Quantitation of Diltiazem and Desacetyldiltiazem in Dog Plasma by High-Performance Liquid Chromatography

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Abstract □ A high-performance liquid chromatographic procedure was developed for the determination of diltiazem and desacetyldiltiazem in dog plasma. Two milliliters of plasma is extracted with a hexane–2-propanol mixture. The assay uses a reverse-phase column maintained at 55°C with a silica saturation column and a pellicular precolumn. The mobile phase is acetonitrile–water (50:50) at pH 6.6 with 1.5-g/L heptanesulfonic acid added as the ion-pair reagent. The procedure is sensitive to 5 ng/mL for both compounds in dog plasma and is linear up to 2000 ng/mL for diltiazem and 1000 ng/mL for desacetyldiltiazem. Preliminary dog mean plasma profiles of diltiazem and desacetyldiltiazem are presented.

Keyphrases □ Diltiazem—quantitation with desacetyldiltiazem, dog plasma, HPLC □ Desacetyldiltiazem—quantitation with diltiazem, dog plasma, HPLC □ HPLC—quantitation of diltiazem and desacetyldiltiazem, dog plasma

Diltiazem hydrochloride, a new antianginal drug being used in Japan for the treatment of angina pectoris, is classified as a calcium antagonist. It affects vascular smooth muscle and dilates coronary vessels, making the drug clinically useful in the treatment of ischemic heart disease (1, 2).

Methods have been published for the determination of diltiazem and/or desacetyldiltiazem, the principal blood metabolite of diltiazem in plasma. A GC method of Rovei *et al* (3), which uses a hexane plasma extract at pH 7 with a nitrogen–phosphorus detector after a complex derivatization procedure, and a TLC–spectrophotometric procedure (4) have been presented. These methods either lack sensitivity (TLC) or are time consuming (GC).

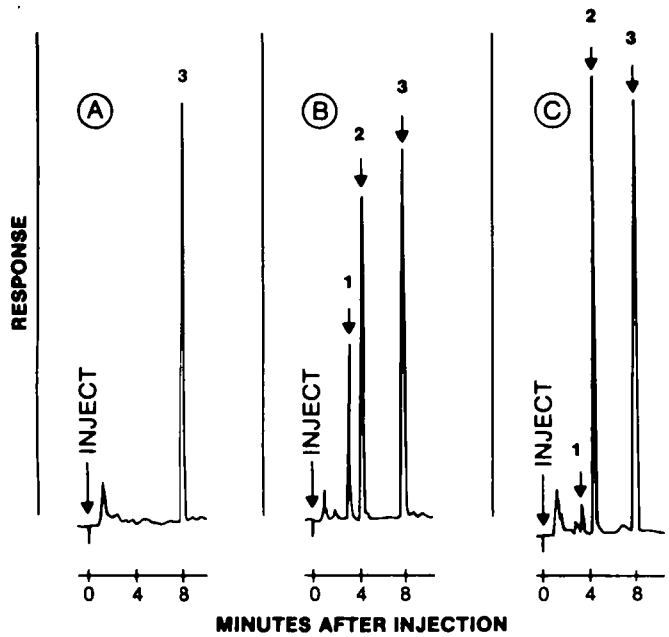

Figure 1—*Chromatograms from (A) blank dog plasma containing 400 ng of prazepam (3), (B) plasma spiked with 100 ng/mL of desacetyldiltiazem (1) and 200 ng/mL of diltiazem (2), and (C) dog plasma following the intravenous administration of diltiazem (2 mg/kg, 1-h postdose).*

Many clinical trials and pharmacology studies have been conducted with this compound; however, few studies have reported the pharmacokinetic parameters in humans or other animal species. The objective of this study was to develop a rapid and sensitive diltiazem and desacetyldiltiazem plasma assay to assess the pharmacokinetics of diltiazem and to present preliminary plasma level data for the beagle dog.

EXPERIMENTAL

Protocol—Six beagle dogs were administered diltiazem hydrochloride in solution at an oral dose of 5 mg/kg and as a bolus dose of 2 mg/kg iv in a crossover design. The dogs were fasted overnight before drug administration and until the 6-h blood collection. Blood samples were collected immediately before dosing and up to 10 h postdose, using sodium heparin as the anticoagulant. The plasma was immediately frozen at −20°C until analysis.

Reagents—Chromatography-grade acetonitrile[1], 2-propanol[1], methanol[1], and hexane[1] were used. Sodium acetate[1] and sodium borate[1] were ACS analytical reagents. Heptanesulfonic acid sodium salt[2] was used as received.

