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Enantioselective *N*-Oxygenation of Verapamil by the Hepatic Flavin-Containing Monooxygenase

JOHN R. CASHMAN

Department of Pharmaceutical Chemistry and the Liver Center, University of California, San Francisco, California 94143-0446

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SUMMARY

The chemical and enzymatic *N*-oxygenation of verapamil was investigated. Verapamil *N*-oxide is readily synthesized by chemical means. It is not indefinitely stable, however, and undergoes Cope-type elimination to produce 3,4-dimethoxystyrene and a hydroxylamine. The major stable metabolite observed during the metabolism of verapamil with rat and hog liver microsomes and purified flavin-containing monooxygenase is 3,4-dimethoxystyrene. 3,4-Dimethoxystyrene is formed at a rate 4 times that of nor-verapamil. Studies suggest that *N*-oxygenation is catalyzed largely by the flavin-containing monooxygenase and *N*-demethylation is catalyzed by cytochrome P-450. This conclusion is based on the effects of cytochrome P-450 inhibitors and positive effectors for the flavin-containing monooxygenase as well as on studies with the purified enzyme. In the presence of rat and hog liver microsomes, significant stereoselectivity in *N*-oxygenation of verapamil is observed (*S*/*R* ratio of 3.1 and 4.1, respectively). With purified hog and rat hepatic flavin-containing monooxygenase, the stereoselectivity for verapamil *N*-oxygenation (*S*/*R* ratio of 10.1 and 6.6, respectively) suggests a role for this enzyme in the stereoselective first-pass metabolism of verapamil.

Verapamil (1) is a clinically effective antiarrhythmic drug that is used for a variety of cardiovascular disorders (1). The metabolism of verapamil has been determined in humans and animals (2, 3) and verapamil undergoes extensive biotransformation. In humans, where only 2% of the parent drug is excreted unchanged (3, 4), only about one half of the drug has been accounted for in terms of identifiable metabolites. Not only in humans, but also in rats and dogs, the major metabolic routes involve *N*-dealkylation (2–4) (Fig. 1). Although both verapamil *N*-demethylation and *O*-demethylation are certainly measurable (2–5), it is not clear why cleavage of the C-N-C system of verapamil is such a dominant route of metabolism. *N*-dealkylations are generally thought to involve cytochrome P-450 enzyme activity (6), but flavin-containing monooxygenase activity has been shown to give rise to *N*-dealkylation products via Cope-type elimination reactions of initially formed tertiary amine *N*-oxides, which are formed by this monooxygenase (7).

(*S*)-Verapamil undergoes first-pass and systemic clearance at a substantially higher rate than does (*R*)-verapamil (8, 9). In humans, (*S*)-verapamil is metabolized much more rapidly than the *R*-enantiomer and the *S*/*R* ratio is greatest for all *N*-dealkylation metabolites (4). In rat and human microsomal preparations, *N*-demethylation and *O*-demethylation demonstrate much more modest stereoselectivity than *N*-dealkylation (4, 5). Because of the relatively minor contribution to the overall metabolism and the modest stereoselectivity observed, it is not likely that the stereoselectivity of these *N*-demethylation and *O*-demethylation processes determines the stereoselective first-pass effect observed *in vivo*. Other factors must be involved for verapamil clearance, including novel metabolic routes leading to stereoselective *N*-dealkylation and also possibly protein binding.

In this study, I describe the *N*-oxygenation of verapamil by rat and hog liver microsomes and by the purified flavin-containing monooxygenase. Verapamil is efficiently *N*-oxygenated to verapamil *N*-oxide, which decomposes via Cope-type elimination to produce 3,4-dimethoxystyrene. As with other tertiary amines (10) that are substrates for the flavin-containing monooxygenase, *N*-oxygenation is accomplished by the enzyme in a markedly stereoselective fashion (11).

Materials and Methods

Reagents. Verapamil was obtained from Sigma Chemical Co.; norverapamil (*N*-desmethyl verapamil) was generously provided by Dr. H. U. Siebeneick (Knoll Pharmaceuticals, NJ). Pure (*R*)- and (*S*)-verapamil were a kind gift of Professor W. L. Nelson (University of Washington). Aminobenzotriazole was a generous gift of Professor P. Ortiz de Montellano of this department. All of the compounds of the NADPH-generating system were obtained from Sigma. All other chemicals, reagents, and solvents used in this study were obtained in the highest quality from commercial suppliers.

Synthesis of verapamil *N*-oxide. A solution of *meta*-chloroperbenzoic acid (41.6 mg, 0.24 mmol) in 5 ml of dichloromethane was added at 4° to an ice-cooled dichloromethane solution (3 ml) of verapamil free base (110 mg, 0.24 mmol). After the mixture was stirred at 0° for 4.5 hr, TLC indicated that the formation of product was complete.

ABBREVIATIONS: TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; EI, electron impact.

