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# Regioselectivity and Substrate Concentration-dependency of Involvement of the CYP2D Subfamily in Oxidative Metabolism of Amitriptyline and Nortriptyline in Rat Liver Microsomes

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## Abstract

Kinetic analysis of the metabolism of amitriptyline and nortriptyline using liver microsomes from Wistar rats showed that more than one enzyme was involved in each reaction except for monophasic amitriptyline *N*-demethylation. The $V_{max}$ values particularly in the high-affinity sites for E-10-hydroxylation of both drugs were larger than those for Z-10-hydroxylations. Their E- and Z-10-hydroxylase activities in Dark-Agouti rats, which are deficient for CYP2D1, were significantly lower than those in Wistar rats at a lower substrate concentration (5 $\mu$M). The strain difference was reduced at a higher substrate concentration (500 $\mu$M). A similar but a smaller strain difference was also observed in nortriptyline *N*-demethylase activity, and a pronounced sex difference (male > female) was observed in *N*-demethylation of both drugs in Wistar and Dark-Agouti rats. The reactions with the strain difference were inhibited concentration-dependently by sparteine, a substrate of the CYP2D subfamily, and an antibody against a CYP2D isoenzyme. The profiles of these decreased metabolic activities corresponded to that of the lower metabolic activities in Dark-Agouti rats.

These results indicated that a cytochrome P450 isozyme in the CYP2D subfamily was involved in E- and Z-10-hydroxylations of amitriptyline and nortriptyline in rat liver microsomes as a major isozyme in a low substrate concentration range. It seems likely that the CYP2D enzyme contributes to nortriptyline *N*-demethylation.

Amitriptyline and nortriptyline are tricyclic antidepressants widely used clinically. Fig. 1 shows major metabolic pathways of amitriptyline in man and rat. 10-Hydroxylated metabolites also have pharmacological activity (Nordin & Bertilsson 1995); thus, in interpreting the pharmacological activities of amitriptyline and nortriptyline, it is important to understand the kinetics of the metabolism of these drugs.

Clinical studies have suggested that pharmacokinetics of amitriptyline and nortriptyline are influenced by debrisoquine-type oxidation polymorphism. In-vitro studies using human liver microsomes (von Bahr et al 1983) also suggested participation of human debrisoquine 4-hydroxylase (CYP2D6) in nortriptyline metabolism. In the present study, oxidative metabolism of amitriptyline and nortriptyline was kinetically analysed in rat liver microsomes.

## Materials and Methods

### *Chemicals*

Amitriptyline, nortriptyline, imipramine as hydrochlorides, and sparteine sulphate were purchased from the Sigma Chemical Co. (St Louis, USA). E-10-Hydroxyamitriptyline, Z-10-hydroxyamitriptyline, E-10-hydroxynortriptyline, Z-10-hydroxynortriptyline, and *N*-desmethylnortriptyline were from H. Lundbeck A/S (Copenhagen, Denmark). NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from the Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals and solvents used were of analytical grade.

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### *Preparation of liver microsomes*

Wistar and Dark-Agouti rats of both sexes (2 months old) were obtained from the Takasugi Experimental Animal (Kasukabe, Japan) and the Japan SLC Inc. (Shizuoka, Japan), respectively. Liver microsomal fractions were prepared according to the method of Omura & Sato (1964). Protein concentrations were assayed by the method of Lowry et al (1951).

### *Incubation of microsomes with amitriptyline and nortriptyline*

A 1-mL incubation mixture contained 0·2 mg $mL^{-1}$ microsomal protein, 10 mM glucose-6-phosphate, 2 units $mL^{-1}$ glucose-6-phosphate dehydrogenase, 0·5 mM NADPH, 20 mM $MgCl_2$, and various concentrations of amitriptyline or nortriptyline in 0·154 M Tris-HCl buffer (pH 7·4). After 5 min preincubation under air at 37°C, the reaction was started by adding NADPH. The incubation was performed for 1 min and was stopped with 1 M NaOH. In kinetic studies, substrate concentrations used were 0·3–500 $\mu$M. Other studies were performed at substrate concentrations of 5 $\mu$M or 500 $\mu$M. In immunoinhibition studies, microsomes were preincubated with various amounts of antiserum against P450BTL as a CYP2D isozyme or preimmune serum at 25°C for 30 min, followed by adding other components of the incubation mixture. The characterization of P450BTL and its antibody were described elsewhere (Suzuki et al 1992).

