Biphasic Kinetics of Imipramine N-Oxidation in Rat Brain Microsomes

Shizuo Narimatsu,⁎,† Shigeo Yamamoto,† Tomomi Koitabashi,‡ Rika Kato,‡ Yasuhiro Masubuchi,‡ Tokuiji Suzuki,‡ and Tosiharu Horie‡

Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Okayama University, 1–1–1 Tsushima-naka, Okayama 700–8530, Japan and Department of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Chiba University, 3–1–1 Yayoi-cho, Inage-ku, Chiba 263–8522, Japan. Received September 24, 1998; accepted November 25, 1998

Imipramine (IMI) N-oxidase activity in brain microsomes from rats of both sexes was determined by high performance liquid chromatography, and compared with the results in rat liver microsomes. Brain and liver microsomal IMI N-oxidation was sensitive to thermal inactivation and had an optimal pH at around 9.0. IMI N-oxidase activity (15.54 pmol/min/mg protein) in brain microsomes was about one-hundredth that of liver microsomes (2.08 nmol/min/mg protein) at a substrate concentration of 5 mM. IMI N-oxidase activities in both brain and liver microsomes displayed biphasic kinetics that associated a low Km-low Vmax element with a high Km-high Vmax component. Furthermore, a significant sex difference was observed in Vmax values (male > female) in both phases, but Km values were similar between male and female rats, resulting in a significant sex difference (male > female) in intrinsic clearance values (Vmax/Km) of the low-Km and the high-Km phases.

Key words: flavin-containing monoxygenase; imipramine N-oxide; rat brain microsomes; rat liver microsomes; biphasic kinetics; sex difference

Imipramine (IMI) is one of the tricyclic antidepressant agents, which are currently widely used for the treatment of major depression. Following oral administration, this drug is well absorbed from the intestine, and undergoes the first-pass metabolism in the liver.1 IMI is biotransformed to several primary oxidative metabolites such as desmethylinipramine (DMI), 2-hydroxynipramine, 10-hydroxynipramine and IMI N-oxide (Fig. 1). IMI N-demethylation and hydroxylation at positions 2 and 10 are catalyzed by cytochrome P450.2,3 Among the oxidative metabolites, DMI has a potent antidepressant activity, and can contribute to the effect as an active metabolite of IMI.1 Compared with DMI, 2- and 10-hydroxynipramines do not seem to be pharmacologically and toxicologically very important as active metabolites of IMI.

IMI N-oxide is also reported to be pharmacologically active,4,5 but its role(s) in vivo remain to be elucidated. Accumulated evidence indicates that the IMI N-oxide is formed from IMI by flavin-containing monoxygenase (FMO, EC 1.14.13.8) mainly in the liver of experimental animals.6,7 Lemoine et al.8 demonstrated using human subcellular fractions that FMO in the liver and kidney exhibited IMI N-oxidation activity in humans. Ravindranath and his colleagues9 reported that human brain contained IMI N-oxidase activity for which brain microsomal FMO is responsible. From these reported results, it is thought that IMI N-oxide formation has some role in the brain as well as in the liver and kidney. This raises a possibility that IMI N-oxide also takes some part as an active metabolite in adverse effects observed after IMI administration, such as antimuscarinic effects, cerebral and cardiac toxicity10 as well as inhibition or inactivation of metabolic enzymes.11–13 We thus started this project to further understand the pharmacological and toxicological roles of IMI N-oxide as an active metabolite of IMI. This paper describes the biphasic kinetics of IMI N-oxidation in rat brain and liver microsomes.

MATERIALS AND METHODS

Materials IMI hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); desipramine hydrochloride, IMI N-oxide and 10-hydroxydesipramine hydrochloride were supplied by Ciba Geigy Co., Ltd. (Basel, Switzerland). NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from the Oriental Yeast Co., Ltd. (Tokyo, Japan).

Enzyme Sources Adult Wistar rats (6–7 weeks old) of both genders were obtained from Takasugi Experimental Animals Lab. (Saitama, Japan). The animals were kept in animal rooms at 22–24°C with a 12 h light and dark cycle for at least one week prior to the experiments. The rats were sacrificed, and livers were perfused with 0.9% NaCl to remove blood. Brains were washed with saline. Microsomal fractions of livers and brains were prepared by a published method.14

* To whom correspondence should be addressed.