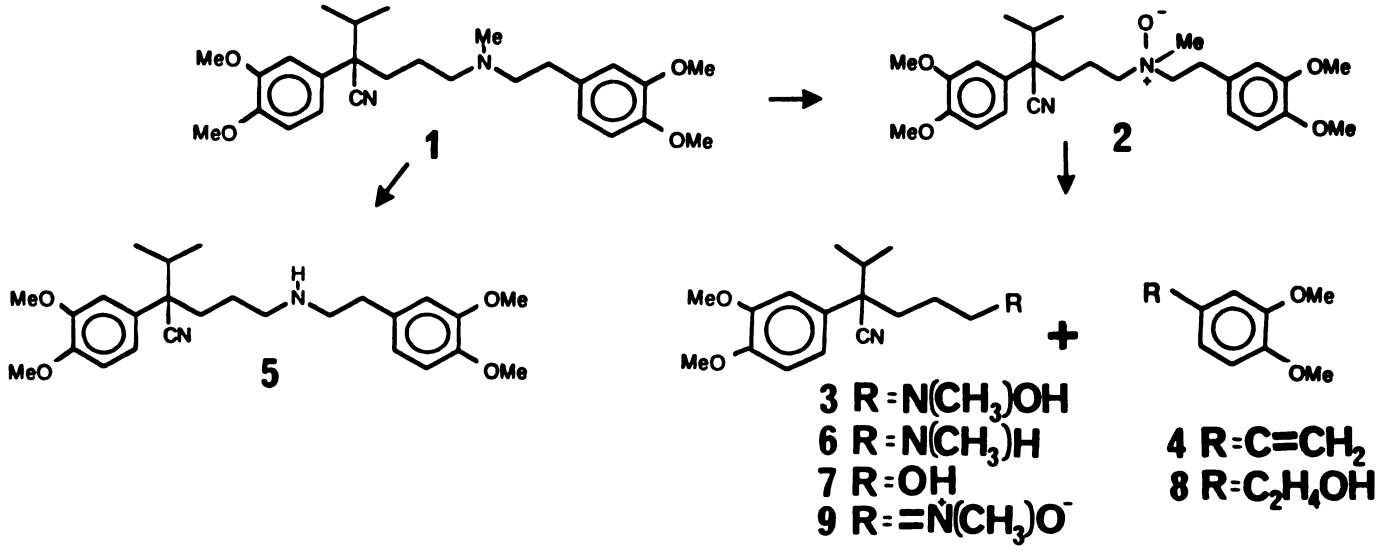

Fig. 1. Proposed overall metabolic pathways of verapamil (**1**). *Me*, methyl.

The crude reaction mixture was chromatographed directly on basic alumina (eluent, CH_2Cl_2/CH_3OH, 9:1, v/v) to give the *N*-oxide (**2**) (100 mg; yield, 91%). ^1H NMR ($CDCl_3$):δ 0.84 (3 H, d), 1.23 (4 H, m), 1.85 (1 H, m), 1.92 (1 H, m), 1.97 (1 H, m), 2.03 (1 H, m), 2.95 (2 H, s), 3.09 (3 H, m), 3.15 (2 H, m), 3.20 (2 H, m), 3.85 (6 H, s), 3.88 (6 H, s), 6.70 (1 H, m), 6.76 (1 H, m), 6.87 (2 H, m); IR (CH_2Cl_2):2229 cm^{-1} (C ≡ N stretch), 818 cm^{-1} (*N*-oxide); liquid secondary ion mass spectrometry (glycerol) *m/z* (relative intensity): 471 (MH^+, 100), 455 (MH^+–0,12.8); UV (methanol): λ_{max} (ε), 278 (5700).

Synthesis of deuterated and tritiated verapamil. The synthesis of [^{2}H]verapamil and [^{3}H]verapamil was based on methods developed previously (12). Verapamil (scrupulously dried free base, 2 mg, 4.4 μmol) was combined with 0.5 ml of heptafluorobutyric acid and either D_2O (500 μl, 100% deuterium) or ^3H_2O (5 μl, 50 mCi) in a 13 × 100 mm screw cap culture tube and was heated to 150° for 4 days. The reaction was cooled to room temperature and opened and 2 ml of CH_2Cl_2 was added, followed by the cautious addition of saturated $NaHCO_3$. The organic phase was separated from the aqueous phase, washed with brine and hexane, and dried over Na_2SO_4. The dichloromethane solution was evaporated, applied to a Whatman LK5DF silica gel TLC plate, and eluted with ethyl acetate/triethylamine (97:3, v/v). This system separated verapamil (R_f = 0.36) from minor amounts of decomposition products that form during the exchange reaction. The same tritium-exchange reaction was performed with (*R*)- and (*S*)-verapamil to afford (*R*)-[^{3}H]verapamil and (*S*)-[^{3}H]verapamil, respectively, in good overall yield. The specific activity of the exchanged material was quite high (approximately 30–63 mCi/mmol) and for metabolism studies dilution with unlabeled starting materials afforded working stocks of 1–10 mCi/mmol. Typical results for deuterium exchange of verapamil are listed in Table 1.

Liver and enzyme preparations. Microsomal fractions were isolated, by the method described previously (13), from homogenates of male Sprague Dawley rats (250–300 g). Hog liver microsomes were generously provided by Professor D. M. Ziegler (the University of Texas at Austin). Purified hog and rat liver flavin-containing monooxygenase was obtained by a slight modification of the method of Sabourin *et al.* (14). Purified hog and rat liver flavin-containing monooxygenase appeared as one band and was homogeneous, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To minimize inactivation of the flavin-containing monooxygenase, all procedures employing the enzymes were carried out as quickly as possible at 4°.

Metabolic incubations and product analyses. The metabolism incubation medium contained 50 mM potassium phosphate, pH 8.0, 0.5 mM $NADP^+$, 2.0 mM glucose-6-phosphate, 1 IU of glucose-6-phosphate dehydrogenase, and 2.1 and 0.3 mg of rat and hog liver microsomal protein, respectively, or, alternatively, 80 and 40 μg of purified rat and hog liver flavin-containing monooxygenase, respectively. In every case the total volume of the reaction was 0.5 ml. After a brief equilibration, the incubation was initiated by addition of substrate and continued with constant shaking at 33°. At time intervals (i.e., generally 3 min), the reaction was stopped and analyzed for products as described below.

