Metabolism of Diltiazem in Hepatic and Extrahepatic Tissues of Rabbits: *In Vitro* Studies

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Diltiazem (DTZ) is a calcium channel blocker widely used in the treatment of angina and hypertension. DTZ undergoes extensive metabolism yielding several metabolites, some of which are active like *N*-desmethyldiltiazem (MA), desacetyldiltiazem (M1) and *N*-desmethyl,desacetyldiltiazem (M2). Due to the nature of its biotransformation, several organs should have the ability to metabolize DTZ, however it is still assumed that the liver is the only organ implicated in its elimination. In this study, the fate of DTZ, MA and M1 was assessed in several organs that could contribute to their biotransformation. To this purpose, DTZ (48.2 μM) was incubated in the 10,000 × g supernatant of homogenates of rabbit tissues for 60 min at 37°C. Multiple samples were withdrawn, and DTZ and its metabolites were assayed by HPLC. The elimination rate constant of DTZ in 10,000×g supernatants varied between the organs: liver 334 ± 45, proximal small intestine 69 ± 11, distal small intestine 25 ± 3, lungs 15 ± 6 and kidneys 8 ± 6 (10^{-4} min^{-1}). The metabolism of DTZ in the liver generated large amounts of MA but no M1, and in the small intestine, modest amounts of both metabolites. When MA (50.0 μM) or M1 (53.7 μM) were incubated in liver homogenates, the estimated elimination rate constant were 166 ± 23 and 468 ± 53 (10^{-4} min^{-1}), respectively. The rate of degradation of the metabolites in the small intestine was much slower. These results demonstrate that, *in vitro*, DTZ is metabolized by several organs, the liver accounting for 75% of the total activity, and that MA is the major metabolite generated.

KEY WORDS: diltiazem; metabolism; liver; extrahepatic tissues.

INTRODUCTION

Diltiazem, a calcium channel blocker of the benzothiazepine family, is widely used in the treatment of hypertension and angina (1,2). When given orally, diltiazem is subjected to an important first-pass effect to undergo an oxidative metabolism mainly via the cytochrome P-450. As a result, diltiazem oral bioavailability is approximately 40% (1,3–5), and less than 4% of an oral dose is excreted unchanged in urine (6–8). The biotransformation of diltiazem generates several acidic and basic metabolites, which are further metabolized through oxidation and conjugation pathways (9–12). Although less potent than diltiazem, some of the basic metabolites retain pharmacological activity as antihypertensives and as coronary vasodilators, i.e., desacetyldiltiazem (M1) 100% and 50%, *N*-desmethyldiltiazem (MA) 33% and 20%, and *N*-desmethyl,desacetyl diltiazem (M2) 33% and 16%, respectively, compared to diltiazem, (13).

Even though the liver has been acknowledged as the major site of drug metabolism, little is known about the role of other organs in the disposition of diltiazem and its metabolites. The presence of cytochrome P-450 and other metabolizing enzymes in many organs suggests that extrahepatic tissues could contribute to the biotransformation of endogenous and exogenous substrates (14-16). In order to determine the extent and the relative contribution of extrahepatic organs in the disposition of diltiazem and its metabolites, *in vitro* studies were carried out with organs known to contain isozymes of the cytochrome P-450, i.e., the liver, the gut, the lungs and the kidneys (14-18).

MATERIAL AND METHODS

Animal Model

Male New-Zealand white rabbits (2.4–2.7 kg) purchased from La Ferme Cunicole (Mirabel, Québec, Canada) were used throughout the study. They were maintained on Purina pellets and water ad libitum in individual well ventilated metabolic cages. The animals were kept in their cages for at least ten days before any experimental work was undertaken.

Homogenate Preparation

Immediately after the sacrifice of the rabbits (n = 7), the liver, small intestine segments (0 to 30 cm and 150 to 180 cm beyond the pylorus), the lungs and the kidneys were removed and washed with a phosphate 0.05M–KCl 1.15% buffer (pH 7.40). To avoid weight distortion, the organs were carefully dried. For both segments of the small intestine, the epithelial cells were obtained scraping off gently the mucosa, after rinsing the lumen with the buffer solution. Renal cortex was dissected manually. All operations were carried out in a cold room at 4°C.

