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IN VIVO OXIDATIVE CLEAVAGE OF A PYRIDINE-CARBOXYLIC ACID ESTER METABOLITE OF NIFEDIPINE

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Abstract—The pharmacokinetics of the primary pyridine metabolite of nifedipine (2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinecarboxylic acid dimethylester) (M-0) and its [$^2H_6$]dimethylester analog ([$^2H_6$]M-0) were studied in male rats. A large, 5.8-fold deuterium isotope effect for the formation clearance of the monomethylester (M-1) was observed, which is strongly indicative for an oxidative reaction mechanism involving the abstraction of a hydrogen atom, presumably by cytochrome P-450. M-0 exhibited a high systemic blood clearance (104 ± 27 ml/min/kg) (mean ± SD) which was not significantly influenced by deuterium substitution (125 ± 13 ml/min/kg). Its systemic clearance is presumably flow limited, and extrahepatic metabolism can be anticipated. The major metabolic pathway for M-0 in male rats seems to be a direct oxidation at the 2-methyl position and subsequently a rapid conversion of the unstable 2-hydroxymethyl-dimethylester to the lactone of the monomethylester (M-2), as has been shown by others *in vitro*. Non-oxidative ester cleavage of M-0 in our rats was negligible. Deuterium substitution of M-0 at the ester methyl groups induced "metabolic switching" in favor of the direct oxidation of M-0 to M-2.

Nifedipine (NF), the archetype of the dihydropyridine calcium entry blockers, is widely used in the treatment of hypertension and angina pectoris. *In vivo*, the compound is rapidly oxidized to the corresponding pyridine derivative, 2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinecarboxylic acid dimethylester (M-0), and further metabolism involves hydrolysis of an ester group (M-1), hydroxylation of the 2-methyl group (M-2_0), and formation of the lactone (M-2_1) from the latter (Fig. 1) [1]. For many other dihydropyridine compounds these reactions are also important metabolic pathways [2–6].

There is good *in vitro* evidence for the involvement of the cytochrome P-450 mixed function oxidase system in the oxidative dehydrogenation of NF and analogues [7–9] which is supported by *in vivo* induction and inhibition studies in animals as well as in man [10–12]. The aromatization is catalysed by specific isozymes of the cytochrome P-450 class III, *in vitro* [8] as well as *in vivo* [11, 13]. The oxidation on the 2-methyl group is also cytochrome P-450 mediated [14]. In humans the pharmacokinetics of nifedipine and M-0 exhibit large interindividual variability after oral administration [15–17].

Recently, evidence was published for the involvement of cytochrome P-450 in the cleavage of the ester bond of some pyridine-carboxylic acid esters *in vitro* in rat liver microsomes and purified cytochrome P-450 isozymes [14, 18]. Involvement of cytochrome P-450 in the *in vitro* cleavage of carboxylic acid esters has only some precedent in cases where also extensive non-oxidative hydrolysis occurs [19, 20].

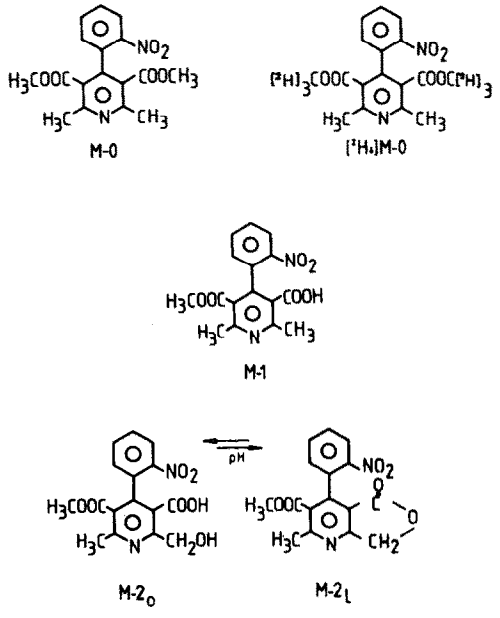

Fig. 1. Structures of M-0, [$^2H_6$]M-0, M-1, M-2_0 and M-2_1.

We have studied the pharmacokinetics and metabolism of M-0 and its [$^2H_6$]dimethylester analog [$^2H_6$]M-0 (Fig. 1) in the rat and provide evidence for the involvement of cytochrome P-450 in the oxidative cleavage of carboxylic acid esters *in vivo*.

