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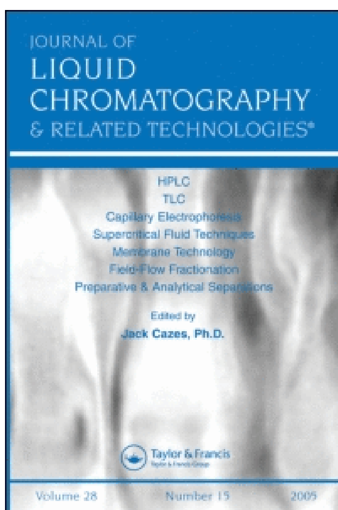

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An HPLC Method for the Determination of Diltiazem and Three of its Metabolites in Serum

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AN HPLC METHOD FOR THE DETERMINATION OF DILTIAZEM AND THREE OF ITS METABOLITES IN SERUM

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ABSTRACT

A high performance liquid chromatographic method was developed for the determination of diltiazem and three of its metabolites in serum. Serum samples are extracted with methyl-*tert.*-butyl ether followed by back-extraction into 0.05N sulfuric acid. Doxepin hydrochloride was used as internal standard. A cyanopropylsilane column in the reverse-phase mode was utilized with a mobile phase of acetonitrile-phosphate buffer-triethylamine (pH 3.5). Using UV detection at 237nm, the lower limit of sensitivity was 1.5-3.0 ng/ml. Recovery and reproducibility results and possible interferences from other compounds are presented and discussed. The assay procedure was applied to a chronic oral dosing study in humans to assess the disposition of diltiazem and its primary metabolites.

INTRODUCTION

Diltiazem, *cis*-(+)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxy-phenyl)-1,5-benzothiazepin-4-(5H)-one hydrochloride, is a calcium-channel antagonist proven effective in the treatment of angina pectoris (1-3).

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Human pharmacokinetic studies have been performed in normal volunteers using both single oral, multiple oral, and intravenous doses. Maximum serum diltiazem concentrations ranged from 50 to 500 ng/ml after administraion of 60 to 210 mg single oral doses (4). Peak serum concentrations following administration of a rapid release loose filled capsule occured at approximately one hour, suggesting rapid absorption. Apparent serum half-lives of diltiazem varied from 2.9 to 4.0 hours (5). Previously determined pharmacokinetic characterstics of diltiazem include a large systemic clearance (15-16 ml/min/kg), approximating hepatic blood flow, large apparent oral clearance (21-24 ml/min/kg), extensive protein binding (80-90%), low absolute bioavailability (30-40%) despite greater than 90% absorption from the G.I. tract, and a large volume of distribution (4-7 L/kg) (4,5). To characterize the pharmacokinetics and pharmacodynamics of diltiazem in various patient populations, a reliable analytical procedure is required.

Chromatographic methods for the determination of diltiazem include gas chromatography (GC) (6-9), and high-performance liquid chromatography (HPLC) (10-12). These methods either do not permit the separation and determination of diltiazem metabolites present in serum or have mobile phase conditions (HPLC) that limit the lifetime of the analytical column and diminish resolution over time.

The present paper describes diltiazem (DTZ), N-desmethyldiltiazem (DMD), desacetyldiltiazem (DAD), and N-desmethyl-desacetyldiltiazem (DMDAD) analysis in serum using reverse-phase HPLC on a cyanopropylsilane column following a simple two-step liquid-liquid extraction.

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MATERIALS

Instrumentation

The system utilized consisted a constaMetric III metering pump (LDC/Milton Roy, Riveria Beach, FL) fitted with a six-port rotary valve (Model 7126, Rheodyne, Cotati, CA) and a 100 μL capillary loop; a spectroMonitor III variable wavelength UV detector (LDC/Milton Roy, Riviera Beach, FL) operated at 237 nm; a C-R3A Chromatopac integrator (Shimadzu, Kyoto, Japan); and a 6 μm cyanopropylsilane column, 4.6 x 250 mm (Dupont, Wilmington, DE). All separations were performed isocratically at a flow rate of 1.2 ml/min (1100 psi) with the column maintained at ambient temperature.

Chemicals and Reagents

Sulfuric acid, phosphoric acid, potassium phosphate monobasic, and triethylamine were reagent grade (Fisher Scientific, Fairlawn, NJ). Acetonitrile and methyl-*tert.*-butyl ether were HPLC

grade (Burdick and Jackson, Muskegon, WI). DTZ, DMD, DAD, and DMDAD were supplied by Marion Laboratories, Kansas City, MO. Doxepin hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO).