Tissues were minced and homogenized in phosphate 0.05M - KCl 1.15% buffer (pH 7.40) with a *Potter-Elvehjem* to obtain a 20% (w/v) homogenate. After centrifugation, several aliquots of the 10,000 × g supernatant fraction (Beckman 13-40, rotor 50.2, Beckman, Palo Alto, CA) were isolated and frozen at −80°C until its use for the kinetic studies. Preliminary studies using 10, 20 and 40% (w/v) homogenates demonstrated that the elimination rate constants of DTZ were comparable in the 20 and 40% (w/v) homogenates, but faster than that in the 10% (w/v) homogenate, suggesting first order kinetics in the 20 and 40% (w/v) homogenates. For this reason, the 20% (w/v) homogenate has been used all along the study.

Chemicals

The NADPH-generating system, including NADP 0.26 mM, glucose-6-phosphate 4 mM, nicotinamide 20 mM (Sigma Chemical Co., St Louis, MO) and magnesium 10 mM (Fisher Scientific Ltd., Fairlawn, NJ) was prepared extemporaneously. Two ml aliquots of the NADPH-generating system were used in each experiment.

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Diltiazem, *N*-desmethyldiltiazem (MA) and desacetyldiltiazem (M1) (Nordic Merrell Dow Research, Montréal, Canada) were used at concentrations of 48.2 μM, 50.0 μM and 53.7 μM, respectively. These concentrations were selected on the basis of predicted levels in human tissues following the intake of 60 mg of diltiazem three times daily, after reaching plasma steady state levels of 200 ng/ml (1) and assuming an accumulation factor in the tissues (19). Other *in vitro* studies used diltiazem at concentrations ranging from 20 up to 1000 μM (20,21). The metabolites concentration was the same to allow for comparisons between the substrates. Stock solutions of diltiazem and its metabolites were prepared weekly in the phosphate buffer 0.05M - KCl 1.15% (pH 7.40) and stored at 4°C, in order to avoid the possible degradation of the substrates.

Experimental Protocol

The kinetic studies were carried out in a shaker bath at a constant temperature of 37°C. After a ten-minute preincubation, 2 ml of the homogenate was mixed with the solution containing the cofactor system (2 ml) and the test compound (1 ml). Samples were withdrawn at 0, 5, 10, 20, 40 and 60 minutes and transferred into tubes containing acetonitrile along with 30 nM of loxapine, the internal standard. The tubes were vortexed vigorously in order to precipitate the proteins and to stop the reaction. Following centrifugation, the samples were immediately assayed by HPLC as described elsewhere (22). The recovery of the assayed compounds was estimated by comparing the peak heights at the beginning of each experiment with standards of either diltiazem, MA, M1 or M2.

Pharmacokinetic Parameters

The pharmacokinetic parameters were calculated by least-square linear regression analysis of log concentrations versus time plots, describing a monoexponential open model (23). The area under the curve ($AUC_{0\rightarrow 60}$) of homogenate concentrations of diltiazem, MA, M1 and M2 as a function of time were estimated by means of the trapezoidal method. The elimination rate constant (K_{el}) was determined by linear least-square regression analysis of the concentrations as a function of time. The percentage of the metabolized substrate was calculated at the end of each experiment using the following equation: % metabolized = $(C_0 - C_{60}) / C_0$, where C_0 and C_{60} are the substrate concentrations at 0 and 60 minutes. The average rate of elimination was estimated by the following equation: $(C_0 - C_{60}) / 60$ min.

Statistical Analysis

Values are expressed as the mean ± S.E.M. Differences between the organs were assessed using the analysis of variance for parallel groups and the significance was determined using Dunnett's distribution table. The minimal level of significance was $p<0.05$.

RESULTS

Preliminary studies were carried out to determine the stability of diltiazem by incubating it with the cofactor system at 37°C in absence of the homogenate. Under these conditions, the degradation of diltiazem into M1 increased with time and averaged 1.3% after 90 minutes of incubation. At the beginning of the experiments, substrate recovery from the homogenates averaged 106.4 ± 1.1% ($n=43$).

