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KINETIC PROPERTIES OF THE METABOLISM OF IMIPRAMINE AND DESIPRAMINE IN ISOLATED RAT HEPATOCYTES

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Abstract—The metabolism of imipramine and desipramine was examined by using isolated rat hepatocytes. The enzyme systems having high-affinity-and-low-capacity and low-affinity-and-high-capacity kinetic properties were found to catalyze aromatic 2-hydroxylations of imipramine and desipramine, and aliphatic N-demethylation of imipramine, respectively. The K_m and V_{max} values for N-demethylation of imipramine (which formed desipramine) were about 5–10 and 5 times larger than those of both 2-hydroxylations respectively. A competitive inhibition between the 2-hydroxylations of imipramine and desipramine ("parallel pathway interaction") (Chiba M, Fujita S and Suzuki T, *J Pharm Sci* 77: 944–947, 1988), observed using liver microsomes, was found also in isolated hepatocytes. It was concluded that the characteristics of imipramine metabolism observed in liver microsomes were well reproduced in isolated rat hepatocytes.

Enzymological analysis of drug metabolism using liver microsomes or isolated hepatocytes is a powerful method to interpret the phenomena observed in *in vivo* pharmacokinetics. Based on comparisons of drug metabolism in perfused liver preparations with metabolism in liver microsomes and in isolated hepatocytes, Billings *et al.* [1] suggested that drug metabolism in isolated hepatocytes correlates better with *in vivo* drug metabolism than does metabolism in microsomes. The liver microsomal assay system for oxidative drug metabolism is an artificial system composed of NADPH-generating system assuring unlimited supply of NADPH during the reaction period and of exogenous divalent cations in an artificial buffer solution in the absence of cofactors for conjugating enzymes. Compared to drug metabolism with isolated hepatocytes, the microsomal assay system lacks plasma membrane, conjugation enzyme activities, the rate-determining step in the electron supply, and endogenous cofactor such as divalent cations. Therefore, information obtained from studies with liver microsomes including the kinetic parameters is not necessarily in accord with that obtained from isolated hepatocytes [2–4]; *in vitro* data obtained from isolated rat hepatocytes could give more realistic information about the kinetic nature of drug metabolism in the liver compared to that obtained from a subcellular fraction such as microsomes.

It was found in an isolated perfused rat liver preparation that the metabolism of a widely used tricyclic antidepressant, imipramine, in the liver does not proceed as a linear function of dose even within the therapeutic concentration range [5]. Imipramine is metabolized extensively in rat liver by cytochrome P-450 to form either desipramine by aliphatic N-demethylation or 2-hydroxyimipramine (2OH-IMI) by aromatic 2-hydroxylation. These primary metabolites are further 2-hydroxylated or N-demethylated, respectively, forming a common secondary metabolite, 2-hydroxydesipramine (2OH-DMI). The hydroxylated metabolites of imipramine and desipramine are conjugated to glucuronides (Scheme 1).† Competitive metabolic interaction has been observed between imipramine 2-hydroxylation and desipramine 2-hydroxylation in rat liver microsomes, suggesting that this may cause in part, a nonlinear disposition of imipramine [6].

In this study, in order to elucidate the cause of dose-dependent imipramine elimination observed in rats [5], we employed an *in vitro* approach using isolated rat hepatocytes. They were used to study the kinetic nature of the enzymes involved in imipramine metabolism, and also to investigate whether the microsomal observations ("parallel pathway interaction") obtained in our previous studies using rat liver microsomes [6] can also be observed in living cells.

MATERIALS AND METHODS

Materials. Imipramine hydrochloride, desipramine hydrochloride, nortriptyline hydrochloride, and β-glucuronidase (type H-1) were obtained

