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DETERMINATION OF DRUGS IN UNTREATED BODY FLUIDS BY MICELLAR CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

MANOP ARUNYANART and L.J. CLINE LOVE*

Seton Hall University, Department of Chemistry, South Orange, NJ 07079 (U.S A)

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SUMMARY

Direct serum and urine injection, without sample extraction or protein precipitation steps, into a liquid chromatographic system using sodium dodecyl sulfate (SDS) with 10% added propanol as the mobile phase, is described for measurement of drug levels. The ability of SDS micelles to form soluble protein—SDS complexes, with no on-column precipitation, provides a simple, rapid method for routine determination of quinine, quinidine, propranolol, morphine and codeine at concentration levels found in serum and urine following administration of therapeutic doses. Absolute limits of detection ranged from 0.2 to 6 ng. Variation of the surfactants mobile phase concentration allows control of selectivity and analysis time, although a minimum concentration is required to prevent protein precipitation. Chromatographic efficiencies are improved by the addition of propanol to the micellar mobile phase, and sensitivities improved by use of fluorescence detection. The sensitivities are more than adequate for therapeutic drug monitoring of concentration ranges normally encountered in serum and urine.

INTRODUCTION

Aqueous solutions containing sodium dodecyl sulfate (SDS) at concentrations well above the critical micelle concentration (CMC) have been proposed as selective mobile phases in reversed-phase liquid chromatography [1, 2]. The effects of micellar mobile phases in high-performance liquid chromatography (HPLC) on the elution behavior of a series of neutral arenes and for the chromatographic behavior of ionizable eluents have been described [3, 4]. In micellar HPLC, there are two major equilibria whose constants govern chromatographic behavior, namely, solute—micelle described by K_{eq} and solute—stationary phase described by K_{sw}. The larger the K_{eq} value, the greater the effect of changes in surfactant concentration on the capacity factors.

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Consequently, relative retention times may be changed and selectivity can be obtained by varying the concentration of micelles in the mobile phase. This paper reports a simple, rapid, sensitive and direct technique which could be very useful for routine monitoring of drugs in biological fluids by micellar chromatography.

Development of selective and sensitive analytical techniques for the analysis of minute quantities of drugs in biological fluids have attracted considerable interest in analytical toxicology and therapeutic drug monitoring. Gas chromatography has been applied for determination of amphetamine and phentermine in biological fluids [5], morphine in opium [6] and heroin in illicit street preparations [7], but these methods require prior extraction of samples and, in most cases, derivatization of the drug before determination. Immunoassay methods such as enzyme-multiplied immunoassay techniques (EMIT®) have been the methods of choice for therapeutic drug monitoring because they are rapid, specific, sensitive and reliable [8], and biological fluids can be analyzed without prior extraction or protein precipitation. Unfortunately, EMIT is limited to only selected drugs. HPLC has also been applied to the determination of morphine and codeine in a variety of matrices [9, 10], and for other drugs in body fluids [11, 12]. For routine drug level monitoring in biological fluids, HPLC has drawbacks such as lengthy analysis time and tedious sample preparation, as it generally requires extraction of the drug from the protein-base sample or protein precipitation [13, 14]. These additional steps considerably increase the possiblity of error. Protein precipitation procedures, for example, may be incomplete resulting in column clogging [15].

In an attempt to eliminate these problems, pre-column technology has been used for partial sample clean-up, which allowed the direct injection of biological samples [16, 17]. Using this approach, no column deterioration or protein-binding effects were observed. Wahlund and Arvidsson [18] have shown that direct injection of blood plasma samples into reversed-phase columns resulted in skewed chromatographic peaks for the drug naproxen. The skew has been shown to be due to strong binding of naproxen to albumin present in the blood plasma.

Micellar HPLC provides an unique solution to these problems by solubilizing the protein components via a surfactant coating, making possible direct injection of biological fluids onto HPLC columns with no column clogging. In addition, the surfactant monomers appear to displace the drug bound to the protein, releasing it for partitioning to the stationary phase. In fact, Granneman and Sennello [19] have shown that surfactant monomers will competitively bind to proteins, thereby releasing protein-bound antibiotics. Also, Hirota and Kawase [20] have used SDS to improve the recovery of ubiquinone-10 in plasma samples. Therapeutic drug monitoring using UV and fluorescence detection for HPLC direct serum injection with micellar mobile phases has been reported recently and the results compared to the EMIT technique [21]. However, the moderate chromatographic efficiency prevented adequate sensitivity for determination of propranolol whose therapeutic range is 0.05–1.0 μg/ml. Dorsey et al. [22] demonstrated that efficiency approaching those with hydro-organic mobile phases can be achieved by addition of at least 3% propanol to the micellar mobile phase and by working at elevated temperature (40°C). In