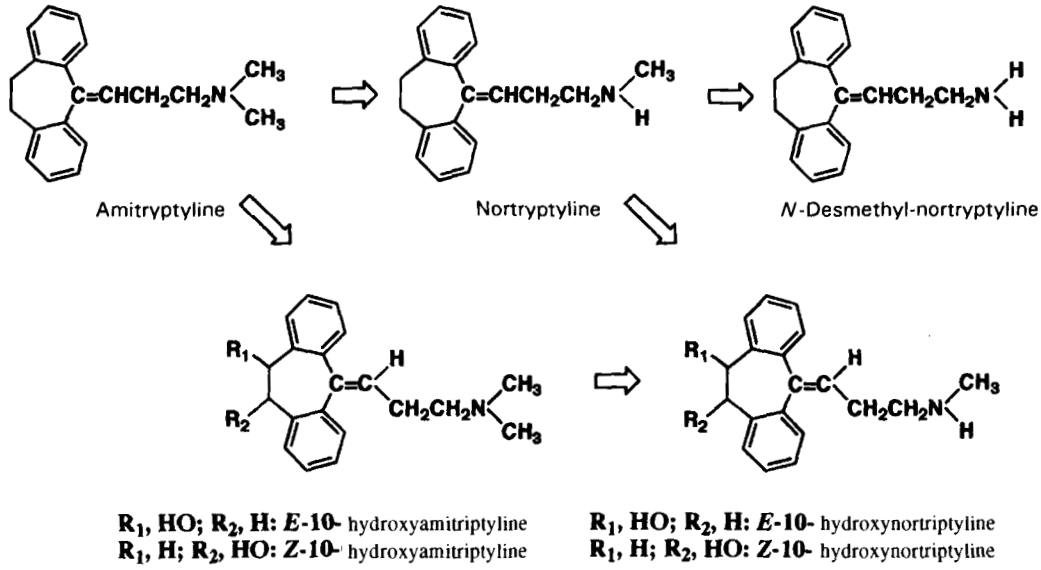


FIG. 1. Metabolic pathways of amitriptyline and nortriptyline in rat liver microsomes.

*Metabolite analysis*

After the termination of the reaction, imipramine was added as an internal standard, and amitriptyline, nortriptyline and their metabolites were extracted into ethyl acetate. The organic layer was evaporated to dryness and the residue was dissolved in 0·1 mL HPLC mobile phase.

The metabolites of amitriptyline were analysed by a reversed-phase column (Inertsil ODS, 4·6 mm i.d. × 250 mm, GL Sciences Ltd., Tokyo) at a flow rate of 1·0 min $mL^{-1}$. The mobile phase was $CH_3OH/CH_3CN/H_2O$/di-*n*-butylamine (130 : 150 : 175 : 6) adjusted to pH 7·4. These conditions allowed the separation of E-10-hydroxyamitriptyline (4·0 min), Z-10-hydroxyamitriptyline (4·6 min), nortriptyline (6·6 min), internal standard (9·5 min), and amitriptyline (11·7 min), but not E-10-hydroxynortriptyline and Z-10-hydroxynortriptyline ( < 3·0 min).

The metabolites of nortriptyline were analysed by a normal-phase column (LiChrospher Si 60, 4·6 mm i.d. × 250 mm, Kanto Kagaku Co., Tokyo) at a flow rate of 1·0 min $mL^{-1}$. The mobile phase was $CH_3CN/CH_3OH$/29% $NH_4OH$ (440 : 80 : 19). These conditions allowed the separation of internal standard (3·6 min), *N*-desmethylnortriptyline (4·5 min), nortriptyline (6·2 min), E-10-hydroxynortriptyline (8·6 min), and Z-10-hydroxynortriptyline (9·6 min), but not amitriptyline, E-10-hydroxyamitriptyline and Z-10-hydroxyamitriptyline ( < 3·0 min). UV intensities of the compounds were monitored at 239 nm.

*Data analysis*

Enzyme kinetic parameters ($K_m$ and $V_{max}$) were analysed according to a nonlinear least-squares regression analysis based on a simplex method (Yamaoka et al 1981). Best fittings of the data were performed by weighting them with the reciprocal of their square. Statistical significance was calculated by Student's *t*-test.