Diltiazem Metabolism

The decline of diltiazem concentrations in tissue homogenates as a function of time is depicted in Figure 1. In liver homogenates, the decline of diltiazem concentrations was much faster than in the other organs, i.e., the estimated *in vitro* elimination rate constant of diltiazem in the liver was almost five times higher than the one calculated in the proximal small intestine, and more than thirteen times the one obtained in homogenates of the distal small intestine (Table I). The ability of the tissues to biotransform diltiazem is well described by the percentage of the dose being metabolized after 60 minutes of incubation (Table I). In liver homogenates, the metabolism of diltiazem generated large amounts of MA, which reached a maximal concentration at 20 minutes, to decrease slightly afterwards (Fig. 2). No other metabolites were detected under the present conditions.

Homogenates of epithelial cells of the proximal intestinal mucosa were able to metabolize diltiazem (Fig. 1), but at a faster rate than did homogenates of the mucosa of the distal intestine; as a consequence, the percentage of the dose metabolized was greater (Table I). The incubation of diltiazem in the proximal intestine homogenate yielded MA, at much lower concentrations than the liver did, while M1 was not detected. The biotransformation of diltiazem in the distal mucosa of the intestine generated small amounts of both MA and M1 (Figs. 2 and 3). The $AUC_{0\text{-}60}$ of MA generated was almost three times smaller than that calculated when diltiazem was incubated in the mucosa of the proximal small intestine (Table II).

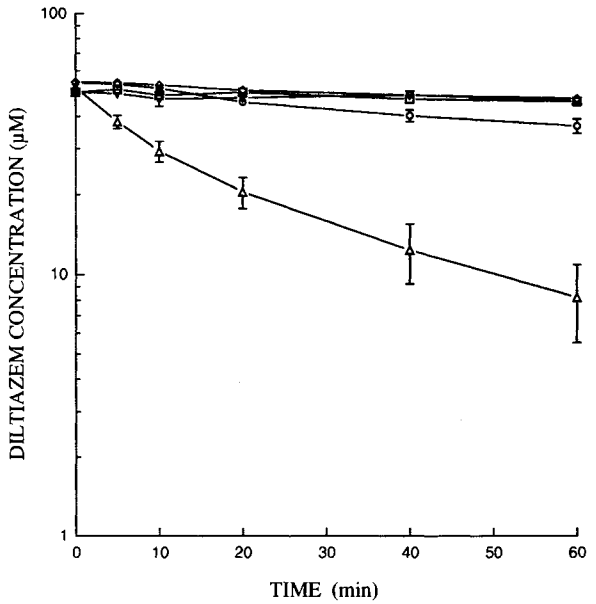

Fig. 1. Disappearance of diltiazem in the 10,000 × g supernatant fractions at 20% (w/v) of liver (△), proximal intestine (○), distal intestine (◇), lungs (□) and renal cortex (▽) of rabbits. Values are expressed as the mean ± S.E.M.

Table I. Diltiazem (48.2 μM) Metabolism in 10,000 × g Supernatant Fractions of Organ Homogenates 20% (w/v)

Homogenate		K_{el}[a] $\times 10^{-4}$ (min^{-1})	Elimination rate (μM/min)	% metabolized
Liver	(n = 6)	334 ± 45[b]	0.76 ± 0.05	84.6 ± 5.2
Intestine (0–30 cm)	(n = 6)	69 ± 11[c]	0.28 ± 0.04[c]	31.6 ± 4.2[c]
Intestine (150–180 cm)	(n = 6)	25 ± 3[d]	0.12 ± 0.02[d]	13.1 ± 1.7[d]
Lungs	(n = 3)	15 ± 6[d]	0.06 ± 0.03[d]	7.2 ± 3.2[d]
Renal cortex	(n = 2)	8 ± 6[e]	0.06 ± 0.02[d]	7.1 ± 1.8[d]

[a] K_{el}: elimination rate constant.
[b] mean ± S.E.M.
[c] $p < 0.05$ compared to values in liver.
[d] $p < 0.05$ compared to values in liver and proximal small intestine.
[e] $p < 0.05$ compared to values in liver, proximal and distal small intestine.

Compared to the liver, the ability of the lungs and the renal cortex to metabolize diltiazem was very small and variable, as reflected by the estimated elimination rate constants (Table I). Only small amounts of M1 were detected after 40 minutes of diltiazem incubation in the lungs and in the renal cortex (Fig. 3). The elimination rate constant of diltiazem in the lungs did not differ from those in the renal cortex, and in the distal segment of the small intestine (Table I).