the present study, SDS micellar mobile phases containing 10% propanol did not induce protein precipitation in serum and urine samples, but did exhibit improved chromatographic efficiencies. The sensitivity of the method was improved by using fluorescence instead of absorbance detection, allowing monitoring of additional drugs. The present study extends the use of micellar HPLC to the determination of quinine, quinidine, propranolol, morphine, and codeine in serum, and demonstrates for the first time the use of micellar HPLC for determination of drugs in urine by direct injection of the sample into the chromatograph.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Technicon FAST LC high-pressure pump (Technicon, Tarrytown, NJ, U.S.A.), a Model 7120 sample injector with a 20-μl injection loop (Rheodyne, Cotati, CA, U.S.A.), and a Model FS970 LC fluorometer (Kratos, Ramsey, NJ, U.S.A.). The HPLC analytical column was either a 10-μm μBondapak C_{18} (30 cm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) or a 5-μm Supelcosil LC-CN column (15 cm × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.). A pre-column (12.5 cm × 4.6 mm I.D.) packed with silica gel (25—40 μm) (Whatman, Clifton, NJ, U.S.A.) was located between the pump and sample injector in order to saturate the mobile phase with silica to minimize dissolution of the analytical column packing. All chromatograms were recorded on a Recordall Model 5000 strip chart recorder (Fisher Scientific, Springfield, NJ, U.S.A.).

Reagents

SDS was electrophoresis grade obtained from Bio-Rad Labs. (Rockville Center, NY, U.S.A.) and was used as received. Serum blank samples were obtained from General Diagnostics of Warner Lambert (Morris Plaines, NJ, U.S.A.) and used as received. Quinidine (Mallinckrodt, St. Louis, MO, U.S.A.), quinine, morphine and codeine alkaloids (S.B. Penic, Lyndhurst, NJ, U.S.A.), propranolol (Warner Lambert), and propanol and methanol (Fisher Scientific) were used as received.

Procedure

Micellar mobile phases were prepared by dissolving the appropriate quantity of SDS in distilled water containing 10% propanol. The mobile phase was filtered through a 0.45-μm Nylon-66 membrane filter (Rainin Instruments, Ridgefield, NJ, U.S.A.), and degassed under vacuum prior to use. A mobile phase flow-rate of 1.0 ml/min was used, and retention times were measured from the injection point to the peak maxima on the chromatogram. Stock solutions of 500 μg/ml of each drug in methanol were diluted to 10 μg/ml or 1 μg/ml with distilled water, and these were added in appropriate quantities to serum or urine. These serum- or urine-base standards were injected directly into the micellar HPLC system to prepare calibration curves of peak height versus concentration of drug, and to determine the precision of the measurements.

RESULTS AND DISCUSSION

Serum and urine blanks were mixed with the micellar mobile phase to determine if any protein precipitation was evident. For concentrations of SDS greater than 0.02 *M* containing 10% propanol, no precipitation was observed. When these blanks were injected into the chromatographic system, there was no evidence of pressure build-up owing to precipitated proteins at the head of the column or clogging of the injector port, even after more than 250 sample injections. However, there may be some strongly retained components binding to the stationary phase which could eventually cause column clogging, so the column was flushed overnight with mobile phase at 0.1 ml/min after analyzing serum or urine samples.

The blank serum or urine produced a rapid elution of unretained species at the solvent front, then returned completely to the baseline within 20 min. This is probably the result of the protein—SDS complex being excluded from the pores of the stationary phase support, preventing partitioning and retention. The background response level of the unretained species could be varied by changing the excitation wavelength along with appropriate changes in detector sensitivity range. Adjustments in SDS mobile phase concentration and detector sensitivity were made such that adequate resolution and sensitivity were obtained for quantitative determination of the drug content in the biological fluids. In most cases, drug elution occurred on the tail of the protein components, which prevented the use of the most sensitive detector ranges. Also, the sensitivity was adversely affected by the low intensity of the deuterium light source at the optimum absorption wavelength maxima of the drugs studied, and because a compromise excitation wavelength is necessary for the analysis of mixtures of drugs. The best sensitivity was obtained using 215—220 nm excitation, probably due to the high intensity of the deuterium light source at this wavelength, even though it does not correspond to the absorption wavelength maximum of any of the drugs studied. The fluorescence intensity was linear with drug concentration using this excitation wavelength. Sensitivity should be dramatically improved by using a light source with a high intensity at the drug's absorption wavelength maximum.