MATERIALS AND METHODS

Compounds. M-0 and [$^3H_6$]M-0 were synthesized via the Hantzsh method with subsequent oxidation,

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hydrolysis and re-esterification [9, 18]. The identity of the resulting compounds was confirmed by melting points, mass spectra (EI, CI), proton NMR spectra and UV spectra [9, 18]. The compounds were identical according to HPLC and GC data (coelution experiments). The purity of M-0 and [$^2H_6$]M-O as determined by HPLC, was >99% compared to the reference substance of Bayer AG (Wuppertal F.R.G.) (data not shown). Nitrendipine (internal standard for the assay of M-0), M-1 (2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinecarboxylic acid monomethylester, Bay o-2820), M-2_1 (2-hydroxymethyl-6-methyl-4-(2-nitrophenyl)-3,5-pyridinecarboxylic acid monomethylester lactone, Bay h-2228) and Bay o-4160 (2,6-diethyl-4-(2-nitrophenyl)-5-methoxycarbonyl-pyridine-3-carboxylic acid; internal standard for the assay of M-1 and M-2) were gifts of Dr K.-D. Rämsch (Bayer AG, Wuppertal, F.R.G.). The structures of these compounds are shown in Fig. 1.

Animal experiments. Male Wistar rats (180–220 g) from laboratory breed were divided into two groups which received either M-0 or [$^2H_6$]M-0. Twenty-four hours before the experiment the rats were cannulated in the right femoral artery under ether anaesthesia for blood sampling. The polyethylene canula was inserted and, to avoid destruction by the rat during the experiment, pulled s.c., emerging on the nape of the neck, thus allowing free movement of the rats during the experiment. The rats were fasted overnight in a metabolic cage and they had free access to water. Blank urine was collected until administration of the compounds. M-0 and [$^2H_6$]M-0 were dissolved in a mixture of ethanol, polyethylene glycol 400 and normal saline (1.25:1:2.5) and a dose of 1.85 mg (250 μl) was administered by bolus injection into the penis vein under light ether anaesthesia. After recovery (2–5 min) the rats were returned into their metabolic cage. Fourteen blood samples (100 μl) were taken at regular intervals up to 150 min and immediately hemolysed with 0.5 ml of water. Half of the blood samples were used for the assay of M-0 and the other half for the combined assay of M-1 and M-2. Urine was collected up to 24 hr after administration. All samples were stored at −20° until analysis within one month.

Assay of compounds. M-0 in blood was measured by reversed-phase HPLC (modification of Ref. 1). Briefly, after addition of nitrendipine and 0.1 M phosphate buffer pH 7.0, the compounds were extracted with a mixture of dichloromethane and pentane (3:7). After separation and evaporation of the organic layer and subsequent dissolving of the residue in mobile phase (methanol/water 53:47, pH 4.3) the compounds were assayed on a 10 cm C18 column with UV detection at 230 nm. The limit of determination was approximately 20 ng/ml.

M-1 and the sum of M-2_0 and M-2_1 in blood and urine were measured by a reversed-phase ion-pair HPLC method (to be published elsewhere). Briefly, after addition of internal standard Bay o-4160, M-2_0 was converted quantitatively to M-2_1 by heating in the presence of hydrochloric acid. Firstly, M-2_1 was extracted with a mixture of dichloromethane and pentane (3:7) and M-1 was subsequently extracted with chloroform. The organic layers were evaporated to dryness separately, and after subsequent dissolving of the residues in mobile phase [water with 25 mM cetrimide/acetonitrile (72:38)] the compounds were assayed on a 10 cm C18 column with UV detection at 275 nm. The limits of determination were approximately 80 and 40 ng/ml for M-1 and M-2 respectively. No (sponaneous) hydrolysis of M-1 to M-2 occurred during this assay procedure.

Data analysis. The terminal elimination rate constant (k) was assessed by applying logarithmic regression analysis to the terminal part of the concentration–time profile. The half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/k$. The area under the blood concentration–time curve (AUC) was calculated with the lin-log trapezoidal method and extrapolated to infinity using the terminal elimination rate constant. The systemic blood clearance (Cl) was calculated as $Cl = D/AUC$, in which D is the dose. The volume of distribution at steady state (V_{ss}) was calculated as $V_{ss} = D \times AUMC/AUC^2$, in which $AUMC$ was the area under the moment curve. The clearance of formation to a specific metabolite ($Cl_{\rightarrow m_i}$) was calculated as $Cl_{\rightarrow m_i} = Um_i \times Cl$, in which Um_i is the fraction of the dose that was recovered in urine as the specific metabolite m_i. Clearances and volume of distribution were normalized to body weight. Statistical analysis was done with two-tailed, two sample t-test.