## Results and Discussion

Because linearity of the formation of the primary metabolites versus incubation time was observed up to 1·5 min (data not shown), the kinetic studies were performed with the incubation time of 1 min. Typical Eadie-Hofstee plots of E-10-hydroxylase activities of amitriptyline and nortriptyline assayed at substrate concentrations of 0·3–500 μM are shown in Fig. 2. The plots demonstrated that the kinetics was not monophasic, indicating more than one enzyme was involved. Similar results were obtained in amitriptyline and nortriptyline Z-10-hydroxylations, and nortriptyline *N*-demethylation, but not in amitriptyline *N*-demethylation.

Table 1 lists kinetic parameters of the activities analysed with one or sum of two Michaelis–Menten equations. The $K_m$ values for both amitriptyline and nortriptyline 10-hydroxylase activities were different by two orders of magnitude between high- and low-affinity components, whereas the $K_m$ values were similar between E- and Z-10-hydroxylase activities. The $V_{max}$ values for amitriptyline and nortriptyline E-10-hydroxylase activities were much larger than those for the corresponding Z-10-hydroxylase activities. The stereoselectivity in the $V_{max}$ values was more pronounced in the high-affinity component.

The high-affinity component for 10-hydroxylation was responsible for over 90% of total activity at a substrate concentration of 5 μM, whereas high- and low-affinity components almost equally contributed at 500 μM.

The contribution of the CYP2D subfamily to the metabolism of amitriptyline and nortriptyline was evaluated with Dark-Agouti rats, poor metabolizers of debrisoquine (Al-Dabbagh et al 1981). Fig. 3 shows sex and strain differences in amitriptyline metabolism in rat liver microsomes. Amitriptyline E-10-hydroxylase activities in Dark Agouti rats were significantly lower than those in Wistar rats for the corresponding sexes at a lower substrate concentration. A similar strain difference was observed for amitriptyline Z-10-hydroxylation, but

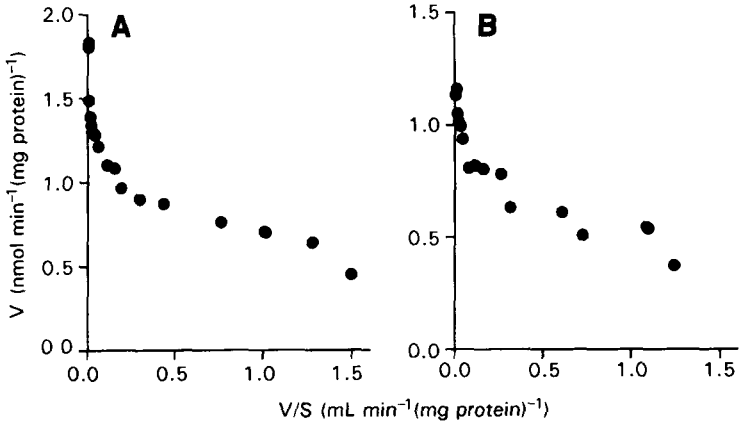


FIG. 2. Eadie-Hofstee plots for E-10-hydroxylase activities of amitriptyline and nortriptyline in rat liver microsomes from male Wistar rats. Substrates used are amitriptyline (A) and nortriptyline (B) at a concentration range of 0·3–500 μM. Plots are typical results of 3 rats..

Table 1. Kinetic parameters for oxidative metabolism of amitriptyline and nortriptyline in liver microsomes of male Wistar rats.

| Reaction | $K_{m1}$ (μM) | $V_{max1}$ (nmol $min^{-1}$ (mg protein)$^{-1}$) | $K_{m2}$ (μM) | $V_{max2}$ (nmol $min^{-1}$ (mg protein)$^{-1}$) |
|---|---|---|---|---|
| Amitriptyline | | | | |
| E-10-hydroxylation | 0·357 ± 0·079 | 0·886 ± 0·087 | 62·3 ± 26·3 | 0·493 ± 0·076 |
| Z-10-hydroxylation | 0·291 ± 0·092 | 0·123 ± 0·018 | 81·2 ± 11·8 | 0·227 ± 0·047 |
| *N*-demethylation | 11·4 ± 1·5 | 11·6 ± 1·60 | | |
| Nortriptyline | | | | |
| E-10-hydroxylation | 0·335 ± 0·075 | 0·941 ± 0·197 | 42·4 ± 16·1 | 0·443 ± 0·049 |
| Z-10-hydroxylation | 0·464 ± 0·256 | 0·162 ± 0·046 | 24·5 ± 8·9 | 0·106 ± 0·010 |
| *N*-Demethylation | 0·579 ± 0·309 | 0·204 ± 0·036 | 131 ± 20 | 2·45 ± 0·30 |

Mean ± s.e. of three determinations.

