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USE OF MICELLAR MOBILE PHASES AND MICROBORE COLUMN SWITCHING FOR THE ASSAY OF DRUGS IN PHYSIOLOGICAL FLUIDS

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SUMMARY

The feasibility of directly assaying drugs in physiological fluids using on-line preconcentration and microbore high-performance liquid chromatography has been demonstrated. The untreated sample is injected onto a hydrophobic pre-column, using micellar sodium dodecyl sulfate (SDS) in the case of serum or phosphate buffer in the case of urine, as the load mobile phase. This traps the components of interest which are then backflushed onto a microbore analytical column using a stronger mobile phase. This procedure was then applied to diazepam in serum and phenobarbital in urine. Recovery was linear and quantitative over the range 30–3000 ng/ml for diazepam in serum and 2–200 μg/ml for phenobarbital in urine. The diazepam method was specific against caffeine and the three major metabolites of diazepam: oxazepam, temazepam, and nordiazepam. The effects of varying pre-column dimensions, pre-column loading time, and SDS concentration volume were evaluated.

INTRODUCTION

The assay of drugs in physiological fluids presents many difficult analytical problems. Frequently the drugs are present in low concentration, are strongly bound to proteins, and are in a complex matrix. High-performance liquid chromatography (HPLC) is frequently the technique of choice for these types of analyses. However, direct HPLC assay of physiological fluids is usually unfeasible due to interference from numerous endogenous compounds in the sample as well as very low analyte concentrations. The high-molecular-mass proteins found in these samples are particularly troublesome since they tend to precipitate within the column leading to rapid loss of chromatographic efficiency. Typically, the drugs must be extracted from their matrix and preconcentrated before assay. The extraction and preconcentration steps are usually labor-intensive, time-consuming, and introduce additional sources of error. While the use of robotics can allow complex sample preparation to be carried out with high precision and minimal labor cost, the equipment costs and development time of such methods are only

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justifiable for cases where high sample throughput over an extended time period is expected.

Several approaches have been used to streamline sample preparation for physiological fluids. One approach was to reduce the sample preparation to the precipitation of proteins [1–5] by organics, trichloroacetic acid or sodium hydroxide or to remove the high-molecular-mass proteins by ultrafiltration [6].

HPLC column switching has also been extensively used as a simple means of automating sample clean-up and preconcentration of the analyte. In one approach [7], the serum sample is loaded onto a pre-column coated with protein (denatured plasma proteins) with a relatively large particle size (20–32 μm), with a buffer or water as the load mobile phase. The analyte is then eluted from the pre-column onto the analytical column in the forward-flush mode with a stronger organic–water mobile phase.

The type of pre-columns which has received the most use in the direct injection of physiological fluids has been the large particle size (20–60 μm), C_8 or C_{18}. This type of pre-column operating in a size-exclusion mode lets serum proteins pass through them but traps the analyte of interest by adsorption chromatography. The sample is usually loaded on with an aqueous buffer and then eluted in the forward-flush mode [8,9] with a stronger analytical mobile phase.

Van der Horst et al. [8] found no difference in the results when the switching was done in forward flush or backflush mode. Nielen et al. [10] found that the detection limit and height equivalent to a theoretical plate (HETP) increased when the switching was done in the forward-flush mode as opposed to the backflush mode. The main difference between the above two approaches was that Nielen et al. [10] used a narrow-bore pre-column (1 mm I.D.).

Sample preparation has also been eliminated by the use of aqueous micelles, usually sodium dodecyl sulfate (SDS), as the mobile phase. The micelles prevent the precipitation of the serum proteins, but give low chromatographic efficiency [11–13].