For assays performed with [^{3}H]verapamil, the reaction was stopped by the addition of 2 volumes of cold methanol containing 1.0 mg of nor-verapamil, 0.75 mg of verapamil, and 0.5 mg of 3,4-dimethoxystyrene. The mixture was maintained at 45–50° for 15 min to afford quantitative Cope elimination of the *N*-oxide to 3,4-dimethoxystyrene. After thorough mixing, insoluble material was separated by brief centrifugation. A 50-μl aliquot of the supernatant was applied to the loading zone of a Whatman LK5DF TLC plate and air dried for about 5 min. The plate was developed in dichloromethane/methanol (98:2, v/v). The band corresponding to 3,4-dimethoxystyrene (R_f 0.83) was scraped into scintillation vials for counting. The plate was developed again two more times and the bands corresponding to verapamil (R_f 0.27) and nor-verapamil (R_f 0.12) were scraped into scintillation vials for counting. Attentive work-up of the TLC plates avoids any possible loss due to evaporation. Based on the specific activity of 3,4-dimethoxystyrene and verapamil, greater than 95% of the radioactivity that was applied to the TLC plate was recovered. The specific activity of 3,4-dimethoxystyrene was determined by careful measurement of the specific activity of tritiated 3,4-dimethoxystyrene (chemically obtained via Cope elimination of tritiated verapamil *N*-oxide). In good agreement with the deuterium exchange studies (Table 1), approximately 58% of the tritium label remained with 3,4-dimethoxystyrene. This value was used in further determinations of metabolic product conversion.

The profile of metabolites was also determined by HPLC analysis of dichloromethane extracts of the reaction mixture, following the general procedure previously described (7, 13). The reaction was stopped by adding 4 volumes of cold dichloromethane and the resulting mixture was heated at 45–50° for 15 min to afford quantitative Cope elimination of verapamil *N*-oxide to 3,4-dimethoxystyrene. After a brief centrifugation, the dichloromethane fraction was passed through a nylon filter, evaporated, and reconstituted with methanol for separation and quantitation by HPLC (IBM model 9000 with a UV detector at 260 nm, fitted with a C-18 precolumn and 5-μm Altex Ultrasphere-ODS reverse phase analytical column). The mobile phase consisted of acetonitrile/50 mM citrate buffer (90:10, v/v) adjusted to pH 4, containing 0.5 mM *N,N*-dimethyloctylamine. This system separates verapamil, 3,4-dime-

thoxystyrene, and nor-verapamil, which have retention volumes of 3.5, 4.1, and 7.32 ml, respectively. Control experiments demonstrated that the recovery of metabolites as judged by HPLC was 95% and 90% of this recovered material was verapamil, 3,4-dimethoxystyrene, or nor-verapamil.

The major metabolite formed in reactions catalyzed by hog liver microsomes was subjected to chemical analysis. The metabolite isolated from large scale incubations was extracted into dichloromethane. The combined extracts were placed on a silica gel preparative TLC plate and developed with ethyl acetate/hexane (30:70, v/v). A major nonpolar UV-active material was isolated. The sample was dissolved in methanol and the UV spectrum revealed a peak at 262 nm and a shoulder at 295 nm. The Fourier transform IR spectra showed bands at 2945, 1609, 1510, 1420, and 1026 cm^{-1}. The EI mass spectra showed a molecular ion *m/z* 164 and prominent ions at 149 and 121. The observed spectra for the isolated metabolite were identical with those for authentic 3,4-dimethoxystyrene.

Other analytical methods. ^1H NMR spectra were recorded on a Varian FT 80 or a General Electric 500 MHz spectrometer. UV spectra were recorded on a Perkin Elmer 559A spectrometer. Liquid secondary ion mass spectra were taken on a Kratos MS 50 that was fitted with a cesium gun. Electron impact spectra were taken with a Kratos MS 25 at 6 kV and a source temperature of 50°. IR spectra were obtained with a Nicolet 5DX Fourier transform IR spectrometer.

Heat inactivation of hog or rat liver microsomes was accomplished by purging the protein with an atmosphere of argon and placing the protein in a bath of 55° water for 60 sec in the absence of NADPH (7, 10).

The concentration of protein was determined by the method of Lowry *et al.* (15).

Results

Chemical oxidation of verapamil. The chemical oxidation of verapamil was studied in order to characterize the chemical properties of the products formed and the mechanism involved. Treatment of verapamil with *meta*-chloroperbenzoic acid produces verapamil *N*-oxide in good yield (see Materials and Methods). Unlike many tertiary amine *N*-oxides, verapamil *N*-oxide undergoes Cope elimination to yield the hydroxylamine, compound **3**, and 3,4-dimethoxystyrene, compound **4** (Fig. 1). A major product detected in dichloromethane extracts of reactions of verapamil *N*-oxide and aqueous buffer eluted identically with authentic 3,4-dimethoxystyrene upon separation by TLC. The EI mass spectrum of this material gave prominent ions at *m/z* (relative abundance) 165 (MH^+, 14), 164 (M^+, 100), and 149 (M^+-CH_3, 45). This EI mass spectrum is virtually identical to that of authentic 3,4-dimethoxystyrene. The NMR spectrum of the major product is identical to that of 3,4-dimethoxystyrene (Fig. 2). No attempt was made to characterize the other products of the reaction. The reaction of verapamil *N*-oxide with various aqueous buffers was investigated. It was determined that basic buffers (i.e., pH 10.0) were more effective at catalyzing the formation of 3,4-dimethoxystyrene from verapamil *N*-oxide.

Verapamil metabolism. The biotransformation of verapamil was studied *in vitro*. When hog or rat liver microsomes, supplemented with an NADPH-generating system, were used, dichloromethane extracts of reactions of verapamil gave a product that eluted with retention volume identical to that of authentic verapamil *N*-oxide, upon separation by HPLC. However, the time course for formation of verapamil *N*-oxide was highly capricious. It was apparent that nonlinear product formation was due to the instability of verapamil *N*-oxide (see above). Although verapamil *N*-oxide formation could be quantitated variable amounts of decomposition to 3,4-dimethoxystyrene during the course of the metabolic reaction was observed. That efficient Cope elimination of verapamil *N*-oxide to 3,4-dimethoxystyrene was observed prompted the design of a new method to analyze verapamil metabolism. Consequently, a radiometric procedure was developed to quantitate 3,4-dimethoxystyrene.