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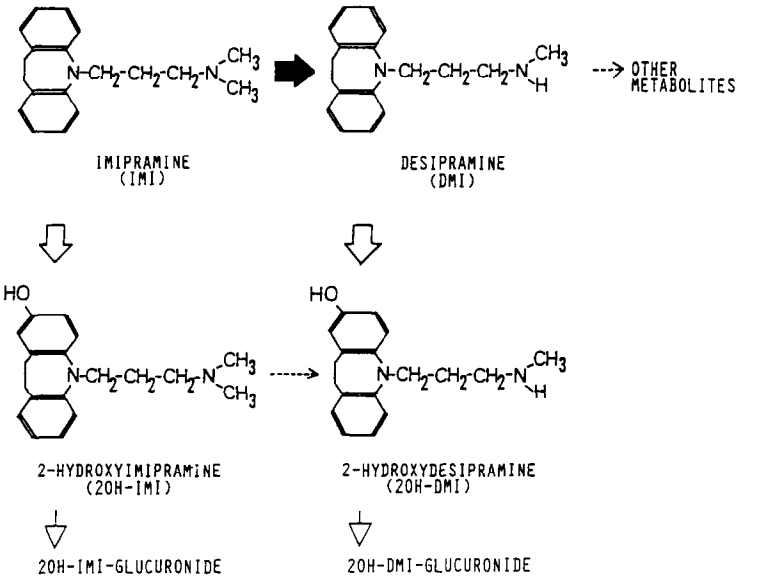
† Preliminary studies revealed that 2-hydroxyimipramine added to the reaction mixture of isolated rat hepatocytes was mainly biotransformed to a conjugated metabolite by glucuronidation (>95%) at a substrate concentration range from 0.1 to 1.5 nmol/mL. Therefore, N-demethylation of 2-hydroxyimipramine is likely to be a minor metabolic pathway especially in living cells.



Scheme 1. Major pathways of imipramine metabolism in isolated rat hepatocytes. Open arrows show aromatic 2-hydroxylations and closed arrows, aliphatic N-demethylation. Broken arrows indicate minor metabolic pathways.

commercially from the Sigma Chemical Co. (St. Louis, MO). 2-Hydroxyimipramine and 2-hydroxydesipramine were gifts from Geigy (Basel, Switzerland). Collagenase was purchased from Boehringer (GmbH, Mannheim, F.R.G.). All other chemicals and solvents were of analytical grade.

Animals. Adult male Wistar strain rats weighing 200–235 g were used. The rats were housed in an air-conditioned room under a 12-hr light–dark cycle and were allowed free access to food (CE-2, Clea Japan Inc., Tokyo, Japan) and tap water *ad lib.*

Preparation and incubation of isolated hepatocytes. Isolated hepatocytes were prepared by the method of Moldeus *et al.* [7] as modified by Seglen [8]. With this method the isolated liver was first perfused for 15 min with 100 mL of Ca^{2+}-free Hank's buffer consisting of NaCl (137 mM), KCl (5.4 mM), NaH_2PO_4–$2H_2O$ (0.5 mM), Na_2HPO_4–$12H_2O$ (0.42 mM), *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (10 mM, pH 7.2), ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) (0.5 mM), $NaHCO_3$ (4.2 mM) and glucose (5 mM). Then the perfusate was changed to buffer consisting of NaCl (137 mM), KCl (5.4 mM), $CaCl_2$ (5 mM), NaH_2PO_4–$2H_2O$ (0.5 mM), Na_2HPO_4–$12H_2O$ (0.42 mM), HEPES (10 mM, pH 7.5), $NaHCO_3$ (4.2 mM), collagenase (0.5 mg/mL) and the perfusion continued for 10–13 min. A flow rate of 30–40 mL/min was maintained throughout the perfusion. The liver was then removed and placed under cold buffer [NaCl (137 mM), KCl (5.4 mM), $CaCl_2$ (1.27 mM), $MgCl_2$–$6H_2O$ (1 mM), $MgSO_4$–$7H_2O$ (0.83 mM), NaH_2PO_4–$2H_2O$ (0.5 mM), Na_2HPO_4–$12H_2O$ (0.42 mM), $NaHCO_3$ (4.2 mM) and glucose (5 mM) buffered with NaOH to pH 7.2]. The capsule was then gently folded back, and the hepatocytes were released by gentle agitation of the lobe. The cell suspension was then filtered through a gauze and centrifuged (approximately 100 *g*, 3 min). Cells were resuspended in the fresh buffer, and the washing procedure was repeated three times. Hepatocytes were then resuspended in Krebs–Ringer bicarbonate buffer (pH 7.4, final volume, 12 mL). Cells were counted in a hemocytometer in the presence of 0.4% trypan blue. All the preparations used in this study were greater than 90% viable as judged routinely by the trypan blue exclusion test.