We have recently demonstrated a highly sensitive technique for assaying drugs in plasma with minimal sample preparation [6]. The sample was subjected to ultrafiltration to remove proteins and the filtrate injected onto a reversed-phase pre-column using water as the mobile phase and then backflushed onto a microbore column using a stronger mobile phase. The increase in analyte concentration which occurred on the pre-column allowed the high mass sensitivity of microbore HPLC to be used to maximum advantage. In this study, the sample preparation has been further simplified by using different load mobile phases in the pre-column to eliminate the need for ultrafiltration. Diazepam in serum and phenobarbital in urine were studied. For the former, aqueous SDS micelles were used as the load mobile phase while aqueous phosphate buffer was used to preconcentrate phenobarbital from urine prior to microbore HPLC determination.

EXPERIMENTAL

Apparatus

The apparatus has been described elsewhere [6]. The pre-column was 15 mm × 3.2 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.) packed with 5-μm

ODS, and the analytical column was 25 cm×1 mm I.D. (Alltech Assoc., Deerfield, IL, U.S.A.) packed with Adsorbosphere, 5-μm ODS. A saturation column, 100 mm×4.6 mm I.D., packed with 37–53 μm silica gel (Whatman, Clifton, NJ, U.S.A.), was placed between the load pump and injector.

The column switching was facilitated by a Rheodyne Model 7126 valve which was pneumatically driven by an in-house column-switching apparatus and triggered by the external event contact closure of the Kratos 783 detector. Detection was at 208 nm for phenobarbital and 242 nm for diazepam and its metabolites. The detector signal was digitized using a Hewlett-Packard Model 18652A analog-to-digital converter and integrated using a Hewlett-Packard Model 3357 laboratory automation system. A DuPont Instruments autosampler allowed the system to be entirely automated.

Reagents

Acetonitrile (Burdick & Jackson Labs. Muskegon, MI, U.S.A.) and methanol (J.T. Baker, Phillipsburg, NJ, U.S.A.) were used to prepare mobile phases. Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), oxazepam (7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), temazepam (7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), nordiazepam (7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one), and caffeine were of USP grade. 5-Ethyl-5-(*p*-hydroxyphenyl)barbituric acid (HPB) and phenobarbital (5-ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine) were obtained from Sigma (St. Louis, MO, U.S.A.). SDS of electrophoresis purity was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) and was recrystalized once from methanol before use.

Sample preparation

Samples of either serum or urine were filtered through a 0.5-μm filter to remove suspended particulates before their direct injection onto the pre-column. Spiked serum samples were prepared from a stock solution of diazepam and its three major metabolites in methanol to produce concentrations over the range 30–3000 ng/ml.

Spiked urine samples were prepared from a stock solution of phenobarbital and HPB in methanol. Appropriate quantities of the stock solution were transferred to a test tube, and the methanol was evaporated to dryness. Urine (2 ml) was then transferred to the test tube to produce concentrations over the range 2–200 μg/ml. The above solutions were used to evaluate the precision and recovery of the methods.

Chromatographic procedures

The following sequence was used in the column-switching technique for the determination of diazepam in serum:

(1) Fill the sample loop, 200 μl, manually or via an autosampler.
(2) Inject the sample onto the pre-column using 0.01 *M* SDS as the loading mobile phase at 1 ml/min for 1.5 min. Linearly ramp the mobile phase to pure water in 0.1 min and hold for 10.5 min.

(3) Switch valve B from the load position to the backflush position at 12.5 min after injection and backflush the pre-column with analytical mobile phase.
(4) Switch valve B from the backflush position to the load position at 20 min.
(5) Wash the pre-column with a minimum of 10 column volumes (about 5 ml) of the loading mobile phase (0.01 *M* SDS) to condition the pre-column for the next sample. The analytical pump flow-rate was 60 μl/min with a mobile phase of methanol–water (65:35, v/v).