MA and M1 Metabolism

After 60 minutes of incubation, liver homogenates metabolized around 60% of the initial dose of MA (Table III and Fig. 3). The elimination rate constant of MA in liver preparations was smaller than that estimated for diltiazem (Table III). The metabolism of MA did not yield M2 (Table II and Fig. 4).

M1 was rapidly metabolized in liver homogenates, and only 7% of the dose was recovered after 60 minutes of incubation (Fig. 3). The elimination rate constant of M1 was elevated, in fact comparable to that of diltiazem in the liver, and much greater than the elimination rate constant of M1 in small intestine homogenates. The metabolism of M1 in liver 10,000 × g supernatant yielded M2 (Table II). Comparing the $AUC_{0\text{-}60}$ of M2 to the theoretical $AUC_{0\text{-}60}$ of M1 assuming no metabolism (53.7 μM times 60 min), it is possible to state that the $AUC_{0\text{-}60}$ of M2 represented 22% of the $AUC_{0\text{-}60}$ of M1.

The sum of the $AUC_{0\text{-}60}$ of M1 and that of M2 yields a value close to the theoretical $AUC_{0\text{-}60}$ of M1 assuming no metabolism (53.7 μM times 60 min), suggesting that the metabolism of M1 in the intestine generates only small amounts of M2 (Table II).

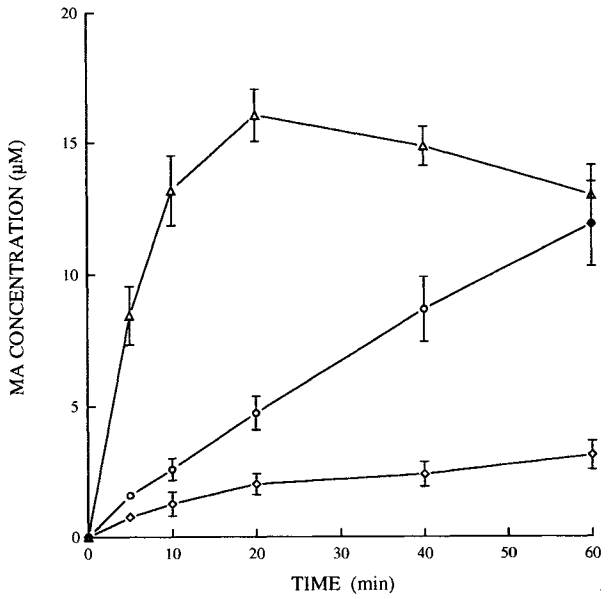

Fig. 2. Production of MA following the incubation of 48.2 μM of diltiazem in homogenates 20% (w/v) of liver (△), proximal intestine (○) and distal intestine (◇). Values are expressed as the mean ± S.E.M.

DISCUSSION

Most xenobiotics are biotransformed through functionalization pathways (phase I) or through conjugations (phase II) in order to facilitate their excretion from the organism (14–17). The majority of phase I reactions are carried out by the cytochrome P-450 family of enzymes (CYP) and through other metabolizing enzymes like esterases, *O*-de-ethylases, *N*- and *O*-demethylases, epoxide hydrolases, xanthine oxydases, decarboxylases, etc (14–16). These enzymes are widely distributed in hepatic and extrahepatic tissues, and could play a relatively important role in the disposition of xenobiotics. Microsomal enzymes (CYP, *O*-de-ethylases, glucuronyl transferases, etc) are found in greater concentrations in the liver, whereas cytosolic enzymes (esterases, gluthatione *S*-transferases, sulphotransferases, etc) have an almost even and wide distribution throughout the organs (15–17).

Using *in vitro* approaches, the true metabolic activity in each organ is very difficult to assess, however assuming that diltiazem is only metabolized in the liver, the intestine, the lungs and the kidneys, that the stability of the enzymes is identical in each organ involved, and that no substances interfere with the metabolism of a substrate, it is possible to compare the activity of an organ to another. In the present study, the ability of an organ to metabolize diltiazem is further complicated by the presence of several parallel routes of metabolism. Therefore, taking into account the net additive effect of these routes of elimination, the present results indicate that the liver accounts for 75% of diltiazem metabo-