As shown in Table 1, efficient acid-catalyzed deuterium exchange of *ortho*- and *para*-aromatic ring protons of verapamil was accomplished. When the reaction was repeated with ^3H_2O, efficient tritium exchange of verapamil was apparent. Except as noted, the following studies were performed with [^3H]verapamil employing the radiometric procedure described above. Preliminary studies showed that rat and hog liver microsomes supplemented with NADPH catalyze formation of 3,4-dimethoxystyrene. In addition, some nor-verapamil could be detected, but its rate of formation was usually 4 times slower under the same normal incubation conditions (Tables 2 and 3). The formation of 3,4-dimethoxystyrene and nor-verapamil was a linear function of protein concentration (0.3–2 mg of protein) and with incubation time for at least 5 min.

As shown in Table 2, *n*-octylamine-treated microsomes stimulate the formation of 3,4-dimethoxystyrene. These results suggest that 3,4-dimethoxystyrene formation is a result of verapamil *N*-oxygenation, because *n*-octylamine is a good inhibitor of cytochrome P-450 (16) and a positive effector for flavin-containing monooxygenase activity (10). Heat inactivation of the microsomes under conditions that completely destroy flavin-containing monooxygenase and that leave the cytochromes P-450 almost intact (10, 13) significantly decreases 3,4-dimethoxystyrene formation. Metabolism inhibitors were employed in order to further distinguish between cytochrome P-450- and flavin-containing monooxygenase-mediated verapamil *N*-oxygenation. Thiourea, an alternate substrate competitive inhibitor of the flavin-containing monooxygenase (10), markedly inhibited 3,4-dimethoxystyrene but not nor-verapamil formation. On the other hand, aminobenzotriazole, a potent mechanism-based inactivator of cytochromes P-450 (17), did not inhibit the formation of 3,4-dimethoxystyrene while it drastically reduced nor-verapamil formation. Data from Table 2 suggest that the flavin-containing monooxygenase is largely responsible for 3,4-dimethoxystyrene formation.

Data in Table 3 show that 3,4-dimethoxystyrene and nor-verapamil formation do not show parallel responses after various changes in incubation conditions. Whereas a typical pattern of flavin-containing monooxygenase involvement may be seen for 3,4-dimethoxystyrene formation, nor-verapamil formation is largely due to cytochromes P-450.

Kinetic constants for 3,4-dimethoxystyrene and nor-verapamil formation by hog and rat liver microsomes were calculated from the rate of 3,4-dimethoxystyrene or nor-verapamil formation, respectively, at variable substrate concentrations (i.e., 8–400 μM verapamil) by the radiometric procedure described in Materials and Methods. The K_m and V_{max} values obtained from double-reciprocal plots of velocity versus substrate are listed in Table 4.

As shown by the kinetic constants of Table 4, verapamil is also a substrate for the purified hog and rat liver flavin-containing monooxygenase. The K_m values listed in Table 4 are in the range observed for other tertiary amine substrates for this enzyme (10, 18). The V_{max} values are consistent with

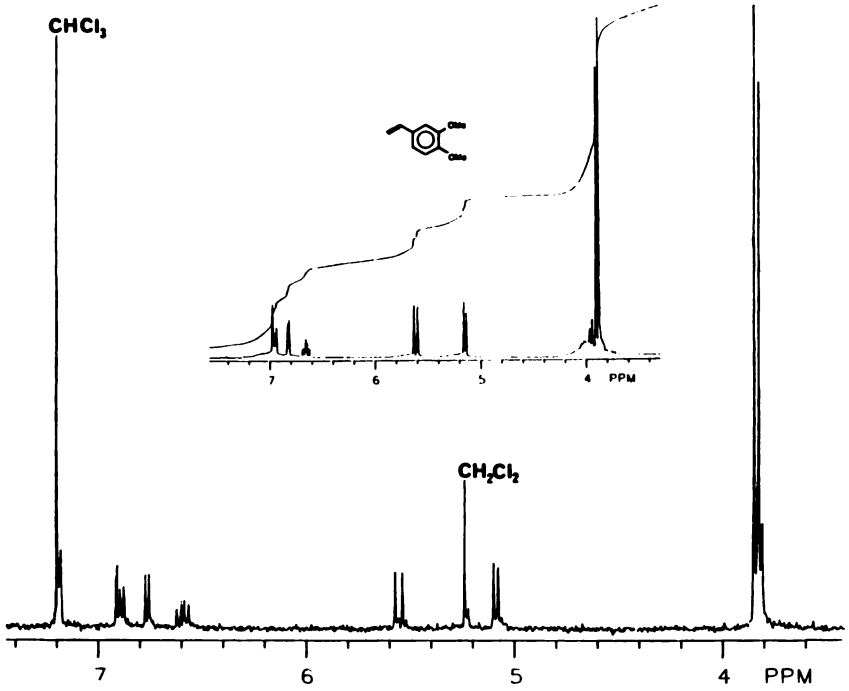

Fig. 2. 500 MHz spectrum of the Cope elimination reaction product of verapamil *N*-oxide. *Inset*, authentic 3,4-dimethoxystyrene.

TABLE 1

Distribution of deuterated species of verapamil

The acid-catalyzed deuterium exchange reactions at various temperatures are described in Materials and Methods. Values reported represent the average of at least three scans. The values for d_1, d_2 . . . d_n are nominal and have not been corrected for contributions due to ignore the natural occurrence of ^{13}C.