For the analysis of phenobarbital in urine the following column switching sequence was used:

(1) Fill the sample loop, 10 μl, manually or via an autosampler.
(2) Inject the sample onto the pre-column using 0.025 *M* phosphate buffer (pH 7.5) as the loading mobile phase at 1 ml/min for 2.0 min.
(3) Switch valve B from the load position to the backflush position at 2.0 min and backflush the pre-column with analytical mobile phase.
(4) Switch valve B from the backflush position to the load position after 10 min.
(5) Wash the pre-column with a minimum of 10 column volumes (about 5 ml) of the loading mobile phase (phosphate buffer, pH 7.5) to condition the pre-column for the next sample. The analytical pump flow-rate was 45 μl/min with a mobile phase of acetonitrile–0.025 *M* phosphate buffer (pH 7.5) (15:85, v/v).

RESULTS AND DISCUSSION

Diazepam in serum

The determination of diazepam was used to demonstrate the applicability of this technique to serum samples. Analytical standards of oxazepam, temazepam, nordiazepam, and diazepam were used over the concentration range 30–3000 ng/ml to evaluate linearity. The linearity and recovery were evaluated for diazepam only. The endogenous peaks in the serum resulted in only a slight interference (5 ng/ml) for the determination of diazepam, while they constituted a considerable interference for the metabolites of diazepam and thus prevented their quantitation. The equation of the calibration curve was $y=0.0434x+0.680$, $r^2=0.9993$. The mean recovery of diazepam in serum was 89.4% over the concentration range 30–3000 ng/ml. Recovery was calculated by comparison of peak areas to that of an external diazepam standard. The less than quantitative recovery may be due to incomplete release of diazepam from the serum proteins. The within-day precision (relative standard deviation, R.S.D.) of the method was determined to be 5.5% ($n=3$) by analysis of serum samples spiked with diazepam (120 ng/ml).

Blank, sample, and standard chromatograms are given in Figs. 1, 2, and 3, respectively. The chromatography was specific against caffeine and the three major metabolites of diazepam. Since one sample can be loaded on the pre-column while another is being chromatographed on the analytical column, the analysis time per sample is 19.5 min.

For the calculation of the capacity factor (k') and separation factor (α), the apparent retention times ($t_{R(A)}$) were corrected by subtraction of the loading time (t_L) to give the corrected retention times, i.e., $t_{R(C)}=t_{(R)A}-t_L$. The cor-

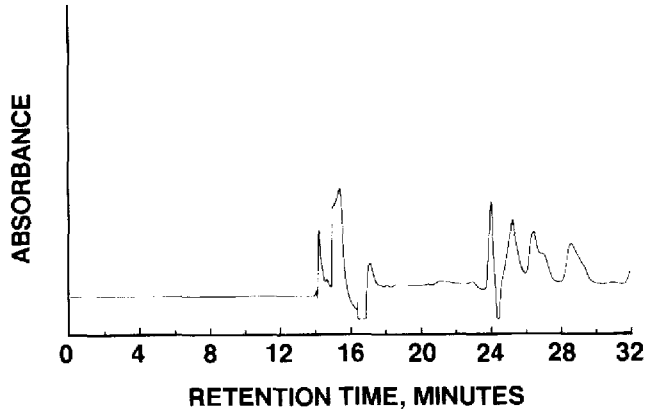

Fig. 1. Chromatogram of serum blank using the apparatus and chromatographic conditions described in the text. The injection volume was 200 μl (0.125 a.u.f.s.).

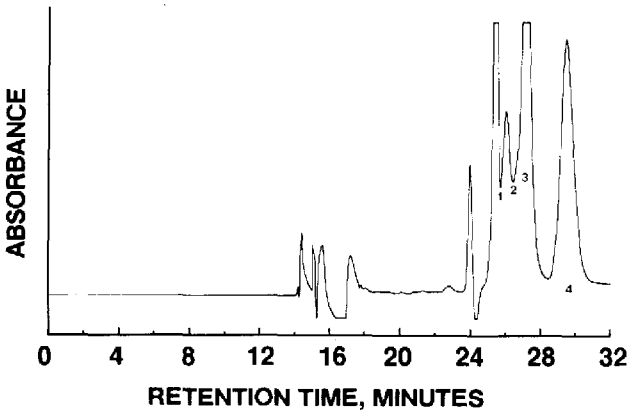

Fig. 2. Chromatogram of diazepam and its three metabolites in seum (120 ng/ml) using the apparatus and chromatographic conditions described in the text. The injection volume was 200 μl (0.125 a.u.f.s.). Peaks: 1 = oxazepam (k' = 7.0); 2 = temazepam (k' = 7.4); 3 = nordiazepam (k' = 8.1); 4 = diazepam (k' = 9.4, α = 1.2).