Table II. Area Under the Curve ($AUC_{0\to60}$) of Diltiazem (DTZ), *N*-Desmethyldiltiazem (MA), Desacetyldiltiazem (M1) and *N*-Desmethyl,desacetyldiltiazem (M2) Following the Incubation of DTZ, MA and M1 in Homogenates 20% (w/v) of Liver, Proximal and Distal Small Intestine and Lungs

Substrate/organ	$AUC_{0\to60}$ DTZ (μM · min)	$AUC_{0\to60}$ MA (μM · min)	$AUC_{0\to60}$ M1 (μM · min)	$AUC_{0\to60}$ M2 (μM · min)
DTZ/Liver	1883 ± 159[a]	809 ± 31	ND[b]	ND
DTZ/proximal int.	2642 ± 85	387 ± 48	ND	ND
DTZ/distal int.	2996 ± 28	114 ± 23	112 ± 12	ND
DTZ/Lungs	2875 ± 12	ND	33 ± 2	ND
DTZ/Kidneys	2856 ± 27	ND	77 ± 8	ND
MA/Liver		1745 ± 80		ND
M1/Liver			1080 ± 115	696 ± 48
M1/proximal int.			3063 ± 71	220 ± 38
M1/distal int.			3255 ± 37	59 ± 14

[a] Values are mean ± S.E.M.
[b] ND, nondetectable under these conditions.

lism, while extrahepatic tissues dispose of the remaining 25%.

In several animal species and in human, the metabolism of diltiazem generates acidic and basic metabolites. Four acidic metabolites are the result of deamination of the dimethylaminoethyl group of diltiazem, reaction that appears to be mediated by microsomal cytochrome P-450; these metabolites do not elicit any important activity (10,12). On the other hand, isozymes of the CYP 3A subfamily have been implicated in the biotransformation of diltiazem to MA a basic metabolite (21). Since CYP 3A subfamily is widely distributed in the organism, with high concentrations in the liver and the gut (12,15), the ability of an organ to metabolize diltiazem should vary according to the amount of CYP 3A locally available. The liver biotransformed diltiazem at a high rate, essentially through *N*-desmethylation, since only MA was detected. These *in vitro* results are compatible with results in rabbits and in human hepatic homogenates showing that MA is the predominant metabolite (21). Assuming first order kinetics, since the liver transformed 61% of the initial amount of MA during 60 minutes, we may estimate that following the incubation of 48.2 μM of diltiazem, 34.5 μM of MA may have been produced to obtain an $AUC_{0\text{-}60}$ of MA of 809 μM · min. M1 was not detected when diltiazem was incubated in hepatic homogenates, which could be due to its high rate of disappearance in the liver. On the other hand, when M1 was incubated in the 10,000 × g liver supernatant, only 20% was recovered as M2. Since no M2 was detected following diltiazem incubation, we may conclude that hepatic tissues metabolize diltiazem generating essentially MA, and small amounts of M1 which are rapidly transformed into M2, which is further metabolized.

The gut accounted for 20% of the overall metabolism of diltiazem, most of which occurs in the proximal segment of the small intestine yielding MA, while M1 was not detected. The activity of the proximal intestine to metabolize M1 was rather slow as compared to the liver, and it yielded only M2. The disposition of diltiazem in the distal intestine occurred at a much slower rate than in the proximal intestine, i.e., around 13% of diltiazem was metabolized *versus* 32%, respectively. In distal portions of the intestine, M1 was easily measurable since its *N*-desmethylation to yield M2 was very slow. Therefore, we must assume that in the proximal small intestine, diltiazem is essentially converted to MA, while in the distal small intestine, both MA and M1 were generated in similar amounts. No attempt was made to incubate MA in intestinal epithelial cells since MA derived from diltiazem did not show any trend to diminish in the incubation media (Fig. 2), suggesting that it was not metabolized in this tissue.