Mobile Phase

Potassium phosphate monobasic and triethylamine were added to deionized water to produce a 0.005M and 0.4% solution. The pH was adjusted to 3.5 with 2M phosphoric acid and filtered. The mobile phase consisted of 22% acetonitrile and 78% phosphate buffer.

Stock Solutions

Stock solutions of DTZ, DMD, DAD, DMDAD, were prepared by dissolving 10 mg of each base in 100 ml methanol. Working dilutions of 1 ng/μL were prepared from the stock solutions. A stock solution of doxepin hydrochloride was prepared by dissolving 10 mg of base in 100 ml of methanol. A working dilution of 6 μg/mL was prepared from the stock solution.

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METHODS

Validation Procedure

The performance of the assay was assessed for a 7 day period to validate between-day reproducibility. This consisted of analyzing 1) a standard curve in serum (blank, 10, 20, 50, 100, and 200 ng/ml of each analyte), and 2) three replicate validation samples at three different concentrations (30, 70, 160 ng/ml of each analyte). Within-day reproducibility was assessed by analyzing a standard curve in serum and seven replicate validation samples at each level.

Preparation and Extraction of Serum Standards and Unknown Samples

To 1.0 ml of serum in a 15 ml screw-capped centrifuge tube, aliquots of drug and metabolite working solutions were added to prepare a standard curve (blank, 10, 20, 50, 100, and 200 ng/ml of each analyte) and validation samples (30, 70, and 160 ng/ml of each analyte). A 100 μL aliquot of internal standard working solution and 5 ml of methyl-*tert.*-butyl ether were added to each spiked serum sample. The tubes were vortexed for one minute and centrifuged at 1200g for 10 minutes. The aqueous layer was frozen in a dry ice/acetone bath. The organic layer was decanted to a 12 ml conical

vortex tube and 100 µL of 0.05N sulfuric acid added. The conical tubes were gently vortexed for one minute and the phases allowed to separate at room temperature for 30 minutes. The ether layer was removed and any remaining ether allowed to evaporate at room temperature. All of the aqueous phase was injected onto the column.

Standard Curve and Concentration Calculations

The ratio of analyte peak height-to-internal standard peak height (PHR), as determined by perpendicular peak height to baseline for each analyte (Shimadzu, Kyoto, Japan), were tabulated for each standard curve concentration and a linear regression analysis was performed. The analyte concentrations of each validation sample were calculated from their resulting PHR and the linear regression function generated from the respective standard curve.

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Sensitivity and Recovery

The lower limit of sensitivity was considered the lowest analyte concentration which yielded a signal-to-noise ratio (analyte peak height/average baseline height) of ≥ 4.0. Percent extraction recovery of the analytes was assessed by comparing the peak heights of chromatograms obtained from extracted serum samples to those of the standard stock methanol solutions at 100 ng/ml on three separate occasions.

Application of Methods

To assess the pharmacokinetics of DTZ, DMD, DAD and DMDAD during chronic oral therapy, eight normal male volunteers (age 20-25) were administered 90 mg diltiazem orally every eight hours. After eight days of treatment, blood samples were collected for 8 hours and serum concentration-time plots constructed for each analyte.

RESULTS AND DISCUSSION

DTZ, DMD, DAD, DMDAD, and doxepin are well resolved from each other. Chromatograms of blank serum, a serum standard and a normal volunteer sample are shown in Figure 1. Doxepin was chosen as internal standard as it was available in high purity, was coextractable, did not react with any components of the sample, eluted near and was resolved well from the analytes of interest, and absorbed adequately at the analytical wavelength. In addition, the following compounds, injected as unextracted