Apparatus—A high-performance liquid chromatograph[3] with an automatic sampler[4] and a reverse-phase C_{18} column[5] was used. A silica saturation column[6] was installed in the oven in front of the injector and a precolumn of pellicular C_{18} packing[7] was inserted after the injector. The column effluent was monitored with a variable-wavelength UV absorbance detector[8] using a dual-pen recorder[9].

Standards—Primary standard solutions of diltiazem hydrochloride [10] (500 μg of free base/mL) and desacetyldiltiazem[10] (500 μg of free base/mL) were prepared in distilled water. A secondary standard solution containing both diltiazem (100 μg/mL) and desacetyldiltiazem (50 μg/mL) was prepared in water. Eight dilutions were made such that 0.1 mL of each dilution would be used to spike 2 mL of blank dog plasma to the concentrations used for the standard curve. The resulting plasma standard curve concentrations were 50, 100, 200, 400, 800, 1600, and 2000 ng/mL of diltiazem and 25, 50, 100, 200, 400, 800, and 1000 ng/mL for desacetyldiltiazem. The internal standard, prazepam[11] (200 μg/mL), was prepared in methanol and then diluted 1:500 in 0.1 M sodium borate buffer (pH 9).

Extraction—A 2.0-mL plasma aliquot from a control dog (standard curve) or unknown sample was placed in a 16 × 150-mm glass culture tube and 1.0 mL of internal standard buffer and 7 mL of hexane–2-propanol (98:2) was added. The tube was closed with a polyef-lined screw-cap and shaken 30 min on a horizonal shaker[12].

Each sample was centrifuged 10 min at room temperature, and 6 mL of the upper hexane layer was transferred to a clean 13 × 100-mm culture tube. The tube was placed in a 40–50°C water bath and evaporated to dryness under a nitrogen stream. The residue was reconstituted by adding 200 μL of mobile phase. The tube was vortexed[13] 30 s to ensure dissolution and centrifuged 2 min to collect the solution. The resulting concentrate was transferred to a microcentrifuge tube[14] and injected onto the HPLC column by the automatic sampler.

Chromatographic Conditions—The mobile phase consisted of acetoni-

[1] Chrom AR; Mallinckrodt, Paris.
[2] Eastman Kodak Co., Rochester, N.Y.
[3] Series 3 with Model LC65T Oven-detector; Perkin-Elmer, Norwalk, Conn.
[4] WISP 710B; Waters Associates, Milford, Mass.
[5] μBondapack C_{18} (300 × 3.9 mm. i.d.); Waters Associates, Milford, Mass.
[6] Pre-SAT Kit; Applied Science, State College, Pa.
[7] 60 × 2 mm Perisorb RP18, 30–40 μm; EM Reagents, Darmstadt, W. Germany.
[8] LC-75; Perkin-Elmer, Norwalk, Conn.
[9] Omniscribe B5217-SI; Houston Instruments, Austin, Tex.
[10] Marion Laboratories, Kansas City, Mo.
[11] Warner-Lambert, Ann Arbor, Mich.
[12] Model 6010; Eberbach Corporation, Ann Arbor, Mich.
[13] Multi-Tube Vortexer, Model 2600; SMI Industries, Emeryville, Calif.
[14] 250-μL polyethylene; Brinkman Instruments.

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Table I—Standard Curve Summary for Diltiazem and Desacetyldiltiazem in Dog Plasma

Standard Curve	Diltiazem			Desacetyldiltiazem		
	r	Slope $\times 10^2$	Intercept	r	Slope $\times 10^2$	Intercept
1	0.9999	0.4464	0.0001	0.9999	0.5003	−0.020
2	0.9999	0.4328	0.003	0.9999	0.4864	−0.003
3	0.9999	0.4460	−0.003	0.9999	0.5100	−0.006
n	3	3	3	3	3	3
Mean	0.9999	0.4417	0.0003	0.9999	0.4989	−0.010
SD	0	0.008	0.003	0	0.012	0.009
CV	0	1.8%	917%	0	2.4%	94.9%

trile–water (50:50) containing 1.5 g of heptanesulfonic acid sodium salt/L and 8 g of sodium acetate/L. The final solution was adjusted to pH 6.6 with glacial acetic acid and filtered[15]. The flow rate was 1.5 mL/min, the column temperature 55°C, the detector wavelength 240 nm, and the injection volume 100 μL.

Quantitation—All determinations were performed by hand measuring the peak heights and computing the peak height ratios of each compound as compared with the internal standard. This ratio was calculated and compared with the slope of the standard curve. Replicate and quality control samples (Table II) were processed from blank dog plasma with each set of samples, and a computer program was developed to calculate the ratios, slopes, and intercepts of the standard curves. The quality control samples were spiked and frozen before study initiation, and the replicate samples were spiked at the time of extraction.

