High-Performance Liquid Chromatographic Determination of Diltiazem and Six of Its Metabolites in Human Urine

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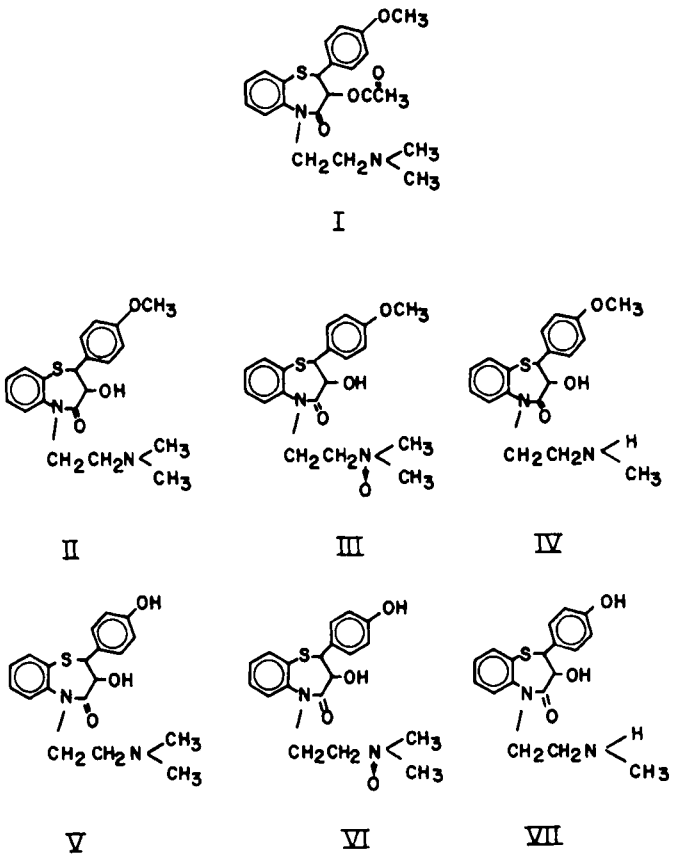
Abstract □ The use of the calcium antagonist diltiazem (I) is increasing, particularly in treating unstable angina. This study describes a simple and specific high-performance liquid chromatographic method for the determination of I and six of its metabolites in urine. Diltiazem and its conjugated and unconjugated metabolites were assayed in the urine of patients treated with a dose of 120 mg po of diltiazem tid. Sensitivity of the method was 100 ng/mL for I and the metabolites desacetyldiltiazem (II), *N*-desmethyldesacetyldiltiazem (IV), *O*-desmethyldesacetyldiltiazem (V), *O*-desmethyl-*N*-desmethyldesacetyldiltiazem (VII), *N*-oxide desacetyldiltiazem (III), and *O*-desmethyl-*N*-oxide desacetyldiltiazem (VI). In the urine of patients, the extent of conjugation of six metabolites was >80%.

Keyphrases □ Diltiazem—HPLC determination, six metabolites, human urine □ HPLC—diltiazem and six metabolites, human urine

Diltiazem, (+)-*cis*-3-(acetyloxy)-5-[2-(dimethylamino)-ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)one monohydrochloride, is a new calcium antagonist which has been shown to be useful in the treatment of unstable angina (1, 2). Limited data are available on the metabolism and pharmacokinetics of this new drug, but the recent development of sensitive and specific analytical methods will advance understanding in these areas. A GC method (3, 4) has been published, but it did not separate diltiazem (I) from its six metabolites and required a derivatization of the principal metabolite, desacetyldiltiazem (II). A spectrophotometric method has also been published (5), but it has limited specificity and sensitivity.

The present article describes a simple method for the determination of I and its six metabolites in urine using high-performance liquid chromatography (HPLC). This method has been applied to the measurement of urine metabolites (conjugated and unconjugated) of I in two patients receiving 120 mg po of I tid.



EXPERIMENTAL

Materials—Diltiazem[1] (I), the six metabolites (II–VII)[1], and loxapine[2] (the internal standard) were used as hydrochloride salts. All solvents and reagents were of analytical grade except acetonitrile and water, which were HPLC grade.

