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Short Communication

High-performance liquid chromatographic method for the determination of diltiazem and two of its metabolites in human plasma: application to a new sustained release formulation*

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Introduction

Diltiazem (D), (+)-*cis*-3-(acetyloxy)-5-[2-(dimethylamino)-ethyl)]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5H)one monohydrochloride (Fig. 1), is a calcium antagonist which has been shown to be useful in the treatment of angina pectoris, hypertension and supraventricular arrhythmias [1–4]. As shown in Fig. 2, D may be either demethylated metabolically to MA (*N*-monodemethyldiltiazem) or deacetylated to DAD (desacetyldiltiazem). These two metabolites may undergo further biotrans-

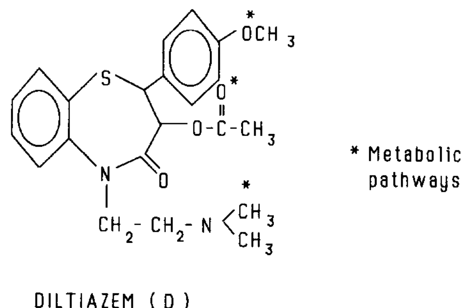

Figure 1
Structure of diltiazem.

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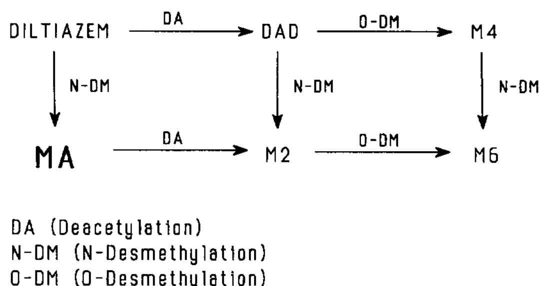


Figure 2
Metabolism of diltiazem.

formation to M4 (*O*-demethyldesacetyldiltiazem), M2 (*N*-demethyldesacetyldiltiazem) or M6 (*N,O*-didemethyldesacetyldiltiazem). Since D and metabolites are unstable molecules, attention must be given during the manipulations before the determination. In fact, DAD was found to be the major metabolite of D, while recently it was demonstrated that it may also be produced by degradation of D [5].

The present paper describes a simple and specific method for the determination of D and two of its metabolites in human plasma using high-performance liquid chromatography (HPLC). This method has been applied to the determination of the pharmacokinetic profile of a new sustained release (SR) formulation of D in healthy volunteers.

Experimental

Materials

D, MA, DAD and loxapine (the internal standard) were used as hydrochloride salts. All solvents and reagents were of analytical grade except acetonitrile, methyl t-butyl ether, phosphoric acid and water, which were HPLC grade.

Apparatus

The HPLC system consisted of a LDC/Milton Roy (Riviera Beach, FL, USA) ConstaMetric III solvent delivery system, a Waters Associates (Milford, MA, USA) Model 441 absorbance detector with fixed wavelength at 214 nm, a Rheodyne (Cotati, CA, USA) Model 7120 injector with 50-μl sample loop. The separation system was a Hichrom (Reading, UK) 10 cm × 4.9 mm stainless steel, 5 μm C8 Spherisorb reversed-phase column. Mobile phase, 0.005 M phosphate buffer (pH 3.0)–acetonitrile (57:43, v/v) containing 1.25 μM of dibutylamine was run at a flow rate of 1.4 ml min^{-1}. All analyses were performed at room temperature.

Extraction

Two millilitres of plasma containing D, MA and DAD were transferred to a screw-capped culture tube. Two hundred microlitres of the internal standard solution (200 ng) were added and the tube was vortexed for 15 s. Then 3 ml of 0.05 M phosphate buffer (pH 7.5) and 6 ml of methyl t-butyl ether were added and the mixture agitated for 10 min on a reciprocating shaker and then centrifuged for 10 min at 800*g*. The organic layer was transferred to a conical tube where 100 μl of 0.05 N sulphuric acid were added. This solution was vortexed for 45 s and centrifuged for 4 min at 800*g*. The organic phase was discarded and a volume up to 50 μl of the acidic solution was injected directly into the sample loop.

