also accomplished by column switching using a phosphate buffer loading mobile phase (pH 7.5) and acetonitrile–0.025 *M* phosphate buffer (pH 7.5) (15:85, v/v) as the analytical mobile phase (Fig. 6). The use of an SDS loading mobile phase was

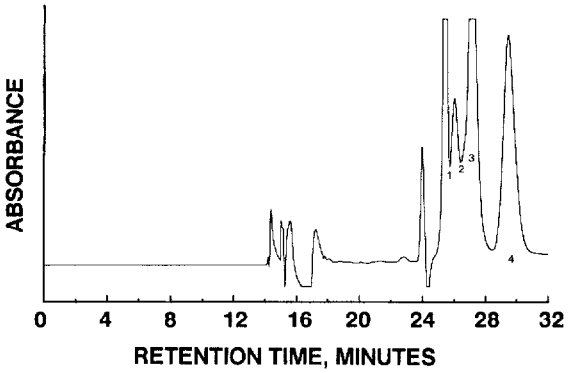

Fig 5 Chromatogram of diazepam and its three metabolites in serum (120 ng/ml) Column-switching conditions were as follows pre-column, 15 mm × 3 2 mm I D (C_{18}), analytical column, 250 mm × 1 mm I D (C_{18}), loading mobile phase, 0 01 *M* SDS, analytical mobile phase, methanol–water (65 35, v/v), flow-rate, 60 μl/min. Injection volume, 200 μl UV detection, 242 nm (0.125 a u.f s.) Peaks 1 = oxazepam (k' = 7 0), 2 = temazepam (k' = 7 4); 3 = nordiazepam (k' = 8 1), 4 = diazepam (k' = 9 4, α = 1 2) [39]

found to be unnecessary since the amount of proteinaceous material in urine is far less than in serum [39,48]. The technique has since been extended to conventional columns (250 mm × 4.6 mm I.D.) and a variety of drugs in serum, *i.e.* diltiazem (Fig. 7), diazepam, phenobarbital, chloramphenicol, and phenytoin [49].

Posluszny and Weinberger [50] have determined chloramphenicol, carbamazepine, procainamide, quinidine, furosemide and propranolol in serum by column switching and micellar mobile phases (Figs. 8 and 9). All the drugs gave good

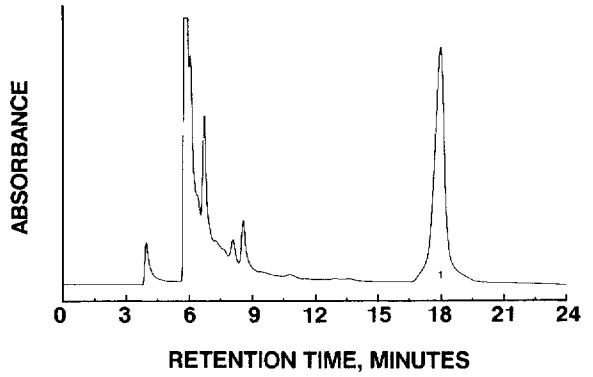

Fig 6 Chromatogram of phenobarbital in urine (50 μg/ml) Column-switching conditions were as follows pre-column, 15 mm × 3 2 mm I D (C_{18}), analytical column, 250 mm × 1 mm I D (C_{18}), loading mobile phase, 0 025 *M* phosphate buffer (pH 7 5), analytical mobile phase, acetonitrile–water (15 85, v/v), flow-rate, 45 μl/min. Injection volume, 10 μl UV detection, 208 nm (1 0 a u f s) Peak 1 = phenobarbital (k' = 8 1) [39]