Apparatus—The assays were performed on a liquid chromatograph[3] equipped with a variable-wavelength UV detector set at 210 nm with attenuation at 128 AU × 10^{-4} cm. The 25 × 0.46 cm i.d. C_{18} column[4] packed with 5-μm Spherisorb ODS was eluted with acetonitrile–0.01 M phosphate buffer, pH 3 (72:28). The mobile phase had a flow rate of 2.5 mL/min from 0 to 5.5 min, increasing to 3 mL/min from 5.5 to 13 min.

Extraction—*Unconjugated Metabolites*—To 1 mL of urine, contained in a culture tube, were added 1 mL of 0.1 M acetate buffer adjusted to pH 5 and 100 μL of internal standard solution (100 μg/mL). The solution was vortexed for 30 s and extracted with 10 mL of chloroform containing 1% isoamyl alcohol. The mixture was agitated for 10 min on a reciprocating shaker and then centrifuged at 2500 rpm. The organic phase was transferred to a second culture tube and evaporated to dryness on a hot plate at 60°C under a nitrogen stream. The aqueous phase was then passed over a C_{18} cartridge[5] (previously prepared with 2.5 mL of H_2O, 2.0 mL of methanol, and another 2.5 mL of H_2O, fol-

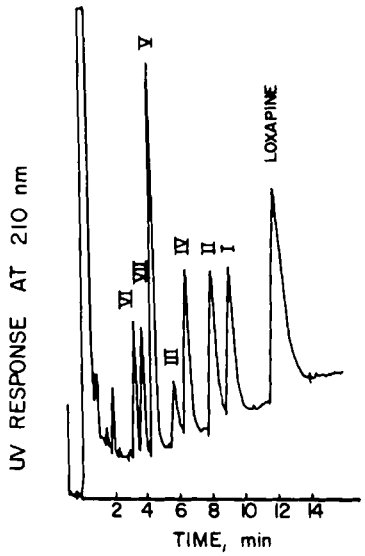


Figure 1—*Typical chromatogram of a urine extract.*

[1] Tanage Seigaku Ltd. Japan.
[2] Lederle.
[3] Hewlett-Packard 1080B.
[4] Cosyns and Spain.
[5] SEP-PAK; Waters Associates.

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Table I—Retention Time, Lower Limit of Detection, and Calibration Curve of Diltiazem and Its Six Metabolites in Urine

Compound	Retention Time, min	Limit of Sensitivity[a], μg/mL	Calibration Curve	r^2
Diltiazem (I)	10.09	0.100	$y = 0.2004x - 0.0046$	0.9999
II	8.94	0.100	$y = 0.2259x - 0.0029$	0.9999
IV	7.37	0.100	$y = 0.2210x - 0.0028$	0.9991
V	6.59	0.100	$y = 0.2134x - 0.0027$	0.9996
VII	5.25	0.100	$y = 0.2205x - 0.0031$	0.9964
III	4.63	0.100	$y = 0.2199x - 0.0038$	0.9885
VI	4.13	0.10	$y = 0.2105x - 0.0026$	0.9905

[a] Limit of detection 0.050 μg/mL.

Table II—Recovery of Diltiazem and Its Metabolites at Different Urine Concentrations

Urine conc., μg/mL	Recovery, %[a]						
	Diltiazem (I)	II	IV	V	VII	III	VI
0.1	99.6 ± 3.4	100.0 ± 2.5	91.5 ± 4.68	74.3 ± 10.1	73.8 ± 4.9	91.4 ± 3.0	85.0 ± 3.3
0.2	100.0 ± 2.7	100.0 ± 2.7	93.8 ± 5.2	78.5 ± 9.4	74.0 ± 5.3	92.4 ± 3.9	84.5 ± 3.1
0.4	101.4 ± 3.3	99.8 ± 2.8	90.9 ± 5.0	77.8 ± 8.5	73.8 ± 6.0	90.9 ± 4.0	82.3 ± 2.4
0.8	98.5 ± 2.9	99.5 ± 2.5	92.1 ± 4.9	75.2 ± 7.8	75.5 ± 5.5	94.5 ± 3.8	85.0 ± 3.0
1.6	100.0 ± 3.1	100.0 ± 3.1	91.6 ± 4.5	77.4 ± 8.5	76.0 ± 4.8	95.0 ± 3.7	84.8 ± 3.0
4.0	100.0 ± 2.9	98.2 ± 3.5	93.8 ± 6.0	76.5 ± 7.4	74.9 ± 4.8	92.9 ± 4.5	86.2 ± 3.4
4.8	99.5 ± 3.2	95.8 ± 4.0	94.5 ± 5.4	76.9 ± 7.0	78.5 ± 5.6	94.5 ± 3.0	85.0 ± 2.0

