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TRACE ANALYSIS OF DIAZEPAM IN SERUM USING MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ON-LINE PRECONCENTRATION

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SUMMARY

The feasibility of determining trace analytes in human serum using on-line preconcentration and microbore high-performance liquid chromatography has been demonstrated. The serum is subjected to ultracentrifugation and injected onto a hydrophobic pre-column using water as the mobile phase. This traps the components of interest which are then backflushed onto a microbore analytical column using a stronger mobile phase. The column-switching apparatus was evaluated using highly dilute aqueous paraben solutions and sample enrichment factors as high as 1500 were obtained. The procedure was then applied to diazepam in serum. Recovery was linear and quantitative over the range from at least 4 to 1000 ng/ml. The 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 sample volume were evaluated.

INTRODUCTION

The determination of trace levels of drugs and their metabolites in physiological fluids frequently involves lengthy sample preparation. Moreover, to obtain sufficient concentration of analyte in the working sample for chromatographic

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analysis, preconcentration steps such as solvent extraction and evaporation to dryness are needed. Although microbore high-performance liquid chromatography (HPLC) offers very high mass sensitivity, its concentration sensitivity is no higher than that of conventional HPLC. Column switching can be used for both sample clean-up and preconcentration of trace analytes. This procedure is also amenable to automation. Scott and Kucera [1] used a column-switching apparatus to load serum samples on a microbore pre-column prior to microbore HPLC analysis. Other investigators, employing conventional columns, have used buffers [2,3], size exclusion, and/or affinity chromatography to trap the analytes in serum on a pre-column prior to injection onto the analytical column, thus preventing the precipitation of the serum proteins [4,5]. Takeuchi and co-workers [6,7] have trapped analytes in serum off-line for injection onto a microbore column.

Cotler et al. [8] determined diazepam and its three major metabolites in man and cat by HPLC with a detection limit of 50 ng/ml in plasma and blood. The sample preparation required several extraction, evaporation to dryness, and reconstitution steps. Rao et al. [9] used Bond-Elut C_{18} columns to isolate diazepam and its metabolites from blood. Reversed-phase HPLC with methylnitrazepam as an internal standard was then used for quantification.

We have recently developed an alternative approach for the determination of trace analytes in serum. The technique involves pretreatment via ultrafiltration to remove serum proteins followed by injection of the ultrafiltrate onto a short reversed-phase pre-column using water as the mobile phase. The components of interest are trapped at the head of the pre-column and subsequently backflushed onto a microbore column using a stronger analytical mobile phase. The use of a relatively wide-bore (3.2 mm I.D.) pre-column in conjunction with a microbore analytical column (1.0 mm I.D.) enables large enrichment factors to be obtained without a concomitant decrease in sensitivity. Since all system components, including columns and valves, are commercially available, this system can be assembled by any experienced chromatographer. This technique has the advantage over previously reported techniques in that no irreversible instrumental modifications are necessary [10,11], neither buffers nor internal standards are needed [9,12], and there is only a relatively small decrease in chromatographic efficiency compared to that obtained by direct sample injection.

The only sample pretreatment necessary was removal of serum proteins by ultrafiltration. Separations of methyl, propyl, and butyl parabens were used to evaluate the performance of the system. The effects of column switching on chromatographic performance and the influence of sample volume, pre-column load time, and pre-column dimensions are discussed.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), methanol and HPLC-grade water (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-

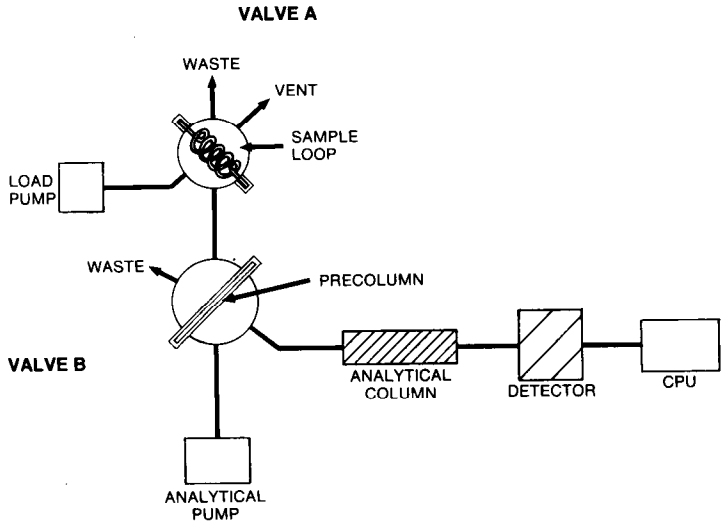

Fig. 1. Microbore column-switching apparatus described in the text. CPU = computer.