The detection and determination of morphine in body fluids is most frequently performed using urine because a large portion of this drug is excreted [23]. When using urine samples greater than 10 ml in total volume and obtained less than 48 h after ingestion of morphine, the sensitivity limit of 0.5 μg/ml has been recommended [24]. Fig. 1 shows the chromatogram of a urine blank and two urine samples containing 0.5 and 1.0 μg/ml of morphine, respectively. The fluorescence intensity increases linearly with morphine concentration, and the relative standard deviation (R.S.D.) of the calibration curve slope was 7.4%. Similar results were obtained for codeine. Table I lists the drugs examined in the present study, the calibration curve concentration range monitored in the urine samples, calibration curve linearity data, precision, and limits of detection for the drugs in urine.

Direct serum injection with unmodified micellar mobile phase HPLC for therapeutic drug monitoring of quinidine has been described, and the limit of detection was well below the therapeutic range normally monitored [21].

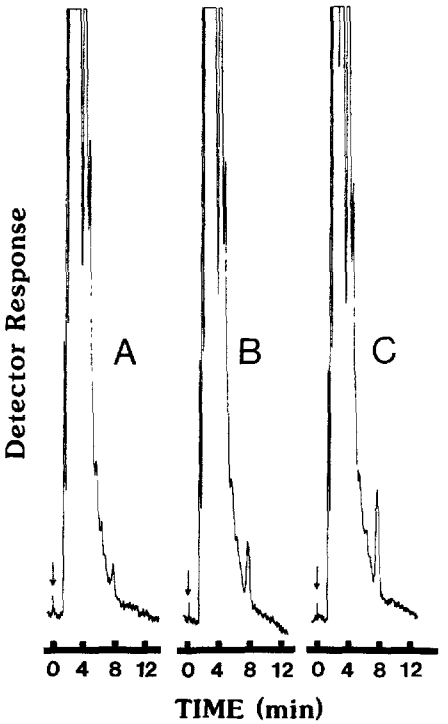

Fig. 1. Chromatograms of (A) urine blank, (B) urine blank with 0.5 μg/ml morphine, and (C) urine blank with 1.0 μg/ml morphine Chromatographic conditions: column, 10-μm μBondapak C_{18}; mobile phase, 0.03 *M* SDS + 10% propanol; flow-rate, 1 0 ml/min; detector voltage, 700 V; sensitivity, 0.05 μA; excitation wavelength, 215 nm; emission cut-off filter, 300 nm.

TABLE I

CALIBRATION RANGE OF URINE DRUG STANDARDS, PERCENTAGE RELATIVE STANDARD DEVIATION (R.S.D.) OF SLOPE, PRECISION, AND LIMITS OF DETECTION (L.O.D.) FOR SELECTED DRUGS IN URINE SAMPLES

All concentrations in μg/ml except those listed for absolute L.O D. values.

Drug	Calibration curve range in urine	Linearity* (% R.S.D. of slope)	Precision** (% R.S.D., n = 5)	Relative L.O.D.***	Absolute L.O.D.§
Morphine	0.4 —1.2	7.40	7.10	0.3	6
Codeine	0.5 —2.0	3.90	3.10	0.3	6
Propranolol	0.04—1.2	3.24	1.89	0.01	0.2
Quinidine	0.20—1.0	3.44	2.75	0.03	0.6
Quinine	0.20—1.0	10.36	6.27	0.03	0.6

*At least four different concentrations of urine drug standards were used.

**Concentrations of urine drug standards used for precision evaluation were 0.4, 1.0, 0.06, 0.6 and 0.2 μg/ml in order of each drug listed above.

***Limit of detection equal to concentration where signal = 3 × R.S.D. of noise. The noise is the standard deviation of several measurements of the response from blank serum measured at the retention time of the drug.

§Absolute L.O.D. = (relative L.O.D.) (injection volume) reported in ng.