Table III. *N*-Desmethyldiltiazem (MA) (50 μM) and Desacetyldiltiazem (M1) (53.7 μM) Metabolism by 10,000 × g Supernatant Fractions of Organ Homogenates at 20% (w/v)

Homogenate		K_{el} (10^{-4}) min^{-1}	Elimination rate (μM/min)	% metabolized
MA in liver	(n = 6)	166 ± 23[a,b]	0.48 ± 0.05[b]	61.3 ± 5.0[b]
M1 in liver	(n = 5)	468 ± 53[c]	0.88 ± 0.02[c]	93.4 ± 2.1[c]
M1 in intestine (0–30 cm)	(n = 5)	38 ± 8[d]	0.19 ± 0.05[d]	19.5 ± 4.6[d]
M1 in intestine (150–180 cm)	(n = 4)	11 ± 3[e,f]	0.05 ± 0.02[e,f]	5.7 ± 1.3[e,f]

[a] Mean ± S.E.M.
[b] $p < 0.05$ compared to values for diltiazem in liver (Table I).
[c] $p < 0.05$ compared to values for MA in liver.
[d] $p < 0.05$ compared to values for M1 in liver.
[e] $p < 0.05$ compared to values for M1 in liver and proximal intestine.
[f] $p < 0.05$ compared to values for diltiazem in distal intestine (Table I).

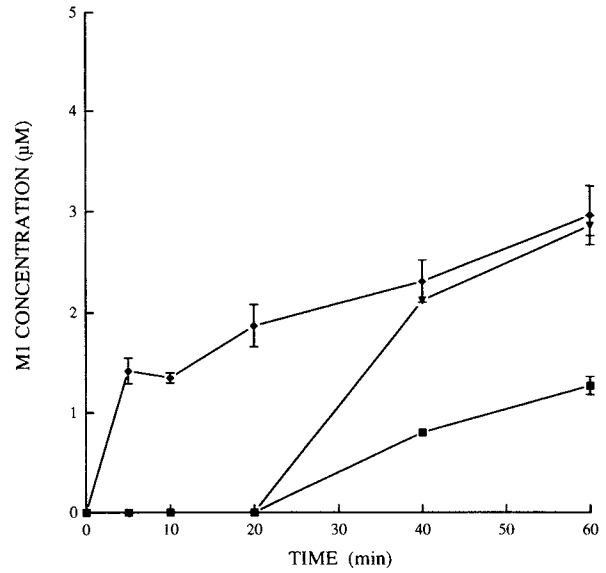

Fig. 3. Production of M1 following the incubation of 48.2 μM of diltiazem in homogenates 20% (w/v) of distal intestine (♦), lungs (■) and renal cortex (▼) of rabbits. Values are expressed as the mean ± S.E.M.

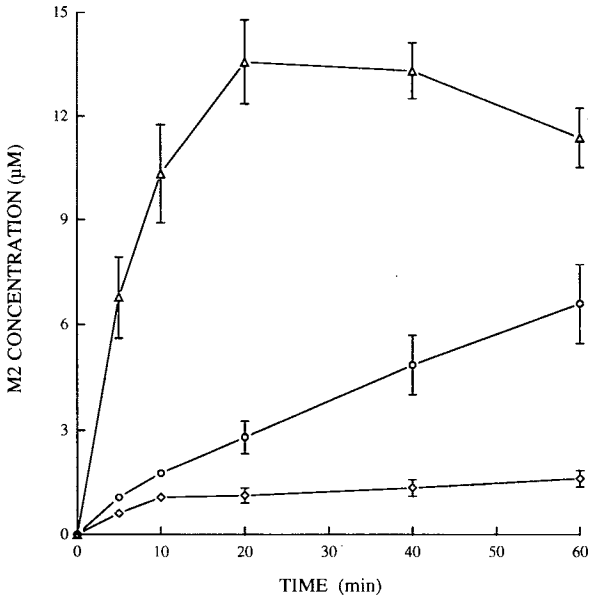

Fig. 5. Production of the metabolite M2 following the incubation of 53.7 μM of M1 in homogenates 20% (w/v) of liver (△), proximal intestine (○) and distal intestine (◇). Values are expressed as the mean ± S.E.M.

These results suggest that the gut may contribute to diltiazem pre-systemic biotransformation. Furthermore, the ability of the intestinal tractus to biotransform diltiazem seems to be site-dependent, with a decreasing activity in distal sections of the small intestine. This observation is in agreement with previous reports indicating decreasing cytochrome P-450 content distally to the pylorus (14,17,18). We may predict that a similar phenomenon would occur in humans, since the amount of CYP 3A decreases progressively towards the ileum (15). This site-dependent metabolism may have clinical repercussions on diltiazem bioavailability, specially when comparing immediate and delayed release formulations, where pre-systemic metabolism and enzyme saturation could be affected by the rate of substrate release and by the site of release within the gut.