RESULTS

Mean blood concentration–time profiles of M-0, M-1 and M-2 after administration of 1.85 mg M-0 and [$^2H_6$]M-0 are shown in Fig. 2. Comparing M-0 and [$^2H_6$]M-0 treated animals, there were large differences in the AUC of M-1 and its urinary recovery, resulting in a 5.8-fold difference in calculated formation clearance of M-1. The systemic clearances of M-0 and [$^2H_6$]M-0 however were not significantly different. The AUC and urinary excretion of M-2 were not significantly different for both treatments. The calculated formation clearance of M-2 was just significantly higher for [$^2H_6$]M-0 than for M-0. There were no differences in volume of distribution of M-0 and [$^2H_6$]M-0. Also the half-lives of the parent compound and its metabolite M-2 were not different for both treatments.

The results and statistical analysis are summarized in Table 1.

DISCUSSION

Large differences in AUC of M-1 and in its urinary recovery were observed after administration of M-0 and [$^2H_6$]M-0, resulting in a 5.8-fold difference in calculated formation clearance of M-1. After administration of [$^2H_6$]M-0, M-1 concentrations in blood were only just above the limit of determination in some rats, indicating that the AUC of M-1 did not exceed approximately 0.2 μg/hr/ml in any of the animals in this group. Although the interpretation of *in vivo* isotope effects in terms of the underlying mechanism should be done with caution, the large deuterium isotope effect reported here is strongly indicative for the previously proposed mechanism involving the abstraction of a hydrogen atom from

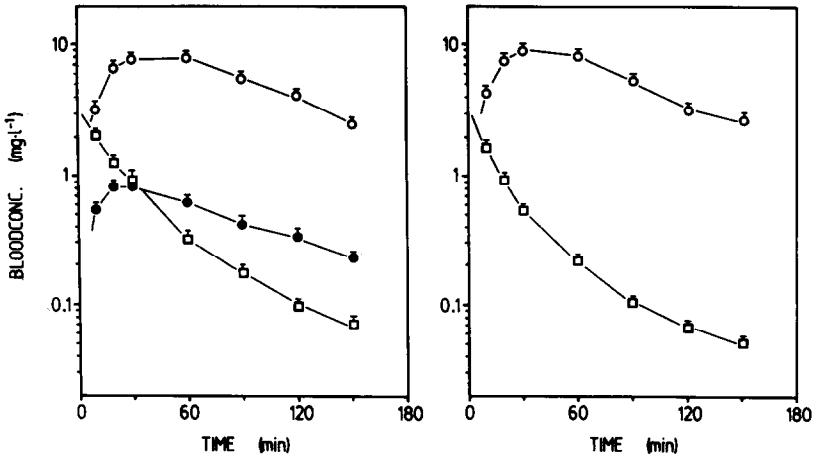

Fig. 2. Mean blood concentration–time profiles of M-0 (□), M-1 (●) and M-2 (○) after administration of 1.85 mg M-0 (left) and [$^2H_6$]M-0 (right). Values are mean ± SE, N = 6.

Table 1. Pharmacokinetic parameters of iv M-0 and [$^3H_6$]M-0 in male rats

	Administered compound		
	M-0	[$^3H_6$]M-0	P-value
Body weight (g)	206 ± 20	212 ± 15	0.6
AUC of M-0 (μg/hr/ml)	1.52 ± 0.39	1.16 ± 0.07	0.05
AUC of M-1 (μg/hr/ml)	1.57 ± 0.44	ND	<0.001
AUC of M-2 (μg/hr/ml)	18.5 ± 8.2	18.4 ± 2.7	0.9
Cl of M-0 (ml/min/kg)	104 ± 27	125 ± 13	0.1
V_{ss} of M-0 (l/kg)	3.9 ± 1.2	4.5 ± 0.7	0.4
$t_{1/2}$ of M-0 (min)	40.7 ± 5.0	44.0 ± 5.5	0.3
$t_{1/2}$ of M-1 (min)	57.1 ± 13.3	ND	—
$t_{1/2}$ of M-2 (min)	57.5 ± 8.8	59.0 ± 7.5	0.8
Ue of M-1 (% of dose)	12.0 ± 2.2	1.7 ± 0.2	<0.001
Ue of M-2 (% of dose)	28.0 ± 3.8	31.2 ± 2.7	0.1
$Cl_{\rightarrow M\text{-}1}$ (ml/min/kg)	12.4 ± 3.2	2.16 ± 0.40	<0.001
$Cl_{\rightarrow M\text{-}2}$ (ml/min/kg)	29.0 ± 8.2	39.1 ± 6.0	0.03