© 1999 Pharmaceutical Society of Japan

Fig. 1. Primary Oxidative Pathways of IMI

© 1999 Pharmaceutical Society of Japan
Enzyme Assay A typical reaction mixture (final volume of 1.0 ml, pH 9.0) consisted of microsomes (1 mg protein), NADPH (1.25 μmol), and Tris-HCl buffer (150 μmol) in a brown conical glass tube with a stopper (10 ml volume). The mixture was preincubated at 37 °C for 5 min and IMI HCl (0.5—5000 μM as final concentrations) was added and incubated at 37 °C for 0.5 min (liver microsomes) or 5 min (brain microsomes). At the end of the incubation, 1.0 ml of aqueous 1 N NaOH solution as a reaction terminator and 1 μmol of 10-hydroxydesipramine as an internal standard were added. Ethyl acetate (6 ml) was then added, and the tube was vigorously shaken and centrifuged (1500 × g for 10 min). The organic layer (5 ml) was taken, evaporated to dryness, and the residue was dissolved in 100 μl of the mobile phase for high-performance liquid chromatography (HPLC), as described below. IMI N-oxide was determined by the internal standard method on the basis of the calibration curve. The calibration curve was made up by adding known varying amounts of synthetic IMI N-oxide and 1 nmol of 10-hydroxydesipramine to ice-cold incubation medium containing various ingredients.

In experiments on thermostability, brain and liver microsomes (each 1 mg protein) were preincubated with or without NADPH (1.25 μmol) at 37 °C for 5 min, and then IMI (5 μM) was added to both preincubation mixtures and NADPH (1.25 μmol) was added to the preincubation mixture without NADPH, and incubation was performed at 37 °C for 5 min for brain microsomes and 0.5 min for liver microsomes. To search for optimal pH for IMI N-oxidation, 150 μM Tris–HCl buffers (pH 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5) were used.

HPLC Conditions The HPLC system consisted of a Shimadzu LC 3A pump, a Rheodyne 7125 injector and a Shimadzu SDP-2A spectrophotometric detector; Column, Li Chrospher Si 60 (Merck, Darmstadt, Germany); mobile phase, CH₃CN–MeOH–29%NH₃ (75:25:4, by volume); flow rate, 1.5 ml/min; detection, UV 254 nm.

Others Protein concentrations were determined by the method of Lowry et al. with bovine serum albumin as a standard. Enzyme kinetic parameters (Kₘ and Vₘₐₓ) were analyzed according to a non-linear least-square regression analysis based on a simplex method. Statistical significance was calculated by Student’s t-test.

RESULTS AND DISCUSSION

IMI (5 μM) was added to the reaction medium (pH 8.5) containing rat liver or brain microsomes (1 mg protein) and NADPH (1.25 μmol), and incubated at 37 °C for 0.5 and 5 min, respectively. IMI N-oxide formed was extracted with an organic solvent under basic conditions, followed by HPLC determination. A typical chromatogram of IMI N-oxide formation in brain microsomes is shown in Fig. 2.

In preliminary experiments, we confirmed that the formation of IMI N-oxide from IMI was NADPH-dependent, and was linear during the incubation time of 5 min for rat brain microsomes and 0.5 min for rat liver microsomes. Enzymatic formation of 10-hydroxydesipramine could not be detected during the incubation time (5 min for brain microsomes and 0.5 min for liver microsomes). Furthermore, there were no peaks corresponding to desipramine or 2-hydroxydesipramine under the conditions employing rat brain microsomes as the enzyme source. We thus compared the formation of IMI N-oxide between the brain and liver microsomal fractions.

IMI N-oxidation is mainly catalyzed by NADPH-dependent FMO. NADPH can be easily lost its catalytic capacity during the incubation even at 37 °C, and co-incubation of NADPH as a cofactor with the microsomes protects the loss of the enzyme activity. The rat brain microsomes lost IMI N-oxidase activity during the incubation without NADPH at 37 °C for 5 min, and co-incubation with NADPH exhibited a remarkable protective effect against the thermal inactivation (Fig. 3). Similar thermal inactivation and protective effects with NADPH were observed in rat liver microsomes (data not
shown).

Figure 4 exhibits a profile for the formation of IMI N-oxide from IMI by rat brain microsomes at various pH points. In the range examined (pH 7.0 to 9.5), pH 9.0 was optimal for IMI N-oxide activity. An essentially similar pH profile was shown in rat liver microsomal IMI N-oxidation (data not shown). When 5 mM IMI was employed as a substrate, IMI N-oxidase activities of rat brain microsomes were 15.54 ± 0.49 pmol/min/mg protein, whereas those of liver microsomes were 2.08 ± 0.19 nmol/min/mg protein (mean ± S.D., n = 3). From these results, IMI N-oxidation was kinetically analyzed at pH 9.0 both in brain and liver microsomal fractions.