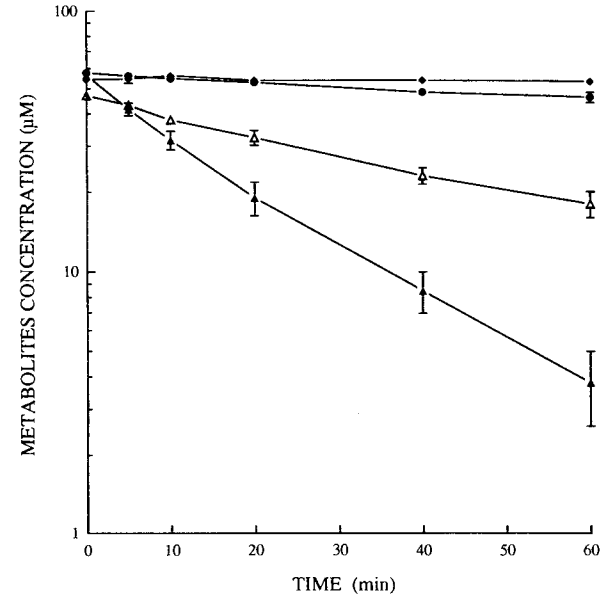

Fig. 4. Disappearance of 50.0 μM of MA (hollow symbols) and of 53.7 μM of M1 (filled symbols) in the 10,000 × g supernatant fractions at 20% (w/v) of liver (△), proximal intestine (○) and distal intestine (◇) of rabbits. Values are expressed as the mean ± S.E.M.

Although the lungs and the kidneys contain cytochrome P-450 (14,15,17), the present study demonstrates that their ability to biotransform diltiazem is very low, since their contribution to overall diltiazem elimination is approximately 3% and 2%, respectively. The metabolism of diltiazem in these organ preparations yielded only M1, and that after 40 minutes of incubation. These results could be explained by the low amounts of cytochrome P-450 found within the lungs and the kidneys (15,17,25), and by reports indicating that extrahepatic organs are more frequently implicated in phase II conjugations (15,16,25,26). For these reasons and because MA concentrations did not show any trend to decrease (Fig. 2), MA was not incubated in these tissues.

When comparing the present *in vitro* observations to results obtained *in vivo*, some similarities are apparent. As we observed, MA is the major metabolite found in plasma and in urine in either rabbits (8,21) or humans (9,11,21,27), while the more potent M1 is present in smaller amounts. However, not all studies agree concerning the importance of the *N*-desmethylation relative to the deacetylation of diltiazem, since M1 has also been reported to be the major metabolite of diltiazem (1,28). This apparent contradiction may be explained on the basis of our results; effectively, when diltiazem is given orally, MA will be the predominant metabolite due to a high hepatic production, and a concomitant rapid biotransformation of M1 produced by the gut or by the liver itself. However, following intravenous administration of diltiazem, the lungs and the kidneys will generate M1 that will be detected systemically. Therefore, we may predict that the route of administration, as well as the rate of elimination of the metabolite, are factors determining the amounts of

MA or M1 present in blood. This speculation is supported by *in vivo* reports showing that M1 was the predominant metabolite following parenteral injection of diltiazem (1,29), and MA was essentially recovered when diltiazem was given orally (9,11,21,27,29).

There is some concern in the literature as to whether M1 is a true metabolite or a degradation by-product, since a) its presence is quite variable in the plasma of patients receiving diltiazem (1,9,11,28) and b) the deacetylation of diltiazem could occur over time without any enzymatic presence (22,30). The fact that fresh solutions of diltiazem or its metabolites were prepared weekly, and that the incubation of diltiazem with tissue homogenates for 90 minutes generated only 1.3% of the dose as M1, allows us to assume that the M1 assayed in tissue homogenates originated from the metabolism of diltiazem.

In summary, several organs are able to metabolize diltiazem *in vitro*, the liver showing the greatest ability to transform diltiazem. MA is the major metabolite due to a great production and slow rate of elimination in the liver, while M1 is biotransformed much more readily. If, *in vivo*, the ability of the proximal intestine to extract diltiazem is around 20% to that of the liver, as in the present study, then the role of the small intestine in diltiazem presystemic metabolism may be important, since the entire dose is exposed to the small intestine.

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