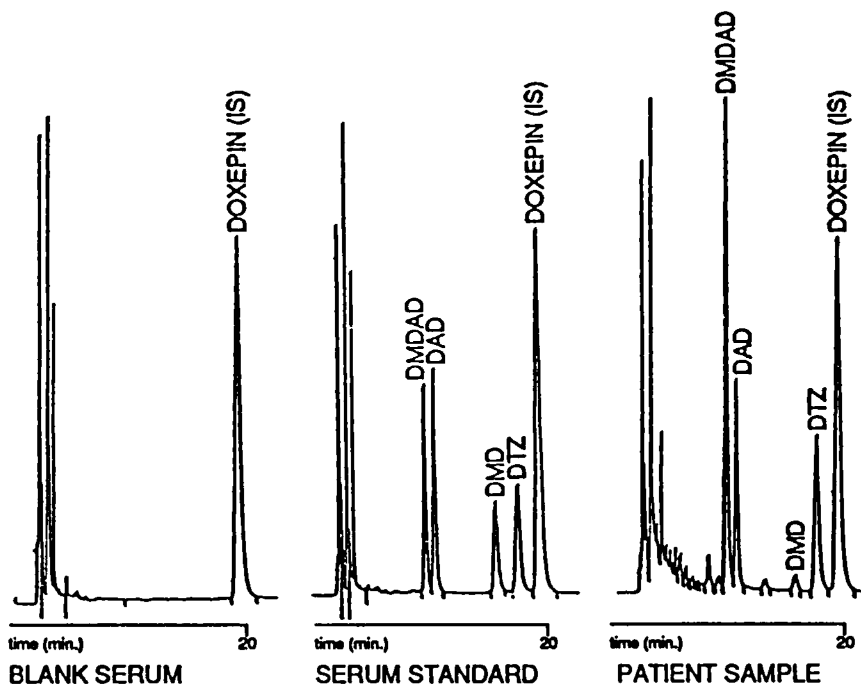

FIGURE 1. Chromatograms of blank serum, a serum standard and a patient sample with doxepin added as internal standard (IS). DTZ = diltiazem, DMD = N-desmethyldiltiazem, DAD = desacetyldiltiazem, DMDAD = N-desmethyl-desacetyldiltiazem.

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dilute aqueous acid solutions, have significantly different retention times than the analytes of interest: carbamazepine, D517, imipramine, 2-hydroxy-imipramine, gallopamil, quinidine, lidocaine, MEGX, GX, trimeprazine, chlorpromazine, prochlorperazine, and thioridazine. Mean recovery (n=3) for the described method was 77 ± 2.6%, 65 ± 4.2%, 79 ± 2.6%, 74 ± 7.8%, and 79 ± 3.5% for DTZ, DMD, DAD, DMDAD, and doxepin respectively. The lower limit of sensitivity was 3.0, 3.0, 1.5, and 1.5 ng/ml for DTZ, DMD, DAD, and DMDAD respectively. The chromatographic conditions did not appear to limit the lifetime or performance of the cyanopropylsilane stationary phase and a decrease in resolution or sensitivity was not observed during the validation procedure.

The standard curve was linear from 10 to 200 ng/ml, for each analyte, during the entire validation procedure. The correlation coefficients and y-intercept values are presented in Table 1 and a typical standard curve plot is illustrated in Figure 2. Continued experience with this method has shown it to be linear over a wider range of 5 to 400 ng/ml for each analyte.

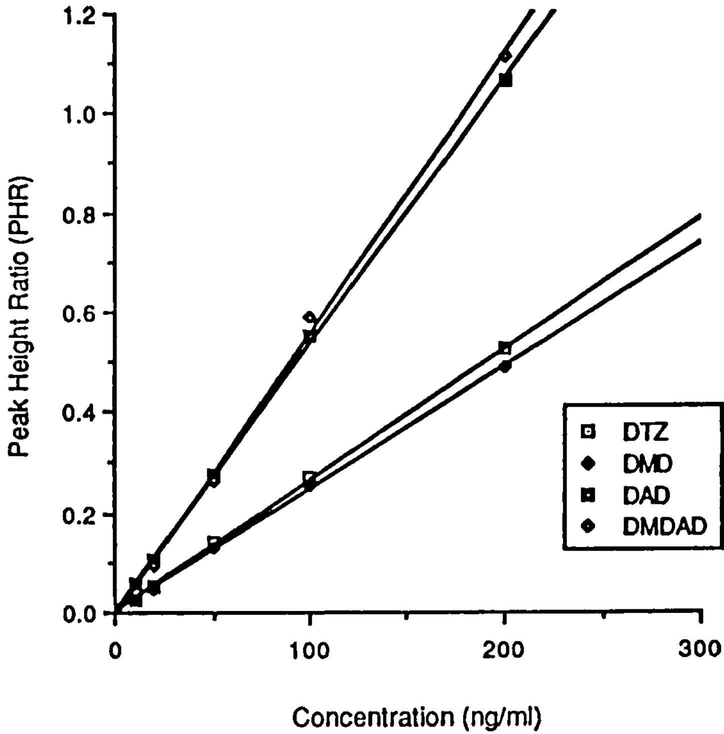

FIGURE 2. Typical plot of analyte serum standards. PHR = ratio of analyte peak height-to-internal standard peak height. DTZ = diltiazem, DMD = N-desmethyldiltiazem, DAD = desacetyldiltiazem, DMDAD = N-desmethyl-desacetyldiltiazem.