(Mean ± SD), N = 6 in each group. ND = not detectable.

the ester methyl group [14, 18, 21, 22]. This is apparently the first experimental proof for oxidative ester cleavage *in vivo*. *In vivo* cleavage of M-0 in male rats by non-oxidative pathways (esterases or nonemzymatically) seems to be negligible as indicated by the very low formation clearance of M-1 from [$^2H_6$]M-0. Preliminary *in vitro* studies with M-0 (Male Wistar rat liver microsomes) indicate a kinetic deuterium isotope effect $^d(V)$ of about 3.* No data about oxidative ester cleavage of M-0 by individual cytochrome P-450 families and isozymes have been published up to now. *In vitro* studies with structural analogues of M-0 [14, 18] may indicate that the isozymes P-450$_{PB\text{-}B}$ and P-450$_{\beta NF\text{-}B}$ are likely to be of major importance for the oxidative ester cleavage of M-0, whereas the isozymes which appear to be more proficient at oxidizing the dihydropyridine ring of nifedipine (P-450$_{UT\text{-}A}$ and P-450$_{PCN\text{-}E}$) may be less adept at cleaving the ester bond.

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No significant differences were observed in the half-lives and the systemic clearance of M-0 and [$^2H_6$]M-0. Since these clearances were higher than the total liver blood blow that could be expected in these rats [23], extrahepatic metabolism should be anticipated. Since the systemic clearance is presumably flow limited, it is therefore not a sensitive indicator of metabolic activity. As shown, the rate of formation of a specific metabolite is a more appropriate indicator of isotope effects if the parent compound is highly cleared. It is very unlikely that these pharmacokinetic differences between the non-labelled and deuterated M-0 arise from differences in distribution or protein binding because no differences in volume of distribution were observed.

In pilot experiments the 24-hr urinary excretion of the metabolites M-1 and M-2, after administration *per se*, was shown to amount to approximately 75% of the dose administered. Thereafter neither M-1 nor M-2 was detected in blood and urine. Also in the present experimental design, neither M-1 nor M-2 can be detected in urine and blood after 24 hr. Although not significant, the urinary excretion of M-

2 tended to be higher for the deuterated compound, but no differences in *AUC* of M-2 were observed. The calculated formation clearance of M-2 was 35% higher for [$^2H_6$]M-0 than for M-0 ($P = 0.03$). The increase in formation clearance of M-2 as a consequence of deuterium labelling is compatible with the observed "metabolic switching" *in vitro* with some other pyridine carboxylic esters [14]. The half-life of the metabolite M-2 was the same in both cases.

From our data it can be concluded that *in vivo* in male rats the major metabolic pathway of this pyridine carboxylic ester is a direct conversion to the metabolite M-2, presumably by oxidation of the 2-methyl group and subsequent rapid conversion of the highly unstable 2-hydroxymethyl diemethylester to M-2_1 [14]. This seems to be also the major *in vitro* metabolic pathway for the primary pyridine metabolite of felodipine in some species [24]. The details of the metabolism of M-0 in man remain to be elucidated.

Since most oxidative pathways are subject to genetic and environmental influences [25], the involvement of cytochrome P-450 in the cleavage of the ester bond of M-0 may, besides variable formation from NF, partially explain the large interindividual variability in plasma kinetics of M-0 as observed in healthy volunteers [16, 17].

To summarize, using deuterium isotope effects, evidence is presented for *in vivo* oxidative ester cleavage of a pyridine carboxylic acid ester metabolite (M-0) of nifedipine. For the metabolic elimination of M-0 in male rats this pathway is of secondary importance to the direct oxidation of the 2-methyl group M-0 to form the lactone of M-2. Non-oxidative ester cleavage of M-0 was negligible.

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