Calibration curves

Standard samples were prepared by spiking human plasma with increasing concentrations of D and metabolites using loxapine as internal standard (200 ng). Three samples were run for each of the following concentrations: 2.5, 5, 10, 15, 25, 50 and 75 ng ml^{-1} for DAD, MA and D; 100 ng ml^{-1} for MA and D; 150, 200, 250, 300 and 350 ng ml^{-1} for D. Calibration curves were constructed by plotting the peak–height ratios (compound/internal standard) of each compound against the respective concentrations.

Analytical recovery

Analytical recoveries of D and its metabolites were determined by comparing the peak–height obtained by direct injection of standard aqueous solution with those obtained after the extraction procedure using loxapine as external standard. Fifty spiked samples of different concentrations of D, MA and DAD were assayed during the study to validate the method of analysis.

Pharmacokinetic study

Seventeen plasma samples from 0 to 36 h were taken from each of the 21 healthy volunteers who received a single oral dose of a new SR 300 mg D formulation. D and its two metabolites were assayed to determine the pharmacokinetic parameters.

Results and Discussion

A typical chromatogram showing the separation of D and metabolites is presented in Fig. 3. Under the chromatographic conditions used, DAD, MA, D and loxapine gave symmetric, well-resolved peaks at 3.4, 4.2, 5.6 and 6.4 min, respectively. Blank plasma samples did not show any significant interferences from endogenous compounds at the retention times corresponding to those of the drug and its metabolites. Moreover, the other known metabolites of D, being more polar, did not interfere with the assay. The results obtained for the calibration curves are presented in Tables 1 and 2. The standard curves were linear (correlation of 0.9978, 0.9921, 0.9921 and 0.9983) throughout the

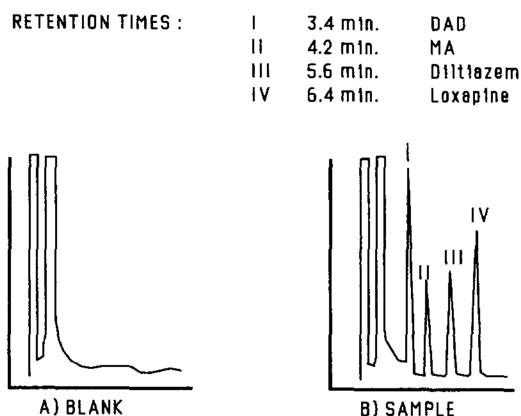


Figure 3
Typical HPLC chromatograms of extracts of human plasma.

Table 1
Calibration curves for diltiazem

Diltiazem					
Conc. (ng ml^{-1})	Ratio ± SEM × 10^{-3}	RSD (%)	Conc. (ng ml^{-1})	Ratio ± SEM × 10^{-3}	RSD (%)
2.5	26 ± 6.4	(12.0)	100	1061 ± 15.0	(2.5)
5.0	54 ± 2.7	(8.8)	150	1549 ± 10.1	(1.1)
10.0	105 ± 6.1	(10.0)	200	2065 ± 45.4	(3.8)
15.0	154 ± 3.1	(3.4)	250	2654 ± 40.1	(2.6)
25.0	265 ± 1.2	(0.8)	300	3092 ± 27.8	(1.6)
50.0	514 ± 3.1	(1.0)	350	3586 ± 103.7	(5.0)
75.0	755 ± 7.9	(1.8)			
100.0	1061 ± 15.0	(2.5)			
Correlation: 0.9978			Correlation: 0.9921		
$y = 0.0104x - 0.0004$			$y = 0.0102x + 0.0407$		

Table 2
Calibration curves for metabolites

	MA			DAD	
Conc. (ng ml^{-1})	Ratio ± SEM × 10^{-3}	RSD (%)	Conc. (ng ml^{-1})	Ratio ± SEM × 10^{-3}	RSD (%)
2.5	25 ± 5.1	(8.2)	2.5	45 ± 4.6	(6.8)
5.0	53 ± 1.5	(5.0)	5.0	87 ± 2.6	(5.3)
10.0	101 ± 4.6	(7.9)	10.0	187 ± 5.4	(5.0)
15.0	148 ± 2.5	(2.9)	15.0	276 ± 6.5	(4.1)
25.0	236 ± 6.1	(4.5)	25.0	462 ± 5.0	(1.9)
50.0	481 ± 6.8	(2.5)	50.0	915 ± 7.2	(1.4)
75.0	668 ± 10.8	(2.8)	75.0	1338 ± 17.8	(2.3)
100.0	984 ± 29.0	(5.1)			
Correlation: 0.9921			Correlation: 0.9983		
$y = 0.0095x + 0.0025$			$y = 0.0179x + 0.0094$		