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), caffeine, methyl paraben, propyl paraben, and butyl paraben were all of United States Pharmacopeia grade.

Apparatus

The apparatus, illustrated in Fig. 1, consisted of a 15 mm×3.2 mm I.D. pre-column from Brownlee Labs. (Santa Clara, CA, U.S.A.) packed with 5-μm ODS, a 25 cm×1 mm I.D. analytical column from Alltech Assoc. (Deerfield, IL, U.S.A.) packed with Adsorbosphere, 5-μm ODS, and a Rheodyne Model 7001 injection valve.

For direct injection experiments a Rheodyne Model 7520 injection valve was utilized, which was connected to the 25-cm analytical column with 4 cm of 0.10 mm I.D., 316 stainless-steel tubing. For the column-switching experiments an additional Rheodyne Model 7126 valve was used to facilitate the backflushing of the analyte from the pre-column onto the analytical column. The detector was a Kratos Model 783 variable-wavelength detector equipped with a 2.4-μl (6 mm pathlength) flow cell. Detection was at 254 nm for the parabens 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 by using the integration event "R", which instructs the computer to reset the baseline at all valleys. A DuPont Instruments autosampler allowed the system to be entirely automated.

The Brownlee pre-column was connected to the Rheodyne switching valve with 3 cm and 15 cm of 0.127 mm I.D., 316 stainless-steel tubing, at the inlet and outlet ends, respectively. A Spectra-Physics Model SP8700XR pump, was used to load the sample onto the pre-column and a Gilson Model 303 pump, fitted with a 5SC

low-volume pump head, was used to backflush the analyte from the pre-column onto the analytical column. For some experiments, an in-house packed microbore pre-column, 3 cm × 1 mm I.D., packed with 5-μm Spherisorb ODS-2 (Phase Separations, Queensferry, U.K.), was used in place of the Brownlee column.

Sample preparation

A 1.0-ml aliquot of serum was transferred to a Centrifree (Amicon, Danvers, MA, U.S.A.) ultrafiltration cartridge and centrifuged at 1200 *g* in a 35° fixed angle rotor centrifuge for 30 min. A 0.5-ml aliquot of the ultrafiltrate was then injected into the liquid chromatograph. Recovery and precision data were generated using samples of ultrafiltrate spiked with a stock solution of diazepam and its three major metabolites to produce concentrations of 4–1000 ng/ml.

Chromatographic procedures

The following sequence was used in the column-switching technique for the initial studies with the parabens. (1) Fill the sample loop, 1.0 ml, manually or via an autosampler. (2) Inject the sample onto the pre-column using water as the loading mobile phase at 1 ml/min for 1.25 min. (3) Switch valve B (Fig. 1) from the load position to the backflush position after 1.25 min, and backflush the pre-column with analytical mobile phase. (4) Switch valve B from backflush position to load position after 10 min. (5) Wash the pre-column with a minimum of ten column volumes (about 5 ml) of the loading mobile phase (water in this study) to condition the pre-column for the next sample. The analytical pump flow-rate was 85 μl/min with a mobile phase of acetonitrile–water (35:65, 40:60 or 50:50).

For the analysis of diazepam in serum the following column switching sequence was used. (1) Fill the sample loop, 0.5 ml, manually or via an autosampler. (2) Inject the sample onto the pre-column using water as the loading mobile phase at 1 ml/min for 5.0 min. (3) Switch valve B (Fig. 1) from the load position to the backflush position after 5.0 min, and backflush the pre-column with analytical mobile phase. (4) Switch valve B from backflush position to load position after 15 min. (5) Wash the pre-column with a minimum of ten column volumes (about 5 ml) of the loading mobile phase (water in this study) 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).