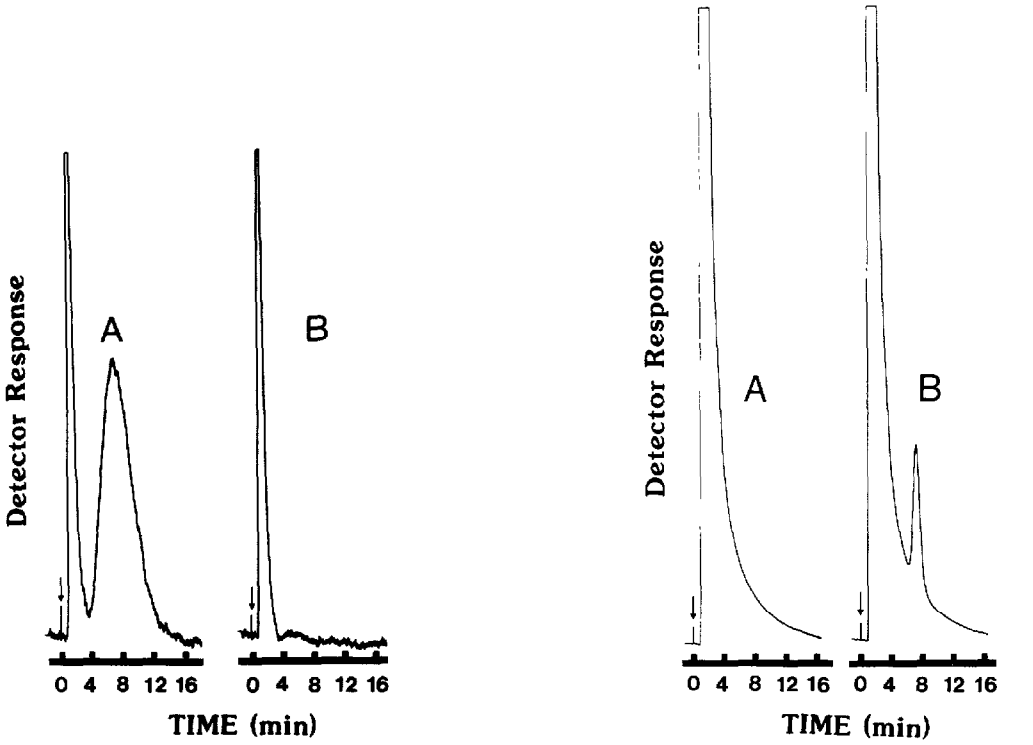

Fig. 2. Chromatograms of (A) serum with 4.0 μg/ml added quinidine and (B) serum blank. Chromatographic conditions: column, 5-μm Supelcosil LC-CN, mobile phase, 0.10 *M* SDS, flow-rate, 1.0 ml/min; detector voltage, 620 V; sensitivity, 0.02 μA; excitation wavelength, 336 nm; emission cut-off filter, 370 nm.

Fig. 3. Chromatograms of (A) serum blank and (B) serum with 3.0 μg/ml added quinidine. Chromatographic conditions: column, 5-μm Supelcosil LC-CN; mobile phase, 0.05 *M* SDS with 10% propanol; flow-rate, 1.0 ml/min; detector voltage, 700 V; sensitivity, 0.5 μA; excitation wavelength, 215 nm; emission cut-off filter, 300 nm.

However, the efficiency of micellar HPLC is moderate compared to hydro-organic reversed-phase HPLC unless a modifier such as propanol is added to the micellar mobile phase. Figs. 2 and 3 show the separation of serum containing known concentrations of quinidine using SDS solutions with and without added propanol, where considerable improvement in efficiency is obtained in the presence of 10% propanol. Note that the concentrations of SDS and quinidine, and the excitation wavelengths used to obtain the data in Figs. 2 and 3, were not the same, but similar retention times were observed. This strongly suggests that the efficiency is improved without drastic changes in selectivity. Although poor sensitivity for quinidine in serum using unmodified SDS mobile phases was found on C_{18} columns [21], good results (Table I) were obtained for determination of quinidine in urine using the propanol-modified mobile phase and C_{18} or cyano columns. Similar improvement in column efficiency with a concomitant decrease in the absolute limit of detection to 200 pg was observed for the determination of propranolol performed by direct injection of serum into the liquid chromatograph.

Figs. 4 and 5 provide a comparison of the separation of mixtures of propranolol and quinidine in serum and urine, respectively. The chromatograms shown in these figures were obtained using the same C_{18} column and 10% propanol in the SDS mobile phase, but the SDS concentrations and detector sensitivities were different. Inspection of the chromatograms in Figs. 1—5 clearly indicates that the body fluid background signal was the limiting factor in the limits of detection. Optimization of the signal-to-noise ratio could be performed by adjustment of detector sensitivity and surfactant concentration or by extraction of the drugs from the biological sample. The linearity of peak height response versus the drug concentration in urine samples, expressed as relative standard deviations of the slopes, ranged from 3.2 to 10.4%. The precision, given by the percentage R.S.D. of five replicate determinations of each drug in urine samples, was 1.9 to 7.1% (Table I). Although propranolol elutes on the tail of the serum protein components, obviating the use of the most sensitive detector range, a 200 ng/ml propranolol sample peak was clearly discerned whose height was proportional to drug concentration (Fig. 4). However, it has been found in other studies that the serum background can be completely eliminated by using a 470-nm cut-off filter [25]. Longer retention