[a] Percent recovery ± *SE* (mean of three assays).

lowed by 2 mL of 0.1 M phosphate buffer at pH 3.0 and finally 3 mL of acetonitrile). The filtrate (acetonitrile) was transferred to the tube containing the residue of the organic phase. The solution was vortexed for 30 s and the solution was evaporated to dryness. The residue was dissolved in 0.3 mL of 0.01 M HCl, and a 150-μL aliquot was analyzed by HPLC.

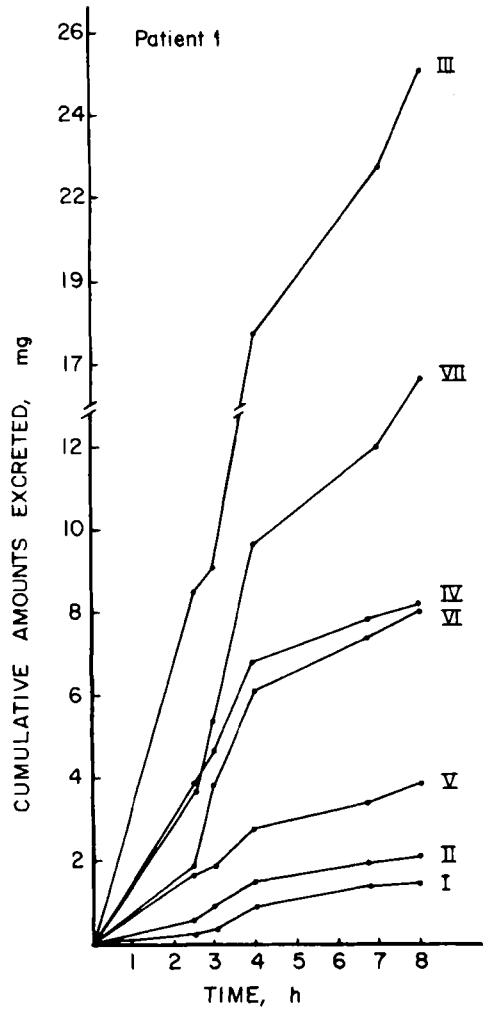

Figure 2—*Cumulative amounts of I and its metabolites excreted in urine after a single oral 120-mg dose.*

Conjugated Metabolites—To determine the total amount of I and metabolites excreted in urine, the conjugated products were hydrolyzed. The optimum conditions necessary for complete hydrolysis were evaluated. The conditions chosen consisted of incubation of 1 mL of urine containing 100 μg of loxapine with 0.1 mL of β-glucuronidase-sulfatase solution[6] at 37°C for 16 h. After incubation, the extraction was done as described above for unconjugated metabolites.

Calibration Curves—Standard samples were prepared by spiking human urine samples with increasing concentrations (0.1, 0.2, 0.4, 0.8, 1.6, 4.0, and 4.8 μg/mL) of I and the six metabolites. Calibration curves were constructed by plotting the ratios of the peak areas of I–VII to that of the internal standard

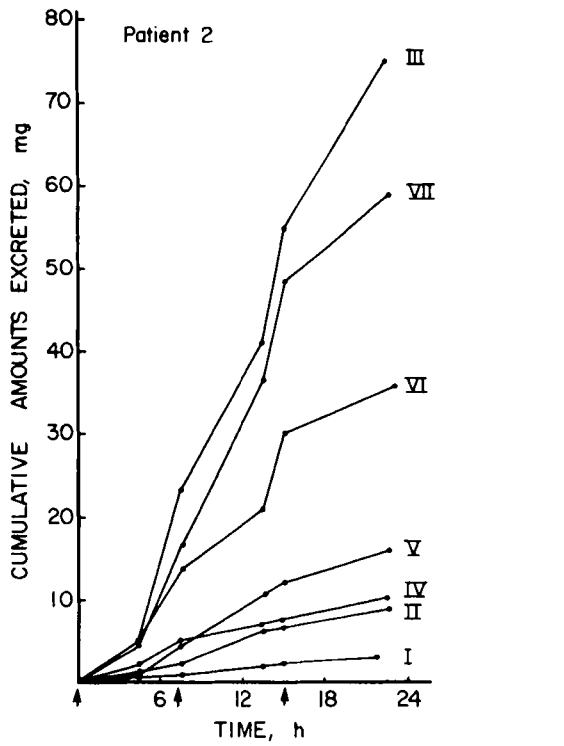

Figure 3—*Cumulative amounts of I and its metabolites excreted in urine after administration of the first three doses (120 mg tid). Arrows indicate the times of dosing.*