RESULTS AND DISCUSSION

Influence of load time on analysis

The load time, the time for which the sample was pumped onto the pre-column, was found to have a significant influence upon recovery. When a 0.5- or 1.0-ml sample is used, sufficient volume of mobile phase must be pumped to move the entire sample from the sample loop onto the pre-column. If too much loading mobile phase is used, a portion of the analyte will break through the outlet of the pre-column. The break through volume of diazepam on the pre-column was determined to be 26 ml using water as the loading mobile phase. Thus, there is an upper limit to the size of sample that could be used in any particular analysis,

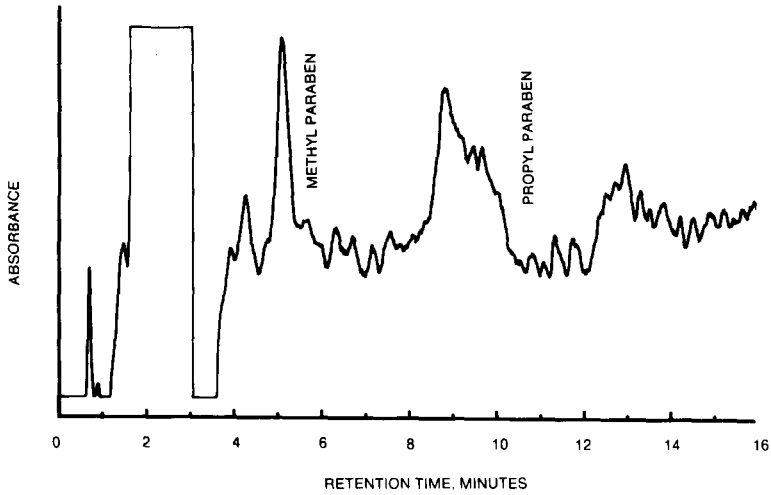

Fig. 2. Chromatogram of methyl and propyl paraben (74 and 150 pg/ml, respectively) using the apparatus and chromatographic conditions described in the text. The injection volume was 1.0 ml and the analytical mobile phase was acetonitrile–water (50:50).

which is dependent upon how strongly the analyte is retained by the pre-column. If too large a sample is used, then a fraction of the analyte will break through the pre-column to waste before the entire sample is loaded. The sample volume which can be loaded onto the pre-column without loss of analyte depends on the hydrophobicity of the species of interest; i.e., the more hydrophobic the analyte, the greater the maximum sample size. The loading time can also have a significant effect upon resolution and baseline quality. The elution of the more viscous components in serum from the pre-column was facilitated by longer loading times.

If quantitative analysis of several species in a given sample is desired, the hydrophobicity of the most weakly retained species determines the maximum sample volume.

Separation of parabens

This technique was initially evaluated by separating a mixture of methyl, propyl, and butyl parabens. Baseline resolution of the three parabens in aqueous solution was obtained with a 1.0-ml sample and a detection limit of 74 pg/ml for methyl paraben was obtained (Figs. 2 and 3). When analyses are performed at these trace levels, it was noted that trace contaminants in the loading mobile phase caused extra peaks in the chromatograms. It was also found that the pre-column should be regenerated with approximately ten column volumes of the loading mobile phase prior to the next sample injection. This insured that the pre-column had a homogeneous mobile phase throughout its length, which resulted in more reproducible retention times and peak areas. The regeneration of the pre-column is simultaneous with the analytical chromatography and thus does not lengthen the overall analysis time.

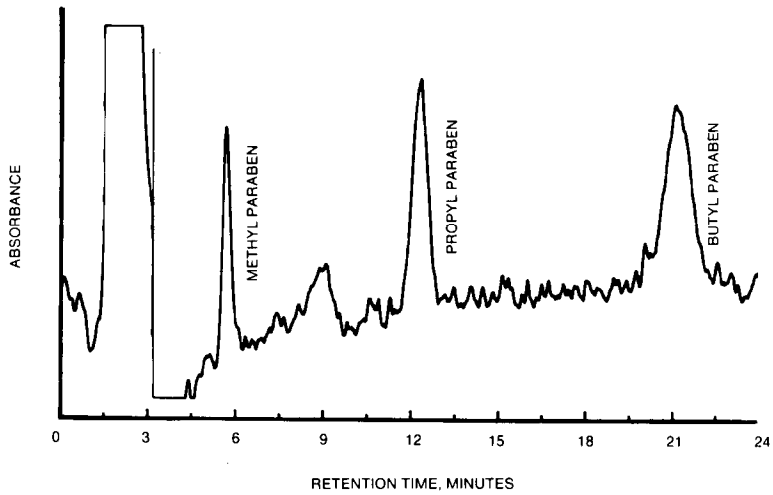

Fig. 3. Chromatogram of methyl, propyl, and butyl parabens (0.74, 1.5, and 1.9 ng/ml, respectively) using the apparatus and chromatographic conditions described in the text. The injection volume was 1.0 ml and the analytical mobile phase was acetonitrile–water (35:65).