Condition	Relative intensity[a]						
	d_0 (*m/z* 455)	d_1 (*m/z* 456)	d_2 (*m/z* 457)	d_3 (*m/z* 458)	d_4 (*m/z* 459)	d_5 (*m/z* 460)	d_6 (*m/z* 461)
Control[b]	71.4	22.5	6.0				
60°	ND[c]	1.9	53.0	37.3	7.7		
150°	ND	0.5	6.3	31.2	37.0	19.1	5.8
205°[d]	ND	0.3	5.4	33.7	41.4	17.5	1.7

Condition	Relative intensity			
	d_0 (*m/z* 303)	d_1 (*m/z* 304)	d_2 (*m/z* 305)	d_3 (*m/z* 306)
Control	84.3	14.5	1.2	
60°	26.9	70.1	3.0	
150°	25.3	43.4	23.1	7.2
205°	16.2	49.3	25.6	8.9

[a] Analysis performed by liquid secondary ion mass spectrometry using a thioglycerol matrix in the positive mode; *m/z* is reported as MH^+.
[b] Undeuterated standards.
[c] ND, not detectable.
[d] Significant decomposition of starting material observed.

previous observations, which suggest that rat liver flavin-containing monooxygenase *N*-oxygenation is less efficient for rat liver than for the hog liver enzyme (19) and that bulky amine substituents may decrease the velocity of the reaction (10). The major products detected in dichloromethane extracts of verapamil oxygenation catalyzed by the purified hog and rat liver flavin-containing monooxygenase eluted with retention volume identical with that of authentic 3,4-dimethoxystyrene, upon separation by HPLC.

Stereoselectivity in verapamil metabolism. The stereoselectivity of verapamil *N*-demethylation and *N*-oxygenation was investigated in order to determine the enzymes involved and the preferred orientation of the substrates. There is a marked preference for *N*-oxygenation of (*S*)-verapamil, when extracts of reactions catalyzed by hog and rat liver microsomes were analyzed by HPLC. As shown in Table 5, the ratio of (*S*)-verapamil to (*R*)-verapamil that is *N*-oxygenated by hog and rat liver microsomes was 4.1 and 3.1, respectively. In marked contrast, the enantioselectivity of verapamil *N*-demethylation was quite modest. As shown in Table 5, there is almost equal preference for (*S*)- or (*R*)-verapamil to be converted to (*S*)- or (*R*)-nor-verapamil.

The stereoselectivity of (*R*)- and (*S*)-verapamil *N*-oxygenation was also examined with purified hog and rat liver flavin-containing monooxygenase. As shown in Table 5, there is a large degree of *N*-oxygenation stereoselectivity. For the purified hog and rat liver flavin-containing monooxygenase, there was a 10.1- and 6.6-fold *N*-oxygenation of (*S*)- in preference to (*R*)-verapamil. The stereoselectivity for *N*-oxygenation can be seen more clearly by an examination of the kinetic constants for the reaction (Table 6).

As shown by the kinetic constants listed in Table 6, (*S*)-

TABLE 2

Effect of metabolism inhibitors on hepatic microsomal verapamil N-oxygenation

The complete system contained 50 mM phosphate, pH 7.4, the NADPH-generating system, 400 μM [^{3}H]verapamil, and 0.4–2.0 mg of microsomal protein. 3,4-Dimethoxystyrene was determined by the radiometric procedure. The results are averages of four determinations (± standard deviations) for reactions performed for 3 min.

Description	3,4-Dimethoxystyrene formed	
	Rat liver microsomes	Hog liver microsomes
	nmol/min/mg of protein	
Complete system	4.1 ± 0.6	12.1 ± 1.1
− NADPH-generating system	0.5 ± 0.1	1.9 ± 0.3
+ Heat inactivation	0.2 ± 0.1	1.4 ± 0.3
+ *n*-Octylamine (4.5 mM)	5.5 ± 0.4	34.6 ± 1.8
+ Thiourea (500 μM)	0.8 ± 0.2	3.4 ± 0.6
+ Aminobenzotriazole (500 μM)[a]	4.3 ± 0.7	12.2 ± 1.1

[a] The inhibitor was preincubated with microsomes containing the NADPH-generating system for 5 min.

TABLE 3

Effect of metabolism inhibitors on hepatic microsomal verapamil N-demethylation

The complete system is as described in Table 2. Desmethyl verapamil was determined by the radiometric procedure. The results are averages of four determinations (± standard deviations) for reactions of 3 min.

Description	Desmethyl verapamil formed	
	Rat liver microsomes	Hog liver microsomes
	nmol/min/mg of protein	
Complete system	1.0 ± 0.2	3.0 ± 0.7
− NADPH-generating system	0.1[a]	0.4 ± 0.1
+ Heat inactivation	1.5 ± 0.3	4.5 ± 0.8
+ *n*-Octylamine (4.5 mM)	0.2[a]	0.4 ± 0.1
+ Thiourea (500 μM)	1.1 ± 0.2	2.5 ± 0.7
+ Aminobenzotriazole (500 μM)[b]	0.2[a]	0.3 ± 0.1

[a] The range of values was 0–0.3 nmol/min/mg of protein.
[b] The inhibitor was preincubated with microsomes containing NADPH-generating system for 5 min.

TABLE 4

Kinetic constants for verapamil N-oxygenation and N-demethylation

N-oxygenation and *N*-demethylation were determined by the amount of 3,4-dimethoxystyrene or desmethylverapamil, respectively, that is formed by using the radiometric procedure.