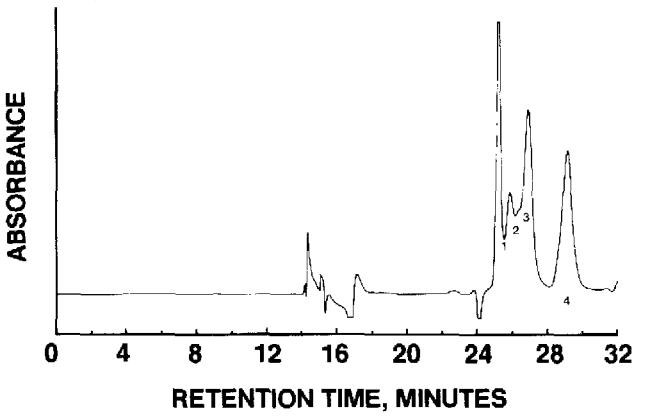

Fig. 3. Chromatogram of a standard solution of diazepam and its major metabolites (100 ng/ml) using the apparatus and chromatographic conditions described in the text. The injection volume was 200 μl (0.25 a.u.f.s.). Peaks: 1 = oxazepam (k' = 7.0); 2 = temazepam (k' = 7.4); 3 = nordiazepam (k' = 8.1); 4 = diazepam (k' = 9.4, α = 1.2).

rected retention time for an unretained peak ($t_{0(C)}$) was determined by a similar fashion. Thus, $k' = (t_{R(C)} - t_{0(C)})/t_{0(C)}$ and $\alpha = k_1'/k_2'$.

The backpressure of the chromatographic system did not increase throughout the study which represented the injection of more than 20 ml of serum. Thus, the SDS in the loading mobile phase appears to be sufficient to protect the columns from contamination by high-molecular-mass components in the matrix. The limit of detection (30 ng/ml) and extraction efficiency (89.4%) of the present technique compares favorably with that obtained by other methods which used off-line extraction and preconcentration procedures for the analysis of diazepam in plasma [14–18].

Phenobarbital in urine

During the initial method development for the determination of phenobarbital in urine, 0.01 *M* SDS was used as the load mobile phase. It was found that the SDS greatly decreased the pre-column's capacity and adversely effected the resolution and peak shape. Since phenobarbital is more polar than diazepam, lower retention of the former on a reversed-phase pre-column is expected. Thus, the SDS was substituted with 0.025 *M* phosphate buffer (pH 7.5). The buffer allowed larger sample volumes to be injected without an increase in column back-pressure or a decrease in chromatographic efficiency. Also, the analyte of interest would be retained on the front of the pre-column while the water-soluble components of the urine would be flushed to waste. The HPB was not retained by the pre-column and thus could not be quantitated by this method. Analytical standards of HPB and phenobarbital were used over the concentration range 2–200 μg/ml to evaluate linearity. Again, since one sample can be loaded while another is being chromatographed on the analytical column, the analysis time per sample is 18.0 min. Blank, sample, and standard chromatograms are given in Figs. 4, 5, and 6, respectively. Linearity was only evaluated for phenobarbital and there were no significant interferences from HPB or endogenous peaks in the urine. The equation of the calibration curve was $y = 0.392x + 1.12$, $r^2 = 0.9987$. The mean recovery for phenobarbital in urine was 102.0%. Recovery was calculated by comparison of peak areas with the standard calibration curve. The within-day precision (R.S.D.) of the method was determined to be 1.6% ($n = 6$) by analysis of urine samples spiked with phenobarbital (50 μg/ml). The limit of detection (signal-to-noise ratio of 2) was 2 μg/ml which compares favorably with other published methods of 2 μg/ml [19] and 5 μg/ml [20].