Influence of column switching on chromatographic efficiency

The number of theoretical plates, N [$N=5.54(t_R/W_{1/2})^2$], calculated using aqueous diazepam solutions in the direct injection and column-switching modes were 1900 and 2000, respectively. This comparison seems to indicate that no extra-column band broadening occurred as a result of using column switching (in fact the switching apparently increased the number of theoretical plates by 5%). This is not accurate, however, since there was an additional time lag before the analytical mobile phase reached the detector due to the volume of the pre-column in the switching mode. If this extra volume is taken into account when calculating theoretical plates, there was a decrease of 15% in the number of calculated theoretical plates. Also, only 75% of the theoretical enrichment factor, when measured by peak area, of 2000 was attained for a 1000-μl column-switching experiment as compared to a 0.5-μl direct-injection experiment. This decrease may have been due to the increased noise level and dilution of analyte from the additional connecting tubing.

In one set of experiments, an in-house packed pre-column (3 cm $\times$ 1.0 mm I.D.) was used. The use of this pre-column produced peaks that were generally less broad than those of the Brownlee column. The major disadvantage of the in-house packed pre-column was its limited sample capacity due to its small size. This greatly limited the degree of preconcentration which could be produced. Further studies on the influence of pre-column dimensions on chromatographic efficiency are in progress.

Diazepam in serum

The determination of diazepam in serum was used to demonstrate the applicability of this technique to physiological samples. Analytical standards of oxazepam, temazepam, nordiazepam, and diazepam were used over the concentration

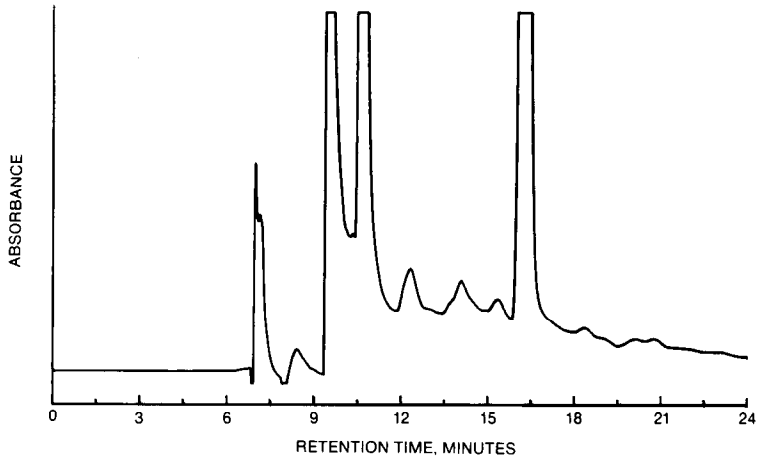

Fig. 4. Chromatogram of serum blank using the apparatus and chromatographic conditions described in the text. The injection volume was 0.5 ml.

range 4–1000 ng/ml to evaluate linearity. The linearity was evaluated for diazepam only. There was no interference from endogenous peaks in the serum or from the metabolites of diazepam. The equation of the calibration curve was $y=0.457x+0.720$, $r^2=1.0000$. The mean recovery of diazepam in serum was 96.6% over the concentration range 4–1000 ng/ml. Recovery was calculated by comparison of peak areas to that of an external diazepam standard. The precision of the method was determined to be 5.2% R.S.D. ($n=3$) by analysis of serum samples spiked with diazepam (160 ng/ml).

Aqueous diazepam standards were treated by ultrafiltration and analyzed by this technique. It was found that the diazepam did not exhibit binding to the ultrafiltration membrane. Ultrafiltration has been used previously for the determination of free phenytoin in plasma by Miller and Pinkerton [13]. The serum samples were spiked after ultrafiltration since the free diazepam (i.e., non-protein bound) was the analyte of interest. Blank, sample, and standard chromatograms are given in Figs. 4, 5, and 6, respectively. The chromatography was specific against caffeine and the three major metabolites of diazepam. It should be noted that since free diazepam is not bound by the ultrafiltration cartridge and since both the preconcentration and backflush desorption steps are quantitative, no internal standard is required.