Assay methods for enzymatic activity. The rates of oxidation for imipramine and desipramine in isolated hepatocytes were measured as follows. After preincubation for 5 min, the reaction was started by adding 0.5 mL of various concentrations of substrates to the cell suspension (the final volume and cell concentration of the reaction mixture were 1.0 mL and 1.0×10^6 cells/mL respectively). The incubations were performed at 37° for 30 sec under air. After incubation, the reaction mixture was supplemented with 1.0 mL of boiling water and placed in boiling water for 1.0 min to immediately terminate the reaction. For determination of conjugated metabolites of 2OH–IMI or 2OH–DMI, the boiled samples were incubated at 25° for 16 hr with 1.0 mL of 1.0 M sodium acetate buffer (pH 5.0) containing β-glucuronidase (2000 units)/sulfatase (98 units), 3% (w/v) ascorbic acid as an antioxidant and 0.5 mL of internal standard (250 ng of nortriptyline). After hydrolysis, 1.5 mL of the sample was used to determine the formation of metabolites by the method described below.

Assay of imipramine and its metabolites. Imipramine and its metabolites were quantitated by a high performance liquid chromatographic (HPLC) procedure described elsewhere [6, 9, 10] with minor modifications. Briefly, samples were transferred to a 10-mL glass-stoppered test tube containing 1.0 mL of 1.0 M carbonate buffer (pH 10.0), and 5.0 mL of

ethylacetate was added. After mixing on a vortex mixer (30 sec), the contents were centrifuged (1200 *g*) for 10 min. The organic layer was transferred to another conical glass tube, and evaporated to dryness in a water bath (approx. 50°) under reduced pressure. The residue was mixed thoroughly with 100 μL of methanol. An aliquot of this solution (50 μL) was injected into the HPLC column.

HPLC conditions. A JASCO model Twincle, equipped with a variable wavelength detector at 254 nm, and a 25 cm × 4.6 mm i.d. SI-60 (5 μm) column (Hibar RT 250-4, E. Merck, Darmstadt, West Germany), was used. The mobile phase [acetonitrile:methanol, 3:1 (v/v)] containing 2.5% (v/v) ammonia water was used at a flow rate of 2.0 mL/min. The calibration plots of the peak height ratio of each drug relative to nortriptyline plotted against the drug concentrations were linear over the range of 0.03 to 15.0 nmol/mL. Statistical analysis by linear least square regression indicated an excellent linearity and reproducibility with a correlation coefficient of over 0.999 and a day-to-day precision of within 10% for each drug. All the recoveries for imipramine and its metabolites were determined to be more than 95% using the assay conditions described. Under these conditions, the typical retention times were: imipramine, 2.6 min; 2OH–IMI, 3.0 min; nortriptyline (I.S.), 4.3 min; desipramine, 5.7 min; and 2OH–DMI, 6.6 min.

Data analysis. Enzyme kinetic parameters (K_m and V_{max}) for each metabolic pathway were analyzed according to the nonlinear least squares regression analysis program MULTI [11] incorporating the Michaelis–Menten equation based on a simplex method. Best fittings of the data were performed by weighing the data with the reciprocal of the square of the initial formation rate.

Metabolic interaction experiments were analyzed by fitting an expression describing a competitive inhibition (equation 1) by the same program mentioned above. Data for all the inhibitor concentration were fitted simultaneously to equation 1:

Formation rate of metabolite

$$= \frac{V_{max} \cdot S}{K_m + (K_m/K_i) \cdot I + S} \quad (1)$$

where V_{max} is the maximum rate of metabolite formation, S is the substrate concentration, K_m is the Michaelis constant, I is the inhibitor concentration, and K_i is the competitive inhibition constant.

RESULTS

Preliminary studies on the effect of cell concentration on the formation rates of metabolites from imipramine and desipramine revealed that the formation rate of each metabolite was almost constant up to about 1.5 × 10^6 cells/mL. Figure 1 shows the time courses of metabolite formation from imipramine and desipramine in isolated rat hepatocytes. The formation of 2OH-IMI (free plus glucuronide) and desipramine from imipramine, and of 2OH–DMI (free plus glucuronide) from desipramine, was linear only within 30 sec. The secondary metabolite of imipramine, 2OH-DMI (free plus glucuronide),