RESULTS AND DISCUSSION

A method has been developed to determine diltiazem and desacetyldiltiazem plasma levels utilizing a 10-min HPLC separation. Typical chromatograms obtained from blank plasma spiked with the internal standard, a standard curve sample, and an actual dog plasma sample are presented in Fig. 1. Blank dog plasma without internal standard indicated no interfering peaks.

Sample extraction is pH, solvent, and time dependent. A pH value of 7 has been shown to be successful for the extraction of diltiazem into hexane (3). However, adjustment to pH 9 and the addition of 2% 2-propanol to the hexane increased recovery from 55 to 77% for desacetyldiltiazem and from 70 to 85% for diltiazem, as compared with the pH 7 extraction. Recovery of diltiazem decreased when the two phases were mixed <30 min on the shaker. No diltiazem degradation has been observed under the pH 9 extraction conditions.

Several drugs were tested for use as internal standards and for possible interference with diltiazem and desacetyldiltiazem. Prazepam, diazepam, oxazepam, clonazepam, chlordiazepoxide, medazepam, and propranolol did not interfere with the HPLC separation. Prazepam was chosen as the internal

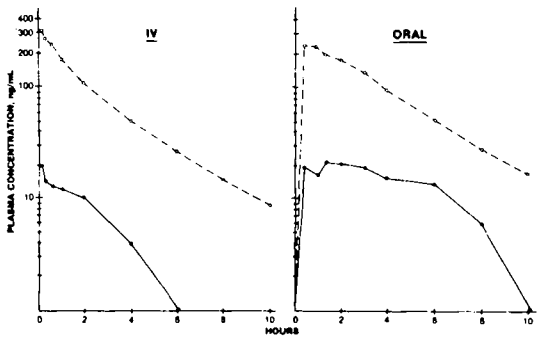

Figure 2—*Mean plasma levels of six beagle dogs administered 2 mg/kg iv of diltiazem or 5 mg/kg of diltiazem orally. Key: (○) diltiazem; (●) desacetyldiltiazem.*

[15] 0.45-μm pore, Nylon 66; Rainin Instruments, Emeryville, Calif.

Table II—Reproducibility for Diltiazem and Desacetyldiltiazem in Dog Plasma

Conc. in Plasma, ng/mL	*n*	Mean, ng/mL	*SD*	*CV*, %
		Diltiazem		
5[a]	5	4.9	0.3	6.5
10[a]	11	12.0	1.4	11.9
25[a]	6	28.0	1.2	4.2
100[b]	4	100.9	2.4	2.4
800[b]	4	801.1	2.1	0.3
		Desacetyldiltiazem		
5[a]	6	5.4	0.5	8.6
10[a]	10	10.8	1.3	12.1
12.5[a]	6	14.6	1.4	9.7
50[b]	4	50.6	2.0	4.0
400[b]	4	395.2	6.6	1.7

[a] Within-day replicate samples. [b] Quality control samples.

standard because of its symmetrical peak shape, extraction recovery properties (90%), and appropriate retention time.

Three standard curves were run on successive days (Table I). The accuracy and precision of the method are shown in Table II by the coefficient of variation and mean values for the two quality control samples and two replicate samples. The lower limit of quantitation was calculated after completion of the study by the method of Gabriels (5). The method uses the mean baseline value of the blank plasma samples, a corresponding F value, and the standard deviation. The lower limit of quantitation was 5 ng/mL for diltiazem and desacetydiltiazem.

Stability test samples of diltiazem and desacetyldiltiazem in plasma stored for up to 13 weeks at −20°C indicated no significant breakdown. The tests concentrations of diltiazem ranged from 50 to 500 ng/mL and those of desacetyldiltiazem from 25 to 250 ng/mL.

The mean of the individual plasma diltiazem and desacetyldiltiazem profiles of the six beagle dogs are presented in Fig. 2 for the 2-mg/kg iv and 5-mg/kg oral doses. Both the intravenous and oral data indicate a terminal diltiazem half-life of ~2.2 h and an absolute bioavailability of 55% when the mean of the individual diltiazem areas under the curve from zero to infinity ($AUC_{0\to\infty}$) of the intravenous and oral results are normalized and compared (6). The half-lives were calculated from the elimination rate constant (k) by the equation $t_{1/2} = 0.693/k$. The elimination rate constant (k) was obtained by an intravenous two-compartment model with bolus administration and an oral two-compartment model with first-order absorption and elimination from the central compartment. The data were fitted by the NONLIN (7) regression analysis using subroutines for both sets of data. The $AUC_{0\to\infty}$ values were calculated by the trapezoidal method and normalized for dose assuming linear kinetics.

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