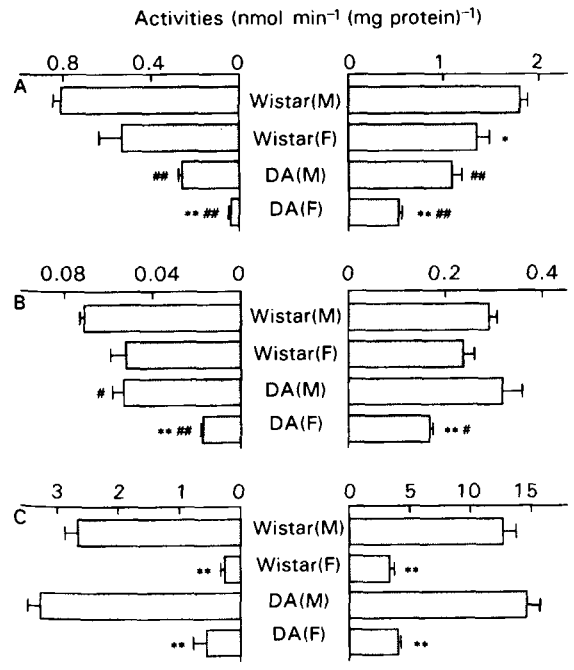


FIG. 3. Strain and sex differences in amitriptyline metabolism in rat liver microsomes from Wistar and Dark-Agouti (DA) rats. M and F indicate male and female, respectively. Amitriptyline E-10-hydroxylase (A), Z-10-hydroxylase (B) and *N*-demethylase (C) activities were assayed at substrate concentrations of 5 μM (left panel) and 500 μM (right panel). Each value represents the mean ± s.e. of three determinations. $*P < 0.05$, $**P < 0.01$ compared with males for the corresponding strains. $\#P < 0.05$, $\#\#P < 0.01$ compared with Wistar rats for the corresponding sexes.

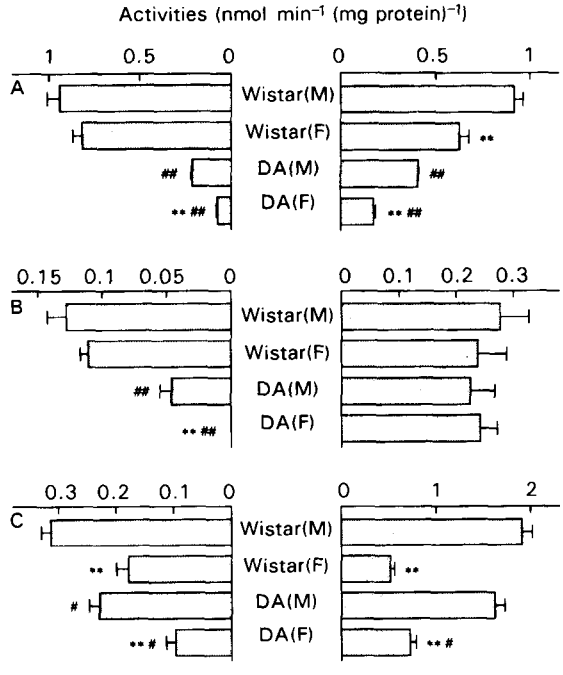


FIG. 4. Strain and sex differences in nortriptyline metabolism in rat liver microsomes from Wistar and Dark-Agouti (DA) rats. M and F indicate male and female, respectively. Nortriptyline E-10-hydroxylase (A), Z-10-hydroxylase (B) and *N*-demethylase (C) activities were assayed at substrate concentrations of 5 μM (left panel) and 500 μM (right panel). Each value represents the mean ± s.e. of three determinations. $**P < 0.01$ compared with males for the corresponding strains. $\#P < 0.05$, $\#\#P < 0.01$ compared with Wistar rats for the corresponding sexes.