Effect of propanol and SDS concentration

The effect of adding *n*-propanol to the load mobile phase, 0.03 *M* SDS with 3% added *n*-propanol, was investigated using diazepam as the test solute. The pre-column was connected directly to the detector for this study. The capacity factor for diazepam decreased from 11.9 to 4.6 and the retention volume on the pre-column decreased from 2.2 to 0.96 ml when *n*-propanol was added to the load mobile phase, although the peak shape was significantly improved.

The use of *n*-propanol in the load mobile phase was abandoned because it greatly

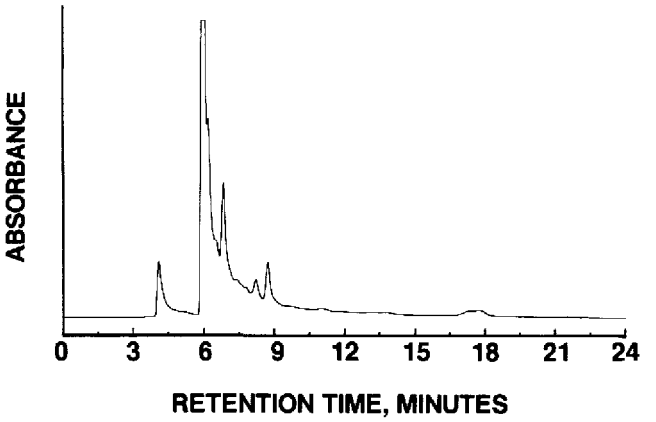

Fig. 4. Chromatogram of urine blank using the apparatus and chromatographic conditions described in the text. The injection volume was 10 μl (1.0 a.u.f.s.).

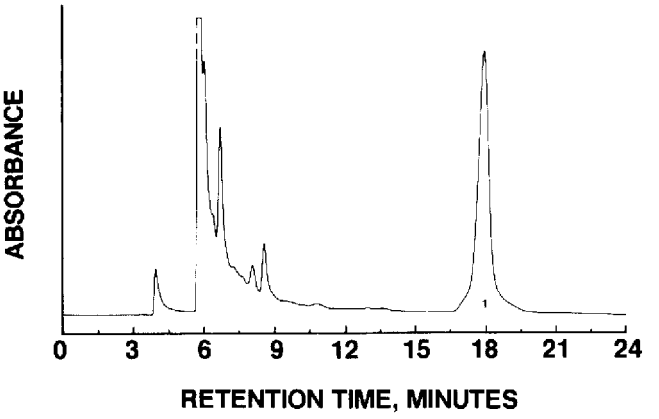

Fig. 5. Chromatogram of phenobarbital in urine (50 μg/ml) using the apparatus and chromatographic conditions described in the text. The injection volume was 10 μl (1.0 a.u.f.s.). Peak 1 = phenobarbital ($k' = 8.1$).

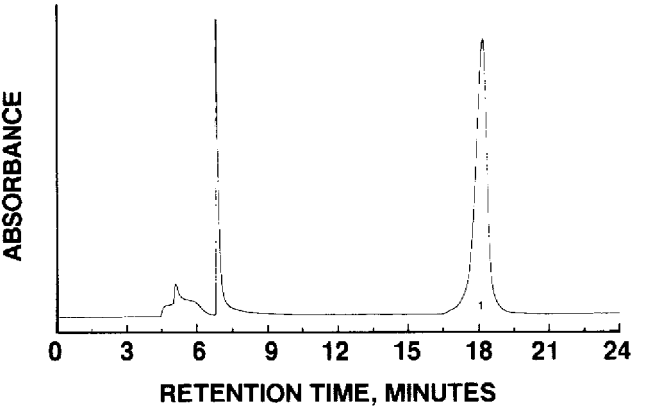

Fig. 6. Chromatogram of a standard solution of phenobarbital (100 μg/ml) using the apparatus and chromatographic conditions described in the text. The injection volume was 10 μl (1.0 a.u.f.s.). Peak 1 = phenobarbital ($k' = 8.1$).

decreased the sample volume which could be used without breakthrough of the analyte peak on the pre-column.