[6] Glusulase (5000 U of arylsulfatase and β-glucuronidase in 0.14 M sodium acetate buffer pH 5); Sigma.

Table III—Reproducibility of the Assay of Diltiazem and Metabolites

Compound	Urine Conc., μg/mL	N	CV, %
Diltiazem (I)	0.8	12	3.0
	4.8	12	3.2
II	0.8	9	4.1
	4.8	9	3.9
IV	0.8	9	4.7
	4.8	9	4.5
V	0.8	9	5.1
	4.8	9	2.8
VII	0.8	9	6.1
	4.8	9	3.7
III	1.6	9	6.2
	4.8	9	4.7
VI	1.6	9	7.4
	4.8	9	3.3

against the respective concentrations I–VII. Five samples were run for each of the seven concentrations.

Analytical recoveries of I and metabolites were determined by comparing the peak areas obtained by direct injection of standard aqueous solution with those obtained after the extraction procedure. This procedure was done for each concentration of I and metabolites used in the calibration curves.

Clinical Study—Urine samples were taken from two patients treated for unstable angina with 120 mg po tid of diltiazem. In patient 1, the urine samples were taken at 2.5, 3, 4, 7, and 8 h after the first dose. In patient 2, the urine samples were taken over a period of 24 h, during and after the first three doses, at 5, 6, 14, 15, and 24 h following the first dose.

RESULTS AND DISCUSSION

A typical chromatogram showing the separation of I and the metabolites is presented in Fig. 1. Table I gives the retention time, sensitivity of the method, and results obtained for the calibration curve of I and its metabolites. The standard curves were linear throughout the range studied, and the limit of sensitivity of I and its metabolites was 100 ng/mL. Analytical recovery of I and its metabolites is presented in Table II. The day-to-day reproducibility of replicate analyses of standard urine samples containing different concentrations of I and metabolites is presented in Table III and shows a very good precision, with a coefficient of variation of 3–7.4%. Standard solutions of I and metabolites were freshly prepared and stored in darkness at low temperature (refrigerator) for short periods of time. New standard solutions were made every week, and particular attention was given to stock solutions of metabolites IV, V, and VII, which showed degradation products after a few hours at room temperature.

Cardiovascular drugs that may be given to patients without producing interferences in the assay of I and metabolites are: digoxin, propranolol, procainamide, nitroglycerin, isosorbide dinitrate, and furosemide. On the other hand, benzodiazepines (flurazepam and diazepam) interfere with the metabolites of I, but not with I itself.

Table IV—Percent of Conjugated Diltiazem and Metabolites in the Urine of Two Patients

	Patient 1	Patient 2
Diltiazem (I)	0%	0%
II	70%	77%
IV	80%	75%
V	90%	96%
VII	95%	96%
III	98%	95%
VI	97%	98%

In Figs. 2 and 3 the cumulative amounts of I and metabolites excreted in urine are presented, and Table IV gives the ratio of conjugated to free products in the urine of two patients. In fact, >80% of each metabolite excreted in urine is conjugated, and only a small amount of I was found unchanged in the urine (Table II). More than 50% of the dose was excreted in the first 8 h.

After a single oral 120-mg dose, metabolites III and VII appeared to be the main metabolites in urine, followed by successively smaller amounts of IV, VI, V, and II. The same metabolic pattern is observed during chronic administration (120 mg tid) with the exception of IV, which did not show a significant increase (Fig. 3). In fact, the cumulative amounts of IV excreted were 8 mg after a single dose and 10 mg after chronic administration. For I and other metabolites, there is a three- or fourfold difference in concentrations between single and chronic dosing.

This method was applied to the measurement of plasma concentrations of I and metabolites, but the lack of sensitivity does not permit adequate pharmacokinetic studies when concentrations are as low as 20 ng/mL.

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