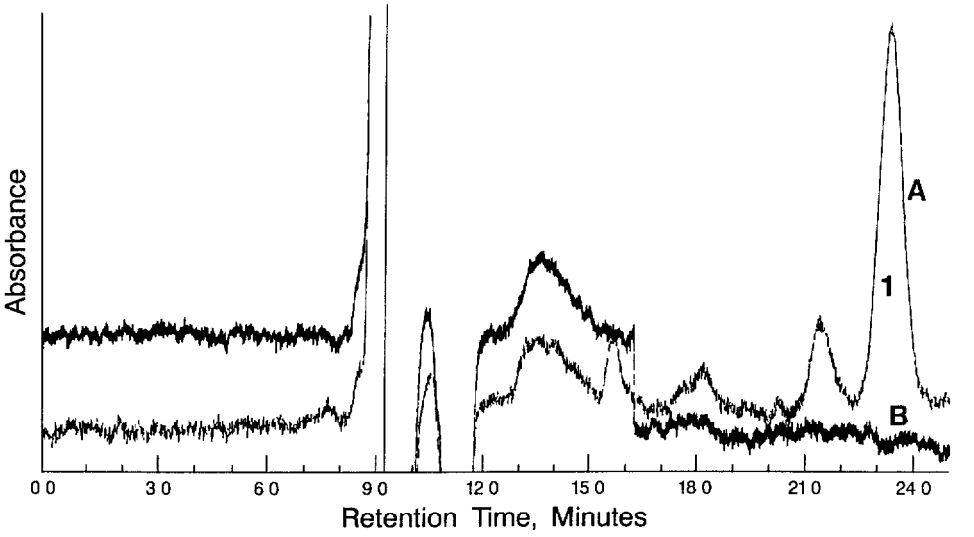

Fig 7 Chromatograms of diltiazem in serum (22 ng/ml) (A) and serum blank (B) Column-switching conditions were as follows pre-column, 30 mm × 4 6 mm I D (C_{18}), analytical column, 250 mm × 4 6 mm I D. (C_{18}), loading mobile phase, 0.01 *M* SDS; analytical mobile phase, methanol–water–trifluoroacetic acid–triethylamine (350 650 5 5, v/v), flow-rate, 1 0 ml/min Injection volume, 100 μl UV detection, 238 nm (0 0012 a u f s) Peak 1 = diltiazem [49]

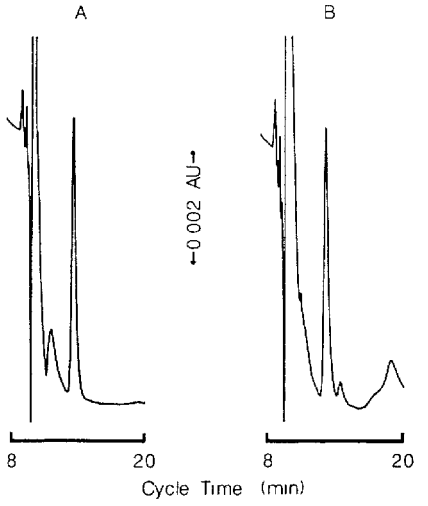

Fig 8 Direct injection analysis, carbamazepine application (A) 100-μl injection of spiked serum at 1 μg/ml concentration, retention time 10 3 min, (B) blank serum; absorbance detection, 278 nm at 0 01 a.u f s , column-switching valve configuration, backflush, analytical mobile phase, methanol–water (55 45, v/v) water containing 0.04 *M* SDS and 0 04 *M* sodium monophosphate, pH 3 0 Flow-rate, 2 0 ml/min (Reproduced with permission from ref 50)

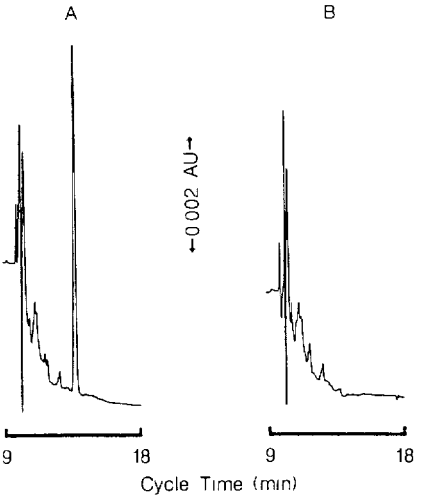

Fig 9 Direct injection analysis, chloramphenicol application (A) 100-μl injection of spiked serum at 1 μg/ml concentration, retention time 13 6 min; (B) blank serum, absorbance detection, 278 nm at 0 01 a u f s , column-switching valve configuration, forward flush, analytical mobile phase, methanol–water (28 72, v/v) containing 0 02 *M* sodium monophosphate, pH 4 6 Flow-rate, 2 0 ml/min. (Reproduced with permission from ref 50)

recoveries (96–104%) and relative standard deviations of 1–6%. They used three different configurations (two forward and one reverse flush) to elute the analytes that were trapped on the pre-column onto the analytical column.

4.2.1. Chromatographic apparatus

A disadvantage of using column-switching techniques is the additional hardware that is needed, *i.e.* an additional pump, column and switching valve. A C_{18} analytical column is typically used, since this seems to offer the best resolution from interferences. The types of pre-column used have had C_8, CN, or C_{18} stationary phases. The dimensions of the pre-columns used varied from 15 to 30 mm in length and between 3.2 and 10 mm I.D. depending upon the application and volume of serum injected.