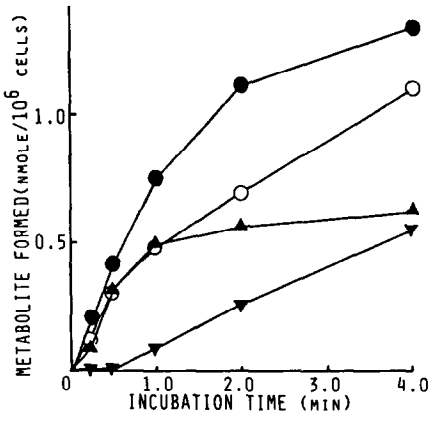


Fig. 1. Time courses of formation of 2-hydroxyimipramine (free plus glucuronide) (▲), desipramine (●), and 2-hydroxydesipramine (free plus glucuronide) (▼) from imipramine, and of 2-hydroxydesipramine from desipramine (○), in isolated rat hepatocytes. Each point is the mean value of three experiments. Incubation was performed in a reaction mixture containing 1 × 10^6 cells. The substrate concentration was 10 nmol/mL.

was formed in significant amounts in the reaction mixture incubated for more than 60 sec. Therefore, the incubation time above 60 sec caused the underestimation of the initial formation rate for each primary metabolite. From these observations, we adopted an incubation time of 30 sec in the reaction mixture containing 1.0 × 10^6 cells as an optimal assay condition for imipramine and desipramine metabolism in isolated rat hepatocytes.

To obtain the kinetic parameters for the formation of 2OH–IMI (free plus glucuronide) and desipramine from imipramine, we examined the effect of imipramine concentration on the formation rates of both of these metabolites. Typical results are shown in Fig. 2. Kinetic studies indicated that the disappearance rate of imipramine was almost completely accounted for by the formation rates of 2OH-IMI (free plus glucuronide) and desipramine at the imipramine concentration range of 0.1 to 2.0 nmol/mL (Fig. 2A). The kinetic parameters in the Michaelis-Menten equation are listed in Table 1. The K_m value for the formation of desipramine was about 10 times larger than that of 2OH–IMI (free plus glucuronide), whereas the V_{max} value of the former was about 4 times as large as that of the latter. Therefore, the formation rate of 2OH–IMI (free plus glucuronide) could be saturable in the imipramine concentration range below 5 nmol/mL, while that of desipramine increased with increasing imipramine concentrations (Fig. 2B).

Figure 3 shows typical results of the effects of various concentrations of desipramine on its disappearance rate and the formation rates of 2OH–DMI (free plus glucuronide) as well as the "other metabolites" in isolated hepatocytes. The formation rate of 2OH–DMI (free plus glucuronide) was about 70–80% of the disappearance rate of desipramine at the corresponding desipramine concentrations. Therefore, the formation rates of "other metabolites" were estimated from the difference between

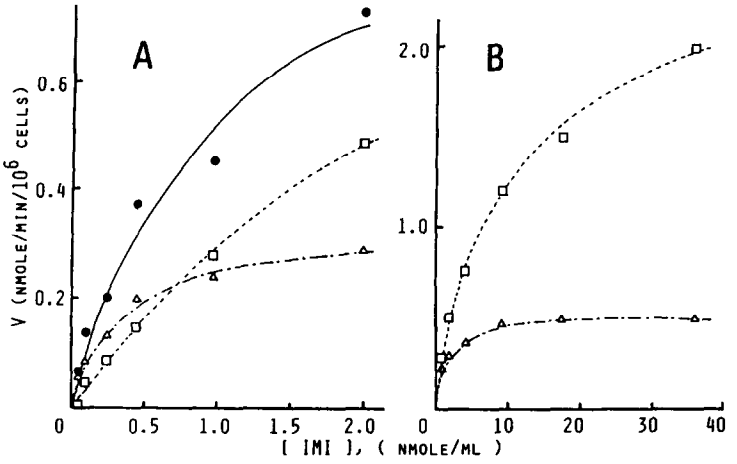


Fig. 2. Effect of imipramine concentration on the rate of disappearance of imipramine (●) and the formation of 2-hydroxyimipramine (free plus glucuronide) (△) and desipramine (□) in isolated rat hepatocytes. Each panel shows a typical result of three experiments.

Table 1. Kinetic parameters of imipramine and desipramine metabolism in isolated rat hepatocytes*

Substrate	Reaction	K_m (nmol/mL)	V_{max} (nmol/min/10^6 cells)
Imipramine	DMI formation	8.19 ± 0.55	2.10 ± 0.14
	2OH-IMI formation	0.706 ± 0.042	0.526 ± 0.059
Desipramine	2OH-DMI formation	1.45 ± 0.20	0.567 ± 0.027
	"Other metabolite" formation	2.38 ± 0.03	0.165 ± 0.019

* Data are the means ± SEM of three experiments. Abbreviations: DMI, desipramine; 2OH-IMI, 2-hydroxyimipramine; and 2OH-DMI, 2-hydroxydesipramine.