Enzyme preparation	*N*-Oxygenation		*N*-Demethylation	
	K_m	V_{max}	K_m	V_{max}
	μM	*nmol/min/mg of protein*	μM	*nmol/min/mg of protein*
Hog liver microsomes	86.9	13.0	101	2.9
Rat liver microsomes	100	3.8	106	1.0
Hog liver FMO[a]	64.4	325		
Rat liver FMO	51	125		

[a] Flavin-containing monooxygenase, purified enzyme.

TABLE 5

Enantioselectivity of (*R*)- and (*S*)-verapamil metabolism

Enzyme preparation	*S/R* Ratio[a]	
	3,4-Dimethoxystyrene	Nor-verapamil
Hog liver microsomes	4.12	1.03
Rat liver microsomes	3.13	1.06
Hog liver FMO[b]	10.11	
Rat liver FMO	6.57	

[a] Average of three determinations by the reverse phase HPLC method. (*R*)- and (*S*)-verapamil (500 μM) were incubated separately.
[b] Flavin-containing monooxygenase, purified enzyme.

TABLE 6

Kinetic constants for the enantioselective metabolism of (*R*)- and (*S*)-verapamil

Kinetic constants were determined by the radiometric method. The results are the average of two determinations.

Enzyme preparation	3,4-Dimethoxystyrene		Desmethyl verapamil	
	K_m	V_{max}	K_m	V_{max}
	μM	*nmol/min/mg of protein*	μM	*nmol/min/mg of protein*
Hog liver microsomes				
+ (*R*)-Verapamil	166	1.5	100	2.2
+ (*S*)-Verapamil	25	10.6	105	3.0
Rat liver microsomes				
+ (*R*)-Verapamil	181	1.8	111	0.4
+ (*S*)-Verapamil	23	4.0	108	0.5
Hog liver FMO[a]				
+ (*R*)-Verapamil	187	390		
+ (*S*)-Verapamil	33	455		
Rat liver FMO				
+ (*R*)-Verapamil	167	110		
+ (*S*)-Verapamil	25	122		

[a] Flavin-containing monooxygenase, purified enzyme.

verapamil is a better substrate for the hog and rat liver microsomal *N*-oxygenase than is (*R*)-verapamil. In contrast, no apparent difference in the kinetic constants for hog and rat liver microsomal *N*-demethylation is apparent. As shown in Table 6, the concentration for half-maximal *N*-oxygenation catalyzed by the flavin-containing monooxygenase for (*S*)-verapamil is significantly lower than that for (*R*)-verapamil. The V_{max} values of the enantiomers are essentially the same, in agreement with previous studies on the mechanism of the flavin-containing monooxygenase (10).

Discussion

Racemic verapamil undergoes extensive metabolism in humans and animals. The percentage of a verapamil dose that is excreted in the urine of humans and animals as unchanged drug is small (1–5%) (3, 4), but, to date, only about 55% of the metabolized drug has been accounted for in terms of identifiable metabolites (2–5). In view of the fact that a high amount of verapamil C-N-C bond cleavage products (i.e., 60–65% of urinary [^{14}C]verapamil) is rather unusual and *N*-demethylation of verapamil (i.e., 20% of urinary [^{14}C]verapamil) is rather unusual and *N*-demethylation of verapamil (i.e., 20% of urinary [^{14}C]verapamil) is a minor route of metabolism, the oxidative biotransformation of verapamil was investigated *in vitro*. The metabolism of verapamil with rat and hog liver microsomes and purified hog and rat liver flavin-containing monooxygenase was investigated in order to determine the products formed and the enzymes involved in its biotransformation.

The possible reason that a greater percentage of verapamil metabolites have not been identified was immediately apparent from chemical investigations of the *N*-oxygenation of verapamil. Although verapamil *N*-oxide is readily synthesized and fully characterized, it does not have an indefinite stability. In the presence of protic solvents and neutral pH, verapamil *N*-oxide gradually decomposes to 3,4-dimethoxystyrene. Although studies are limited, in aqueous solution this Cope-type elimination is probably a bimolecular reaction involving base catalysis (20) (i.e., the reaction is faster at pH 10 than it is at pH 8), although in organic solvents use of heat efficiently promotes the conversion of verapamil *N*-oxide to 3,4-dimethoxystyrene (7). Although the number of examples is not exhaustive, the

data obtained to date suggest that tertiary amine *N*-oxides with allylic (7) or benzylic (this study) proton activation are required for Cope-type elimination. The observation of a Cope elimination reaction for conversion of verapamil *N*-oxide to 3,4-dimethoxystyrene allowed the development of a simple yet highly efficient radiometric procedure to quantitate the metabolism of verapamil. It should be pointed out that verapamil *N*-oxide can be detected as a metabolite of verapamil *in vitro*, but its formation is variable and decomposition to 3,4-dimethoxystyrene does occur during the incubation period (data not shown).

Verapamil, as well as its individual (*R*)- and (*S*)-enantiomers, is readily tritiated employing a method that is similar to one that has been described previously (12). Employing tritiated verapamil, results shown in Table 2 indicate that verapamil is rapidly and extensively converted to verapamil *N*-oxide by microsomes supplemented with NADPH. The formation of verapamil *N*-oxide is not inhibited by cytochrome P-450 inhibitors but is stimulated by *n*-octylamine and inhibited by thiourea, an alternate substrate for the flavin-containing monooxygenase. Together, these observations suggest that verapamil is efficiently *N*-oxygenated by the flavin-containing monooxygenase *in vitro*. The data do not support a major role of cytochrome P-450 (21) in the *N*-oxygenation of verapamil but neither do they conclusively rule out such a role (22–24). With regard to *N*-demethylation of verapamil, the data clearly indicate that cytochrome P-450 is mainly responsible for this transformation (Table 3).