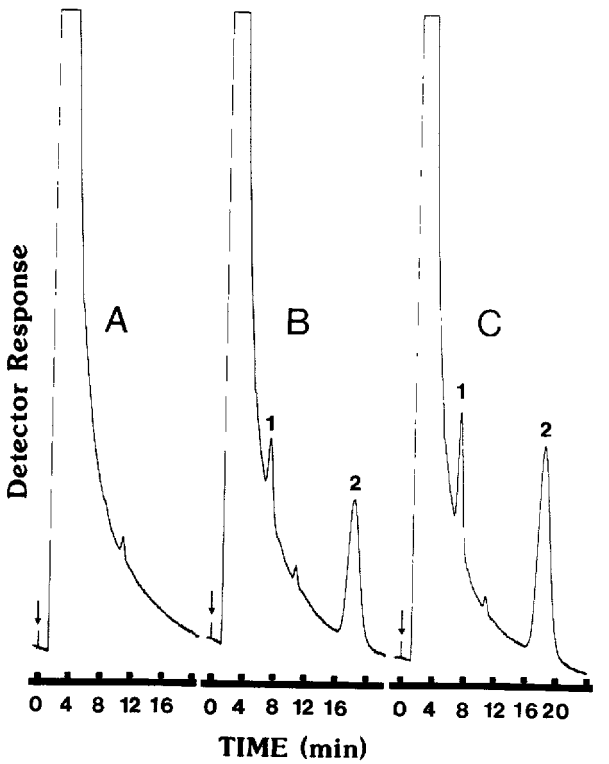

Fig. 4. Chromatograms of (A) serum blank, (B) serum blank with added 200 ng/ml propranolol (1) and 2 μg/ml quinidine (2), and (C) serum blank with added 400 ng/ml propranolol (1) and 3 μg/ml quinidine (2). Chromatographic conditions: column, 10-μm μBondapak C_{18}; mobile phase, 0.03 *M* SDS with 10% propanol, flow-rate, 1.0 ml/min; detector voltage, 700 V; sensitivity range, 0.5 μA, excitation wavelength, 215 nm; emission cut-off filter, 300 nm.

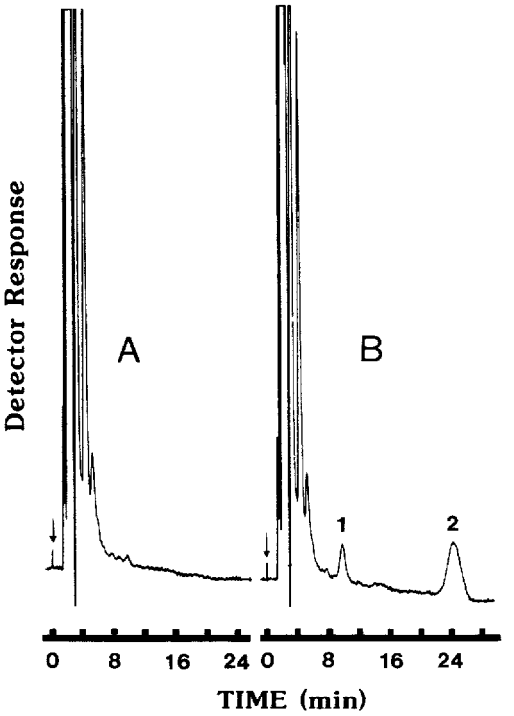

Fig. 5. Chromatograms of (A) urine blank and (B) urine with added 40 ng/ml propranolol (1) and 400 ng/ml quinidine (2). Chromatographic conditions: column, μBondapak C_{18}; mobile phase, 0.02 *M* SDS with 10% propanol; flow-rate, 1.0 ml/min; detector voltage, 700 V; sensitivity, 0.02 μA; excitation wavelength, 215 nm; emission cut-off filter, 300 nm.

times were observed at lower surfactant concentrations, as shown by comparing Figs. 4 and 5, illustrating the degree of selectivity obtainable by varying surfactant concentration at constant propanol content.

Modified micellar mobile phases give remarkably reproducible, sensitive, and rapid results for analysis of drugs in body fluids using direct injection of serum or urine samples. In addition to absorbance and fluorescence detection, it has been demonstrated recently that electrochemical detection works equally well in micellar chromatography [26]. Further studies in progress involve the use of different types of surfactants in the mobile phase for determination of other licit and illicit drugs in body fluids.

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