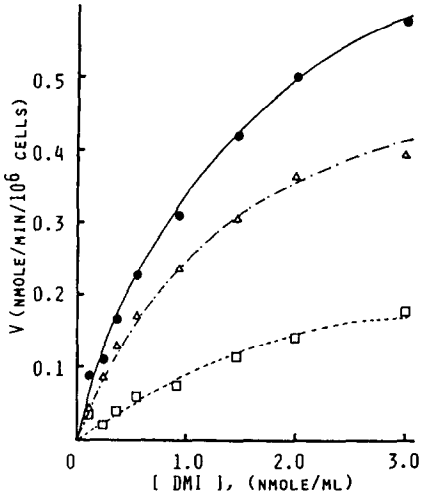


Fig. 3. Effect of desipramine concentration on the rate of disappearance of desipramine (●), the formation of 2-hydroxydesipramine (△) and the estimated formation of "other metabolites" (□) in isolated rat hepatocytes. This figure shows a typical result of three experiments. The formation rate of "other metabolites" was estimated from the difference between the rate of disappearance of desipramine and that of formation of 2-hydroxydesipramine (free plus glucuronide).

the rate of disappearance of desipramine and that of formation of 2OH-DMI (free plus glucoronide). Kinetic parameters for these reactions are also given in Table 1. It was noteworthy that K_m and V_{max} values for 2-hydroxylation of desipramine were approximately equal to those of imipramine 2-hydroxylation.

Figure 4A shows a typical result of the effect of the presence of desipramine on the initial rate of imipramine 2-hydroxylation, and Fig. 4B, that of imipramine on the initial rate of desipramine 2-hydroxylation. These reaction rates were calculated by summing the free forms and the glucuronides of 2-hydroxylated compounds. These figures demonstrate that imipramine and desipramine competitively inhibited the 2-hydroxylation of desipramine and imipramine respectively. A similar interaction has been observed in the assay system using rat liver microsomes [6]. The kinetic parameters (K_m and K_i) obtained from isolated hepatocytes are listed in Table 2. Data obtained from microsomal studies [6] were also given in this table for comparison. A good agreement between kinetic parameters for 2-hydroxylation pathways of imipramine and desipramine obtained from the microsomal assay system and those from isolated hepatocytes was found. The K_m values for imipramine and desipramine were almost equal to their inhibition constants, suggesting that the "parallel pathway interaction" between their 2-hydroxylations

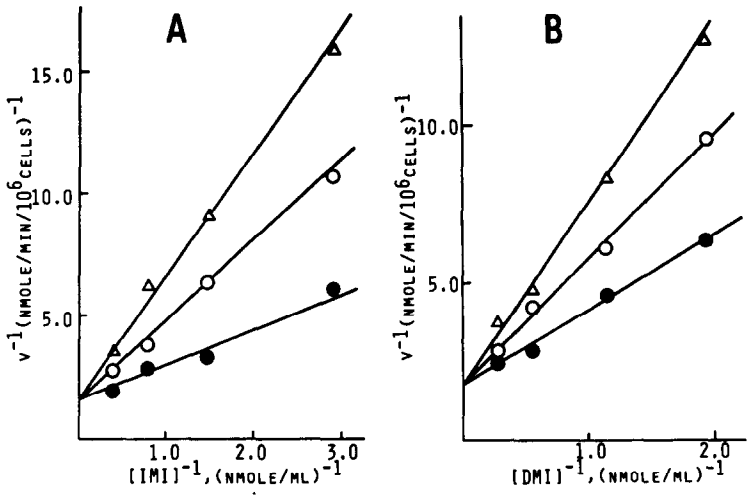


Fig. 4. Lineweaver–Burk plots of imipramine (A) and desipramine (B) 2-hydroxylation activities in the presence of desipramine and imipramine, respectively, in isolated rat hepatocytes. The reaction rate for each 2-hydroxylation was calculated by summing the free form and the glucuronide conjugate of each hydroxylated metabolite. (A) The solid lines were drawn based on computer-derived parameters (K_m = 0.910 nmol/mL, V_{max} = 0.636 nmol/min/10^6 cells, and K_i = 0.998 nmol/mL). Key: (●) control (no desipramine added); (○) 1.27 nmol/mL of desipramine added; and (△) 2.52 nmol/mL of desipramine added. (B) The solid lines were drawn based on computer-derived parameters (K_m = 1.44 nmol/mL. V_{max} = 0.603 nmol/min/10^6 cells, and K_i = 0.897 nmol/mL). Key: (●) control (no imipramine added); (○) 0.710 nmol/mL of imipramine added; and (△) 1.23 nmol/mL of imipramine added.