The kinetic constants listed in Tables 4 and 6 demonstrate that verapamil is a good substrate for the flavin-containing monooxygenase, with a K_m value similar to that of other tertiary amine substrates reported for this enzyme (10). Like other tertiary amines, verapamil can be *N*-demethylated but, as the data suggest, *N*-oxygenation is always 4–5 times faster than *N*-demethylation under the same conditions.

In humans, verapamil undergoes stereoselective first-pass metabolism (8). Although (*S*)-verapamil is 8–10 times more potent than (*R*)-verapamil with respect to its antiarrhythmic activity, the clearance of the *S*-enantiomer is considerably higher (i.e., 3-fold) than that of *R*-enantiomer (9). Because the major route of biotransformation in humans involves the unusual C-N-C cleavage (2–4), the metabolism of (*R*)- and (*S*)-verapamil was investigated *in vitro*.

The *N*-oxygenation of verapamil is highly stereoselective for the *S*-enantiomer of verapamil (Tables 5 and 6). In hog and rat liver microsomes, the *S*/*R* ratio for *N*-oxygenation was 4.1 and 3.1, respectively. Purified flavin-containing monooxygenase from hog and rat liver gave an *S*/*R* ratio for *N*-oxygenation of 10.1 and 6.6, respectively. Because the major route of biotransformation of verapamil in humans involves the unusual C-N-C cleavage, it is possible that the stereoselective C-N-C cleavage observed here for hog and rat hepatic preparations can account for the stereoselective clearance of verapamil in humans. The amount of stereoselectivity observed for verapamil *N*-oxygenation catalyzed by the flavin-containing monooxygenase is similar to that reported for other tertiary amines (11) and, although human flavin-containing monooxygenase has not been examined, it is reasonable to assume that the hog enzyme behaves in a similar fashion. Presently, it is not clear why the enantioselectivity for microsomal and purified enzyme-mediated *N*-oxygenation of verapamil is different. Presumably, nonspecific protein binding may play a role, because the K_m values obtained for verapamil *N*-oxygenation also demonstrate a significant difference between microsomal and purified preparations of the flavin-containing monooxygenase.

Known metabolites of verapamil that are formed *in vitro* by liver preparations have been previously summarized (2–4). Cytochrome P-450-catalyzed *N*-demethylation, *O*-dealkylation, and *N*-dealkylation appears to be responsible for many of the identified metabolites. At the present time, it is unclear how a number of metabolites are formed, including 2-isopropyl-2-(3-methylaminopropyl)-3,4-dimethoxyphenyl) acetonitrile (compound **6**), 5-methyl-4-cyano-4-(3,4-dimethoxyphenyl)-1-hexanol (compound **7**), and 2-(3,4-dimethoxyphenyl) ethanol (compound **8**) (Fig. 1).

One possible explanation for the formation of the rather unusual *in vivo* metabolites of verapamil stems from the observed involvement of the flavin-containing monooxygenase in verapamil metabolism. First, efficient *N*-oxygenation of verapamil produces the unstable tertiary amine *N*-oxide, compound **2**. Compound **2** undergoes Cope elimination to produce the hydroxylamine, compound **3**, and 3,4-dimethoxystyrene. Styrenes may be further biotransformed *in vivo* to phenylacetaldehydes (25) or phenylethanols (25, 26), each of which could be interconverted or further transformed to phenylacetic acids. 3,4-Dimethoxyphenylacetic acid is a major stable metabolite of verapamil (2). The hydroxylamine, compound **3**, could be reduced to the secondary amine, compound **6** (27), or it could be *N*-oxygenated by the flavin-containing monooxygenase to the unstable nitrone, compound **9**, which rapidly decomposes to the aldehyde, which is reduced to the alcohol. Ample precedent exists for this type of biotransformation (10, 27). Of course, other routes of metabolism almost certainly contribute to the formation of compounds **7** and **8**. For example, cytochrome P-450 could produce carbonyl compounds, which would be readily reduced to alcohol **7** or **8**.

Elegant stable isotope metabolism experiments have been performed with verapamil *in vivo* (4, 5, 8) and *in vitro* (4, 5). These studies demonstrate that verapamil undergoes regio- and stereoselective *N*- and *O*-demethylation in rat liver microsomes (4, 5). However, it is unlikely that the small amount of *O*-dealkylated metabolites observed accounts for the large first-pass effect observed *in vivo*. It is possible that the preferential metabolism of (*S*)-verapamil seen in humans is dependent on an as yet undiscovered metabolic pathway(s). However, the results described herein demonstrate that flavin-containing monooxygenase-mediated *N*-oxygenation is highly stereoselective and produces 3,4-dimethoxystyrene as a major initial metabolite. Overall, this constitutes an *N*-dealkylation reaction catalyzed by the flavin-containing monooxygenase. Although Cope-type elimination reactions of tertiary amine *N*-oxides have been reported previously (7, 28, 29), metabolic reactions of this type may be responsible for heretofore unrecognized metabolic pathways.