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 corrected retention time for an unretained peak ($t_{0(c)}$) was determined by a similar fashion to be 2.0 min. 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 ultrafiltrate. Thus, the filtration step appears to be sufficient to protect the columns from contamination by high-molecular-weight components in the matrix.

The sensitivity of the present technique (4 ng/ml) compares favorably with

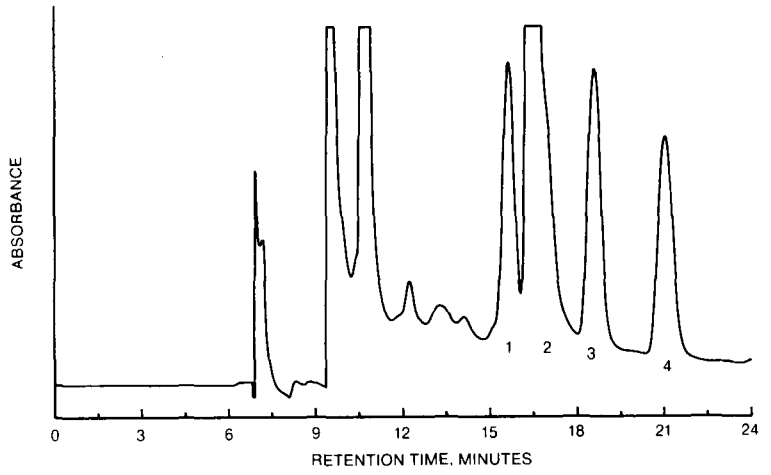

Fig. 5. Chromatogram of diazepam and its three metabolites in serum (160 ng/ml) using the apparatus and chromatographic conditions described in the text. The injection volume was 0.5 ml. Peaks: 1 = oxazepam ($k' = 4.3$); 2 = temazepam ($k' = 4.7$); 3 = nordiazepam ($k' = 5.8$); 4 = diazepam ($k' = 7.0$, $\alpha = 1.2$).

that obtained by other methods which used off-line extraction and preconcentration procedures for the analysis of diazepam in plasma (10 ng/ml) and whole blood (40 ng/ml) [14,15].

CONCLUSION

A generalized technique has been developed and demonstrated for the determination of drugs in physiological fluids. The technique was found to give a 1500-

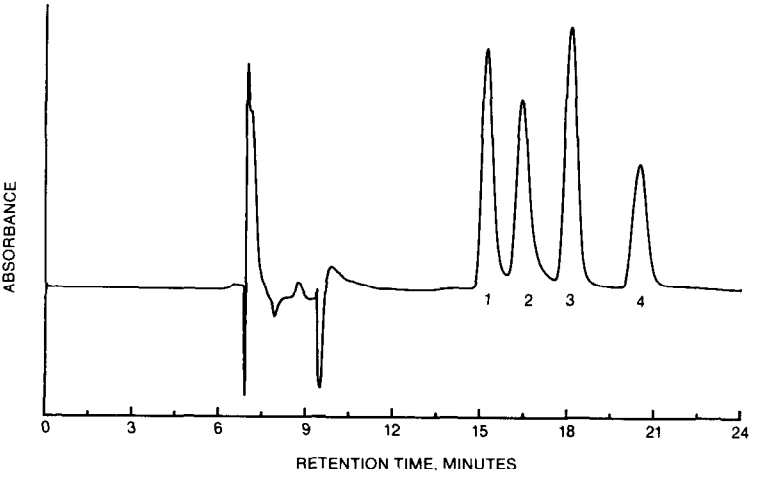

Fig. 6. Chromatogram of a standard solution of diazepam and its major metabolites (100 ng/ml) using the apparatus and chromatographic conditions described in the text. The injection volume was 0.5 ml. Peaks: 1 = oxazepam ($k' = 4.3$); 2 = temazepam ($k' = 4.7$); 3 = nordiazepam ($k' = 5.8$); 4 = diazepam ($k' = 7.0$, $\alpha = 1.2$).

fold increase (75% of that expected based on 0.5-μl injections) in concentration sensitivity while maintaining 85% of the theoretical plates with a 1.0-ml injection volume. The reduction in theoretical plates is presumably due to dispersion of the analyte zone within the pre-column and connecting hardware. The minor efficiency reduction engendered by the wider pre-column is not a serious limitation considering the increase in sensitivity which is obtained. The simplified sample preparation and instrumental design should facilitate the extension of this technique to other analytes at the trace level.

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