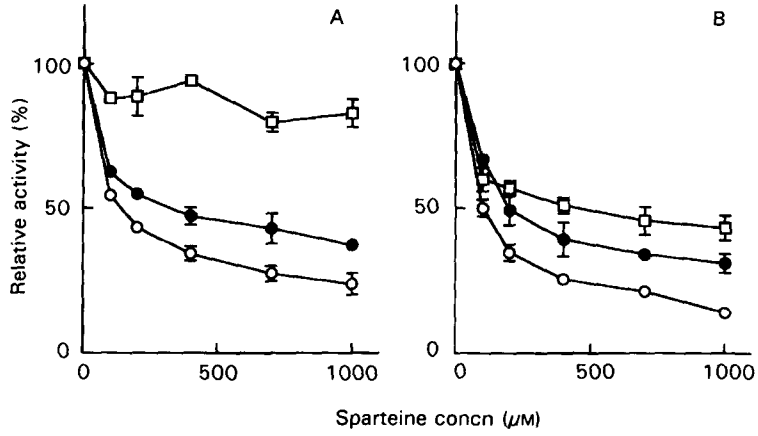


FIG. 5. Effect of sparteine on metabolism of amitriptyline and nortriptyline in liver microsomes from Wistar rats. E-10-hydroxylase (○), Z-10-hydroxylase (●), and *N*-demethylase (□) activities of amitriptyline (A) and nortriptyline (B) are expressed as per cent of the corresponding control activities measured without sparteine. Each value represents the mean ± s.e. of three determinations.

to a lesser extent. At a higher substrate concentration, the strain difference in E- and Z-hydroxylations was reduced. Although there was no strain difference, there was a sex difference for amitriptyline *N*-demethylation (Fig. 3).

For nortriptyline metabolism (Fig. 4), a marked strain difference was observed in E-10-hydroxylation at both substrate concentrations and Z-10-hydroxylation at 5 μM like amitriptyline, but not at 1 mM. Contrary to the results of amitriptyline, a significant strain difference in addition to a sex difference was observed in nortriptyline *N*-demethylation at a substrate concentration of 5 μM.

The regioselective contribution of the CYP2D enzyme to oxidative metabolism of amitriptyline and nortriptyline was confirmed by the inhibition studies with sparteine, a substrate of the CYP2D subfamily and an antibody against a CYP2D enzyme at a lower substrate concentration. Sparteine inhibited amitriptyline E- and Z-10-hydroxylations concentration-dependently, but not *N*-demethylation; nortriptyline *N*-demethylation as well as 10-hydroxylation was inhibited by sparteine (Fig. 5). Preincubation of liver microsomes from male Wistar rats with an anti-CYP2D antibody (Fig. 6) resulted in inhibition of amitriptyline E-10-hydroxylase activity in a concentration-dependent manner. The activity was inhibited by 90% at an antibody/microsomes protein ratio of 7·5. Amitriptyline Z-10-hydroxylase activity was also inhibited by the antibody but less effectively, whereas amitriptyline *N*-demethylase activity was not affected. Similarly, nortriptyline Z-10-hydroxylation as well as E-10-hydroxylation was markedly suppressed by anti-CYP2D antibody (Fig. 7). Contrary to the results of amitriptyline, nortriptyline *N*-demethylation was also suppressed by the antibody, but the extent of the inhibition was only about 30% even at an antibody/microsome protein ratio of 10·0.

Marked sex differences were observed in *N*-demethylation of amitriptyline and nortriptyline, suggesting that male specific isozymes such as CYP2C11 and CYP3A2 are involved in these reactions. We have previously demonstrated that a male specific isozyme, CYP2C11, has a high activity for amitriptyline *N*-demethylation (Fujita et al 1989). It is possible that the contribution of CYP2C11 to amitriptyline *N*-demethylation was so large that the high-affinity site (the activity of the CYP2D subfamily) could not be detected.