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References

1. McAllister, R. G., and E. Kirsten. The pharmacology of verapamil. IV. Kinetic and dynamic effects after single intravenous and oral doses. *Clin. Pharmacol. Ther.* **31**:418–426 (1982).
2. McIlhenny, H. M. Metabolism of [^{14}C]verapamil. *J. Med. Chem.* **14**:1178–1184 (1971).
3. Eichelbaum, M., M. Ende, G. Remberg, M. Schomerus, and H. J. Dengler. The metabolism of DL-[^{14}C]verapamil in man. *Drug Metab. Dispos.* **7**:145–148 (1979).
4. Nelson, W. L., and L. D. Olsen. Regiochemistry and enantioselectivity in the oxidative *N*-dealkylation of verapamil. *Drug Metab. Dispos.* **16**:834–841 (1988).
5. Nelson, W. L., L. D. Olsen, D. B. Beitner, and R. J. Pallow, Jr. Regiochemistry and substrate stereoselectivity of *O*-demethylation of verapamil in the presence of the microsomal fraction from rat and human liver. *Drug Metab. Dispos.* **16**:184–188 (1988).
6. Lindeke, B., and A. K. Cho. *N*-Dealkylation and deamination, in *Metabolic Basis of Detoxication* (W. Jacoby, ed.), Vol. 1. Academic Press, New York, 105–122 (1982).
7. Cashman, J. R., J. Proudfoot, D. W. Pate, and T. Högberg. Stereoselective *N*-oxygenation of zimeldine and homozimeldine by the flavin-containing monooxygenase. *Drug Metab. Dispos.* **16**:616–622 (1988).
8. Vogelgesang, B., H. Echizen, E. Schmidt, and M. Eichelbaum. Stereoselective first-pass metabolism of highly cleared drugs: studies on the bioavailability of L- and D-verapamil examined with a stable isotope technique. *Br. J. Clin. Pharmacol.* **18**:733–740 (1984).
9. Eichelbaum, M., G. Mikus, and B. Vogelgesang. Pharmacokinetics of (+)-, (−)-, and (±)-verapamil after intravenous administration. *Br. J. Clin. Pharmacol.* **17**:453–458 (1984).
10. Ziegler, D. M. Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds, in *Enzymatic Basis of Detoxication* (W. Jacoby, ed.), Vol. 1. Academic Press, New York, 201–227 (1980).
11. Damani, L. A., W. F. Pool, P. A. Crooks, R. K. Kaderlik, and D. M. Ziegler. Stereoselectivity in the *N'*-oxidation of nicotine isomers by flavin-containing monooxygenase. Mol. Pharmacol. **33**:702–705 (1988).
12. Hanzlik, R. P., R. A. Wiley, and T. J. Gillesse. Tritium and deuterium exchange: labeling of aromatic and organometallic compounds in heptafluorobutyric acid. *J. Labelled Compd. Radiopharm.* **16**:523–529 (1978).
13. Cashman, J. R., and D. M. Ziegler. Contribution of *N*-oxygenation to the metabolism of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) by various liver preparations. *Mol. Pharmacol.* **29**:163–167 (1986).
14. Sabourin, P. J., B. P. Smyser, and E. Hodgson. Purification of the flavin-containing monooxygenase from mouse and pig liver microsomes. *Int. J. Biochem.* **16**:713–720 (1984).
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
16. Jeffcoate, C. R. E., J. L. Gaylor, and R. L. Calabrese. Ligand interactions with cytochrome P-450: binding of primary amines. *Biochemistry* **8**:3455–3465 (1969).
17. Ortiz de Montellano, P. R., and J. M. Mathews. Autocatalytic alkylation of the cytochrome P-450 prothetic haem group by 1-aminobenzotriazole. *Biochem. J.* **195**:761–764 (1981).
18. Cashman, J. R. A convenient radiometric assay for flavin-containing monooxygenase activity. *Anal. Biochem.* **160**:294–300 (1987).
19. Cashman, J. R., and J. Proudfoot. A reverse-phase liquid chromatographic assay for flavin-containing monooxygenase activity. *Anal. Biochem.* **175**:274–280 (1988).
20. Cashman, J. R., and S. Pêna. Canrenone formation via general-base catalyzed elimination of 7α-(methylthiol)spironolactone *S*-oxide. *Chem. Res. Toxicol.* **2**:109–113 (1989).
21. Guengerich, F. P., and T. L. Macdonald. Chemical mechanism of catalysis by cytochrome P-450: a unified view. *Acct. Chem. Res.* **17**:9–16 (1984).
22. Duncan, J. D., and A. K. Cho. *N*-Oxidation of phentermine to *N*-hydroxyphentermine by reconstituted cytochrome P-450 system from rabbit liver. *Mol. Pharmacol.* **22**:235–238 (1982).
23. Frederick, C. B., J. B. Mays, D. M. Ziegler, F. P. Guengerich, and F. F. Kadlubar. Cytochrome P-450 and flavin-containing monooxygenase-catalyzed formation of the carcinogen *N*-hydroxy-2-amino-fluorene and its covalent binding to nuclear DNA. *Cancer Res.* **42**:2671–2677 (1982).
24. Cummings, S. W., F. P. Guengerich, and R. A. Prough. Characterization of *N*-isopropyl-*p*-hydroxymethyl benzamide formed during the oxidative metabolism of azocarbazine. *Drug Metab. Dispos.* **10**:459–464 (1982).
25. Mansuy, D., J. Leclaire, M. Fontecave, and M. Momenteau. Oxidation of monosubstituted olefins by cytochrome P-450 and heme models: evidence for the formation of aldehydes in addition to epoxides and allylic alcohols. *Biochem. Biophys. Res. Commun.* **119**:319–325 (1984).
26. Ortiz de Montellano, P. R. Control of the catalytic activity of prosthetic heme by the structure of hemoproteins. *Acct. Chem. Res.* **20**:289–295 (1987).
27. Kadlubar, F. F., E. M. McKee, and D. M. Ziegler. Reduced pyridine nucleotide-dependent *N*-hydroxylamine oxidase and reductase activities of hepatic microsomes. *Arch. Biochem. Biophys.* **156**:46–57 (1973).
28. Weli, A. M., and B. Lindeke. The metabolic fate of pargyline in rat liver microsomes. *Biochem. Pharmacol.* **34**:1993–1998 (1985).
29. Kondo, H., F. Sakamoto, Y. Inoue, and G. Tsukamoto. Studies on prodrugs. 10. Possible mechanism of *N*-dealkylation of *N*-masked norfloxacins having several active methylene groups. *J. Med. Chem.* **32**:679–682 (1989).

Send reprint requests to: John R. Cashman, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446.