Table 2. Metabolic parameters for imipramine (IMI) and desipramine (DMI) 2-hydroxylations and their interaction in rat liver microsomes and isolated hepatocytes*

Reaction	Inhibitor	Preparation	K_m (nmol/mL)	K_i (nmol/mL)
IMI 2-hydroxylation	DMI	Microsomes†	1.21 ± 0.11	0.951 ± 0.110
		Hepatocytes	0.787 ± 0.052	1.07 ± 0.04
DMI 2-hydroxylation	IMI	Microsomes†	0.992 ± 0.108	1.20 ± 0.10
		Hepatocytes	1.44 ± 0.10	0.883 ± 0.021

* Data are the means ± SEM of three experiments.
† Data for microsomal studies were obtained from our previous publication [6].

was a fully competitive type in both systems. These results suggested that competitive inhibition between "parallel pathways", that is, imipramine and desipramine 2-hydroxylations, observed in the kinetic study using rat liver microsomes and also the intrinsic kinetic nature for these metabolic pathways (i.e. K_m and K_i) estimated in rat liver microsomes were well reproduced in studies using isolated rat hepatocytes.

DISCUSSION

It was demonstrated that the enzyme systems having low-affinity-and-high-capacity and high-affinity-and-low-capacity kinetic properties were involved in imipramine metabolism in rats. The metabolic characteristics of the former were well examined in our laboratory. The metabolic activity for imipramine N-demethylation showed a marked sex difference only in young rats (3- to 12-months-old), and age-associated alteration only in male rats [6, 9, 10]. In addition, inhibition studies using antibody to P-450 m_1, a major P-450 isozyme in male rat liver microsomes, revealed that about 80% of imipramine *N*-demethylase activity in 3-month-old male rat liver microsomes was mediated by P-450 m [12]. On the other hand, the metabolic activities of the high-affinity-and-low-capacity enzyme(s) involved in both 2-hydroxylations show no age-associated alterations in both sexes and no sex differences [6, 9, 10]. They were not affected by the presence of the antibody to P-450 m_1 in rat liver microsomes. In human clinical studies, desipramine and debrisoquin phenotype for hydroxylation rate have been clearly demonstrated to segregate together [13–17]. Furthermore, it was reported that a mutually competitive relationship was observed between debrisoquin oxidation activity and 2-hydroxylation activity of desipramine in human and rat liver microsomes [14, 18]. Since debrisoquin 4-hydroxylation was catalyzed by P-450_{UT-H}, which is known to produce the polymorphism of debrisoquin 4-hydroxylation in rats [19], it is likely that the common P-450 isozyme (P-450_{UT-H}) may

participate in both hydroxylations of desipramine and of debrisoquin.

From these observations described so far, the two enzyme systems with different kinetic properties, involved in the perpendicular pathways in the metabolic scheme of imipramine (Scheme 1), are likely to be represented by different P-450 isozymes (i.e. P-450 m_1 and P-450$_{UT-H}$). In contrast, the high-affinity-and-low-capacity enzyme(s) mediating both 2-hydroxylations of imipramine and desipramine, which are parallel to each other in the metabolic scheme, could be catalyzed by a common P-450 isozyme, and this might result in the competitive interaction between these two 2 hydroxylation pathways. Based on these biochemical findings, it is likely that the metabolic characteristics of 2-hydroxylations of both compounds should play an important role in the observed nonlinear disposition of imipramine and desipramine against the imipramine dose in isolated perfused rat liver [5] due not only to their kinetic characters (i.e. high-affinity-and-low-capacity kinetics) but also to the metabolic interactions between them.

The present study demonstrated that the characteristics of imipramine metabolism observed in studies using rat liver microsomes including kinetic parameters and the phenomenon of parallel pathway interaction [6] were well reproduced in studies using isolated rat hepatocytes. Consequently, they should be taken into consideration in elucidating the possible mechanism for the nonlinear disposition of imipramine and desipramine, and in developing a kinetic model for the analysis of *in vivo* pharmacokinetics of both compounds.

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