concentration ranges studied, and the limit of sensitivity for D, MA and DAD was 2.5 ng ml^{-1}. As low as 1.0 ng ml^{-1} could be detected (limit of detection). The limit of sensitivity was defined as the lowest concentration measure with a coefficient of variation less than 12%. The day-to-day reproducibility of replicate analyses shows a good precision with a coefficient of variation of 0.8–12.0%.

Analytical recovery is presented in Table 3. D and DAD were recovered to over 90% while recovery of MA was between 70 and 75%.

The results obtained with the fifty spiked samples are shown in Table 4. The accuracy was found to be good with relative errors ranging from 1.3 to 14.0%.

In Figure 4, mean plasma concentrations of D and metabolites in volunteers were plotted against time. The pharmacokinetic profile of D, after a single dose of a new once a day SR 300 mg, showed a mean maximal plasma concentration (C_{max}) of 214.5 ng ml^{-1} at 3.4 hours (T_{max}) and a mean plasma concentration (C_{min}) of 30.9 ng ml^{-1} after 24 hours. The mean area under the curve to infinity ($AUC\infty$) was 2616.4 ng·h ml^{-1}.

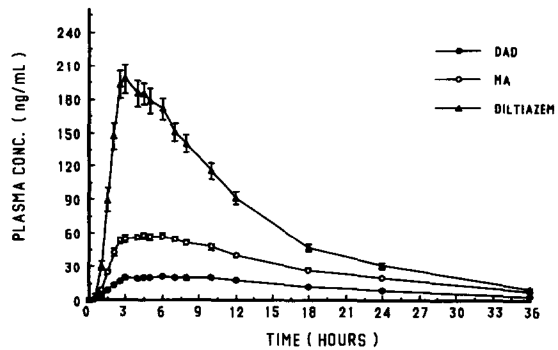
Concerning the two metabolites measured, AUC values of 494.5 and 1189.5 ng·h ml^{-1} were found for DAD and MA respectively. Those results represent about 19% (DAD) and 45% (MA) of the AUC value obtained for D. Values of 5.7 and 4.5 were observed

Table 3
Analytical recovery for diltiazem and metabolites

Conc. (ng ml^{-1})	Diltiazem (%)	MA (%)	DAD (%)
5	93.0	72.5	93.0
25	92.0	70.0	94.0
100	91.8	75.0	85.0

Table 4
Results of 50 spiked samples

	Conc. (ng ml^{-1})	Assay ± SD (ng ml^{-1})	Error (%)
	40	38.6 ± 3.0	3.5
Diltiazem	150	148.0 ± 5.1	1.3
	25	26.1 ± 2.5	4.4
	10	8.9 ± 0.5	11.0
MA	30	29.5 ± 2.2	1.6
	50	54.1 ± 5.1	8.2
	10	11.0 ± 0.6	10.0
DAD	50	52.6 ± 0.4	5.2
	25	28.5 ± 0.2	14.0



	MA	DAD	DILTIAZEM
AUC (ng. h/mL)	1189.5 ± 63.3	494.5 ± 40.6	2616.4 ± 155.6
T max (h)	4.5 ± 0.4	5.7 ± 0.5	3.4 ± 0.3
C max (ng/mL)	62.7 ± 2.9	24.3 ± 1.2	214.5 ± 11.0

Figure 4
Diltiazem 300 mg SR profile of the mean in 21 healthy volunteers.

for the mean T_{max} with corresponding C_{max} values at 24.3 and 62.7 ng ml^{-1}, respectively for the two metabolites.

The results of this study indicate that the present HPLC method is sufficiently rapid, sensitive and reproducible for the simultaneous determination of diltiazem and its two major metabolites within the plasma concentration range observed.

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