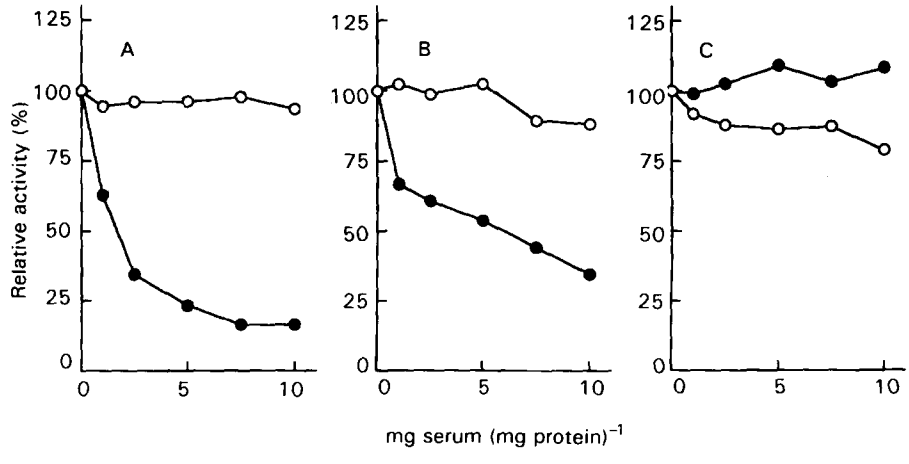


FIG. 6. Effect of anti-CYP2D antibody on amitriptyline metabolism in liver microsomes from male Wistar rats. Liver microsomes from male Wistar rats were preincubated with antiserum against CYP2D (●) or preimmune serum (○) at 25°C for 30 min followed by incubation with amitriptyline (5 μM). E-10-hydroxylase (A), Z-10-hydroxylase (B), and *N*-demethylase (C) activities are expressed as per cent of the corresponding control activities measured without the antibody. Each value represents the mean of two determinations.

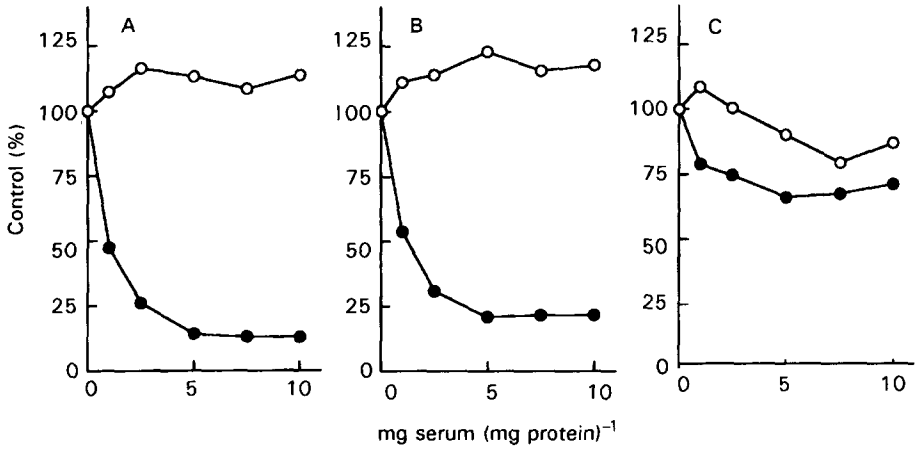


FIG. 7. Effect of anti-CYP2D antibody on nortriptyline metabolism in liver microsomes from male Wistar rats. Liver microsomes were preincubated with antiserum against CYP2D (●) or preimmune serum (○) at 25°C for 30 min, followed by incubation with nortryptyline (5 μM). E-10-hydroxylase (A), Z-10-hydroxylase (B), and *N*-demethylase (C) activities are expressed as per cent of the corresponding control activities measured without the antibody. Each value represents the mean of two determinations.

In clinical studies, CYP2D6 has been reported to be involved in E-10-hydroxylation of amitriptyline and nortriptyline, but not in Z-10-hydroxylation; however, no reliable in-vitro evidence using human liver microsomes is available.

Previous in-vitro studies (Dahl et al 1991; Pfandl et al 1992) have detected E- but not Z-10-hydroxylation of nortriptyline, probably because of its low activity. The present study has demonstrated that a rat CYP2D isozyme was involved in both E- and Z-10-hydroxylations. It is considered that the catalytic activity is much higher for E-10-hydroxylation than for Z-10-hydroxylation, resulting in the marked stereoselectivity in 10-hydroxylation of amitriptyline and nortriptyline in rat liver microsomes.

We conclude that more than one enzyme participates in the oxidative metabolism of amitriptyline and nortriptyline in rat liver microsomes, and that amitriptyline and nortriptyline are useful substances in studying the stereochemistry of the CYP2D isozymes of the rat and the man.

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