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TABLE 1. Statistical Analysis of Standard Curves for Diltiazem and its Metabolites Over a 7 Day Validation Period

	Correlation Coefficient (mean ± S.D.)	Y-intercept Value (mean ± S.D.)
DTZ	0.9997 ± 0.0002	0.0020 ± 0.0094
DMD	0.9994 ± 0.0004	0.0089 ± 0.0180
DAD	0.9997 ± 0.0002	0.0229 ± 0.0709
DMDAD	0.9995 ± 0.0002	-0.0167 ± 0.0214

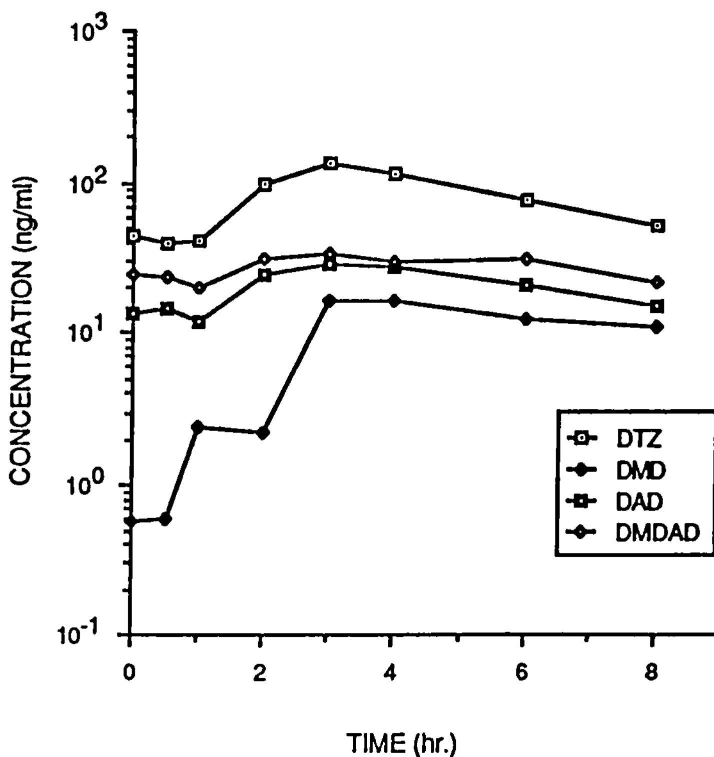

FIGURE 3. Serum concentration-time plot in a normal volunteer receiving chronic oral administration of 90 mg diltiazem every eight hours. DTZ = diltiazem, DMD = N-desmethyldiltiazem, DAD = desacetyldiltiazem, DMDAD = N-desmethyl-desacetyldiltiazem.

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The results of the validation samples are presented as within-day and between-day comparisons (Table 2). The coefficient of variation on replicate DTZ determinations on the same day was less than 4% (within-day). The coefficient of variation was less than 5% for DTZ samples analyzed over 7 consecutive days (between-day). Similar studies with DMD, DAD, and DMDAD revealed coefficients of variation of less than 7% for within-day and between-day comparisons.

A representative serum concentration-time plot from the normal volunteers on chronic oral diltiazem therapy, 90 mg every eight hours, is shown in Figures 3. Mean serum concentration-time data for each of the metabolites are presented in Figure 4. The mean (±SD) apparent oral clearance and half-life of DTZ was 20.9 ± 8.9 ml/min/kg and 3.5 ± 0.9 hr., respectively. The mean (±SD) half-lifes for DMD, DAD and DMDAD were 4.6 ± 1.9 hr. (n=4), 3.76 ± 1.3 hr. (n=7) and 8.1 ± 5.4 hr.