Effect of load time and flow cell size

The effect of load time on the peak width at half height ($W_{1/2}$) as investigated. Diazepam was used as the test solute (0.5 μg/ml). The injection volume was held constant at 20 μl and the load time was varied from 0.2 to 1.0 min in 0.2-min intervals. The analytical mobile phase was acetonitrile–water (50:50, v/v) at 75 μl/min and the load mobile phase was 0.02 *M* SDS at 1.0 ml/min. $W_{1/2}$ linearly increased by 19% when the load time was increased from 0.2 to 1.0 min ($y=9x+41.2, r^2=0.959$). These results indicate that the analyte zone is expanding as it moves down the pre-column and that the stronger analytical mobile phase does not completely compact the analyte zone. Thus, the load time should be only long enough to move the analyte from the sample loop and flush the unretained components of the sample to waste.

The effect of using a 12-μl versus a 2.5-μl flow cell in the Kratos detector was examined. The analytical mobile phase was acetonitrile–water (50:50, v/v) at 75 μl/min and the load mobile phase was 0.02 *M* SDS at 1.0 ml/min. A 100-μl loop was used with diazepam (0.25 μg/ml) as the analyte. The load time was 0.5 min. The number of theoretical plates (N) of the chromatographic system with the 12-μl flow cell was only 93.8% of the N with the 2.5-μl flow cell, vis., 679 versus 743, respectively.

Column lifetime

The pre-column did not exhibit any increase in back-pressure during the study which represented the injection of 50–100 ml of urine or serum. The retention of diazepam on the pre-column did decrease upon repeated injections of serum samples. The breakthrough volume (V_B) of diazepam on a new pre-column with 0.01 *M* SDS was 12 ml. This decreased after repeated injections of serum. When V_B became less than 2 ml or when the peak shape deteriorated to an unacceptable level, the pre-column was changed or rejuvenated by washing with about 60 ml of methanol. This decrease in V_B did not occur for the assay of phenobarbital in urine, probably because the protein content in urine is much less than in serum.

In the initial studies with diazepam, the concentration of SDS in the load mobile phase was varied from 0.01 to 0.05 *M*. The V_B for diazepam decreased when the SDS concentration was increased as one would expect. The SDS concentration of 0.01 *M* was found to give the best separation of diazepam from the serum proteins. When the load mobile phase was maintained at 0.01 *M* SDS, with no step gradient to pure water as in the final method, and the pre-column was backflushed with the analytical mobile phase, the residual SDS which was in the connecting tubing and pre-column was also backflushed onto the analytical column. After about five injections, the efficiency of the analytical column was decreased by 25%. Thus, a step gradient to pure water during the load phase was incorporated. This eliminated the decrease in the efficiency of the analytical column.

CONCLUSIONS

A microbore column-switching technique has been described in which physiological fluids can be assayed for drugs by direct injection onto an HPLC. The technique was demonstrated by the assay of diazepam in serum and phenobarbital in urine. The use of SDS and phosphate buffer as a load mobile phase was discussed for the analysis of serum and urine, respectively. It was found that the addition of *n*-propanol to the load mobile phase significantly improved the analyte peak shape while decreasing k'. Column efficiency was increased when a water wash of the pre-column was used prior to the backflush, since no SDS was then eluted onto the analytical column. The technique allows for increased concentration sensitivity and decreased sample preparation. As with any technique in which the sample extraction and isolation steps have been eliminated or reduced, the possibility of endogenous interferences is increased.

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