4.2.2. Analytical method development

The method development process for column-switching techniques with micellar mobile phases is considerably different than for extraction/reconstitution-based techniques, since the extraction step is being replaced by the pre-column and micellar mobile phase and no reconstitution step is necessary. The chromatography that occurs on the pre-column can be critical to minimizing the endogenous peaks in the chromatogram. The selection of stationary phases on the analytical column and pre-column and the composition of the loading and analytical mobile phases are the controlling factors for the resolution and retention of the

analytes. Koenigbauer and Curtis [39,47–49] utilized identical stationary phases (C_{18}) in the analytical column and pre-column, while Posluszny and Weinberger [50] used more of a multi-dimensional approach. By using different types of stationary phases on the analytical and pre-columns, C_{18} and CN, respectively, a different retention mechanism was operating on each column.

The loading mobile phase should contain a sufficient concentration of SDS to prevent protein precipitation. The addition of acetonitrile and/or buffers may assist in the separation of the drug from the serum proteins. During method development, the eluent from the pre-column should be monitored. This aids in determining when the drug elutes from the pre-column and when the column switching should occur. The drug which has been retained on the pre-column is then either backflushed or forward flushed from the pre-column onto the analytical column using the analytical mobile phase, the loading mobile phase, or some intermediate mobile phase. A 2–3 min heartcut of the eluent (which contains the drug peak) is then diverted to the analytical column. The analytical mobile phase which is stronger than the loading mobile phase compresses the peak. The composition of the analytical mobile phase can be a mixture of organics, buffers, and SDS It should selectively elute the drug from the pre-column. If too strong a mobile phase is used, many endogenous compounds will be eluted with the drug, resulting in potentially interfering peaks in the chromatogram. Between analyses the pre-column is washed with a relatively strong mobile phase to remove the strongly retained serum components.

Posluszny and Weinberger [50] utilized two different forward flush and one backflush valve configurations to selectively elute the drugs from the pre-column onto the analytical column. Backflushing of the pre-column generally results in a less selective elution (but better peak shape) of the drug onto the analytical column and also increases the possibility of plugging of the analytical column by solids from the head of the pre-column [49].

4.2.3. Quantitation and validation

The recovery of most drugs in serum was generally 96–104%, with relative standard deviations of 1–6% [50]. Even the strongly protein-bound drug, diazepam, gave a recovery of 89.4% [39].

Propranolol and chloramphenicol in serum were determined by both DSI and manual liquid extraction procedures. The results of this study showed good correlation between the DSI and manual extraction methods. Linear regression analysis of the data gave correlation coefficients of 0.985 and 0.924 and slopes of 1.02 and 1.12 for propranolol and chloramphenicol, respectively [50].

4.2.4. Detection

For most of the drugs assayed using column switching and micellar mobile phases, the detection limit was limited by artifacts and endogenous serum peaks. When UV detection was used the detection limit was typically in the 30–100

ng/ml range. The use of fluorescence detection with the appropriate cut-off filter enabled detection limits to be reduced to 10 ng/ml or below [39,50]. Optimization of the loading mobile phase and analytical mobile phase and increasing the purity of the reagents should enable fluorescence detection limits in the low ng/ml range.

5 CONCLUSION

Although micellar chromatography was first used in 1980, its application to the determination of drugs in biological fluids has occurred in only the last five years. Micellar mobile phases allow biological fluids (serum or urine) to be directly injected onto an HPLC column without any prior sample clean-up. ISRP columns have offered advantages (elimination of sample preparation) similar to those of micellar mobile phases for therapeutic drug monitoring by direct injection of biological fluids. Micellar chromatography is ideal for the monitoring of drugs at therapeutic concentrations or for neonatal samples, where the sample size is limited. A significant savings in time per sample could be realized by application of micellar mobile phases over the traditional extraction/dilution procedures. The sensitivity limitations of micellar chromatography without column switching preclude its use for pharmacokinetic monitoring. Significantly lower detection limits and larger injection volumes were obtained by using column switching in combination with micellar mobile phases. This relatively recent (1986) advance has made it possible to determine drugs in physiological fluids without any sample preparation at levels applicable to pharmacokinetic studies. The lower detection limits of column-switching techniques were principally made possible by their ability to use greater injection volumes and broader mobile phase/column choices which increased selectivity. The difficulties in developing column-switching techniques are not any more formidable than methods which utilize extraction/reconstitution steps, although they may be more foreign to the method developer.

6 SUMMARY

Although micellar chromatography has been used for the determination of drugs in biological fluids since 1985, relatively few researchers have applied the technique to therapeutic monitoring. The reasons for this are rather unclear. It may be that most of the present extraction/reconstitution techniques are well established or that the method development procedure is unfamiliar. Significantly lower detection limits can be obtained with micellar mobile phases and column switching than with micellar mobile phases alone. Only two groups have used micellar mobile phases in conjunction with column switching for the determination of drugs in biological fluids. Since column switching with micellar mobile phases is a relatively new and untried technique, it will take some time before the full range of its applicability and limitations are known.

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