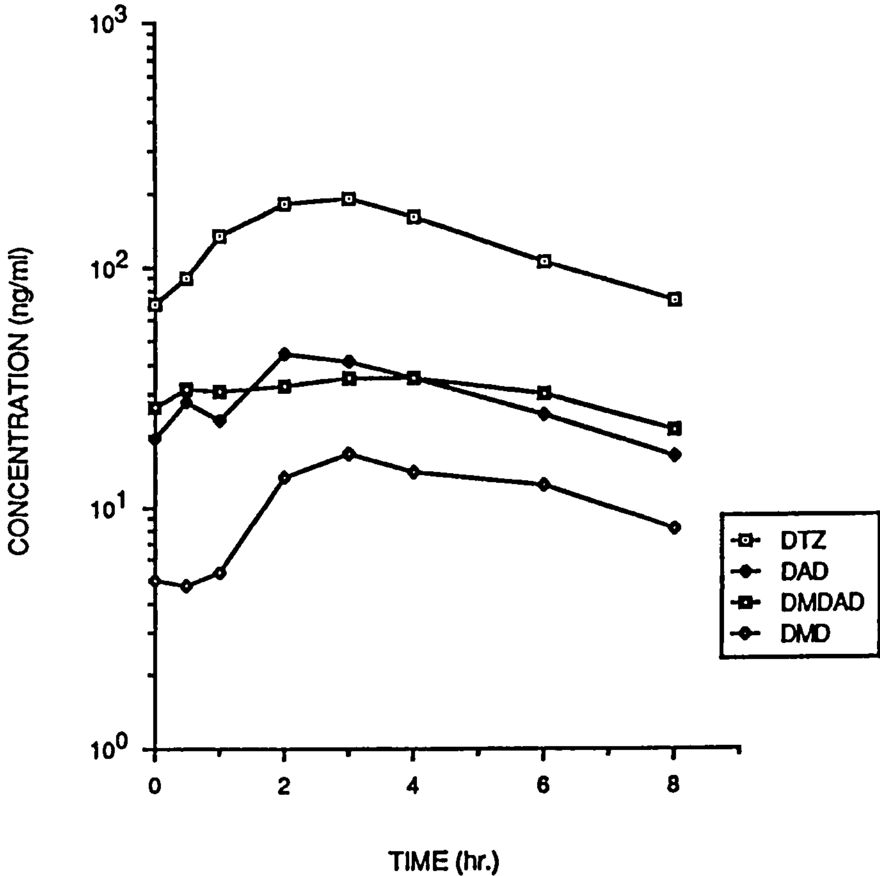

FIGURE 4. Mean (±SD) concentration-time plot of diltiazem and three of its metabolites in serum following chronic oral administration of 90 mg diltiazem every eight hours to a group (n=8) of normal male volunteers. DTZ = diltiazem, DMD = N-desmethyldiltiazem, DAD = desacetyldiltiazem, DMDAD = N-desmethyl-desacetyldiltiazem.

(n=6), respectively. The mean (±SD) ratio of metabolite area under the concentration-time curve (AUC) to DTZ AUC was 0.24 ± 0.06 for DAD and 0.24 ± 0.12 for DMDAD. DMD was only detectable in four of eight subjects. In those four, the mean (±SD) DMD/DTZ AUC ratio was 0.095 ± 0.04. It appears that the rate of decline for DAD and DMDAD parallels DTZ. This would suggest that the rate-limiting step in the elimination of these metabolites is their formation from the parent compound. Other investigators have suggested DMD is the predominant metabolite in serum after chronic oral doses of diltiazem (13). Our data suggest that DMD is systemically present in relatively low concentrations and DAD and DMDAD are the predominant metabolites in serum.

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TABLE 2. Intra-assay Precision and Reproducibility

Drug	Within-day Measured Concentration (ng/ml)	%CV	Between-day Measured Concentration (ng/ml)	%CV
DTZ 30 ng/ml	31.24 ± 0.89	2.85	29.91 ± 1.27	4.2
DTZ 70 ng/ml	74.93 ± 1.46	1.95	71.14 ± 2.35	3.3
DTZ 160 ng/ml	170.66 ± 5.58	3.27	163.40 ± 4.10	2.5
DMD 30 ng/ml	31.15 ± 0.59	1.86	29.17 ± 1.76	6.0
DMD 70 ng/ml	75.42 ± 3.11	4.12	70.81 ± 3.81	5.4
DMD 160 ng/ml	174.87 ± 5.62	3.21	168.41 ± 6.29	3.7
DAD 30 ng/ml	31.18 ± 0.59	1.90	29.51 ± 1.15	3.9
DAD 70 ng/ml	74.60 ± 2.23	2.98	70.68 ± 2.12	3.0
DAD 160 ng/ml	171.87 ± 5.06	2.95	163.57 ± 4.39	2.7
DMDAD 30 ng/ml	30.38 ± 0.69	2.26	28.87 ± 1.98	6.8
DMDAD 70 ng/ml	68.48 ± 4.49	6.55	66.16 ± 2.89	4.4
DMDAD 160 ng/ml	156.21 ± 4.72	3.02	154.22 ± 4.92	3.2

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CONCLUSIONS

The described HPLC method for the determination of diltiazem and three of its major metabolites offers excellent separation and resolution. It is linear and reproducible over a range of concentrations commonly observed after oral administration of diltiazem. The selectivity and sensitivity is suitable for pharmacokinetic investigations involving diltiazem and its major metabolites.

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