The kinetics employing a substrate concentration range from 5 to 5000 μM revealed that IMI N-oxidation was biphasic consisting of a low- Km phase and a high-Km phase. Typical Eadie-Hofstee plots for IMI N-oxidation in brain microsomes are shown in Fig. 5. Calculated kinetic parameters are summarized in Table 1. From intrinsic clearance (CL,int, Vmax/Km) values, the low-Km phases are thought to be the major component in comparison with the high-Km phases in rat brain microsomes from both sexes. Similar results were obtained in rat liver microsomes, although Vmax values were very different between the two subcellular fractions (brain < liver). Moreover, a significant sex difference (male > female) was observed in Vmax and CLint values, whereas no significant difference was seen in Km values.

Ravindranath and his colleagues[18,19] showed that IMI N-oxidation activity was higher in brain microsomes than in liver microsomes, and that brain microsomal IMI N-oxidation was analyzed to be monophasic. In contrast, in the present study brain microsomal IMI N-oxidation activity was much lower than that of liver microsomes, and the IMI N-oxidation was biphasic. This discrepancy may be due to the difference in assay methods of FMO activity employed between their and our experiments, i.e., Ravindranath’s group[18,19] determined the activity for rat brain microsomes in the presence of n-octylamine as an activator of FMO, and sodium cholate, Triton N-101 and Lubrol PX as detergents, whereas we assayed the enzyme activity without any activator or detergent.

In the present study, a significant sex difference was observed in rat brain microsomal IMI N-oxidation as well as in liver microsomes. Lemoine et al.[20] also demonstrated the sex difference in rat liver microsomal IMI N-oxidation. Furthermore, they compared microsomal IMI N-oxidation activities in the lung and the kidney with those in the rat liver and the activities were: liver > lung > kidney in male rats, and lung > kidney > liver in females.[20] In contrast, Ravindranath’s group did not observe a sex-related difference in IMI N-oxidation in rat brain microsomes.[18,19] As shown in Table 2, the sex difference was observed in Vmax values for the low-Km and high-Km phases in brain microsomes as well as in liver microsomes. These activities are thought to be due to microsomal FMO and the contribution of microsomal cytochrome P450 to IMI N-oxidation under the conditions used is little if any, because the IMI N-oxidation in the present experiments was suppressed by heating at 37 °C for 5 min in the absence of NADPH. This phenomenon is well matched to the known features of FMO.

The biphasic kinetics suggest that at least two FMO isozymes are involved in IMI N-oxidation in rat brain and liver microsomes.

Fig. 4. Optimal pH for IMI N-Oxide Formation by Rat Brain Microsomes

A substrate concentration range of 5 to 5000 μM was used. Each point is the mean value of duplicate determinations.

Fig. 5. Typical Eadie-Hofstee Plots for IMI N-Oxidase Activity in Rat Brain Microsomes

A substrate concentration range of 5 to 5000 μM was used. Open circle, brain microsomes from male rat; closed circle, brain microsomes from female rat. Each point is the mean value of duplicate determinations.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Km1 (μM)</th>
<th>Vmax1 (pmol/min/mg)</th>
<th>CLint1 (Vmax/Km1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Male</td>
<td>9.32 ± 2.12</td>
<td>2.94 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>9.56 ± 2.82</td>
<td>0.99 ± 0.25**</td>
</tr>
<tr>
<td>Liver</td>
<td>Male</td>
<td>10.94 ± 2.49</td>
<td>509 ± 154</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>14.92 ± 0.86</td>
<td>51.49 ± 1.31**</td>
</tr>
</tbody>
</table>

CLint intrinsic clearance (μl/min/mg). Each value represents the mean ± S.D. of three determinations. * Significant different from male rats (p < 0.05). ** Significantly different from both male and female rats (p < 0.01, respectively).
liver. Different from other mammalian species such as humans, rabbits and mice, rats do not seem to have many FMO isozymes, i.e., the expression of only two isozymes FMO1 and FMO3 has been reported in this species. Bhamre et al. showed that rat brain FMO immunoreacted to the antibody raised against rabbit FMO2. These findings suggest that three FMO isoforms, FMO1 and FMO3, and also the isoform that immunochemically cross-reacts with rabbit FMO2, may be expressed in the rat, but the present study did not give any evidence about which of these isoforms are responsible for the biphasic IMI \( N \)-oxidation in rat brain and liver.

Lemoine et al. indicated the possibility in their study that rat liver microsomal FMO activity forming IMI \( N \)-oxide is under hormonal regulation. The present results suggest that brain microsomal IMI \( N \)-oxidation is also under hormonal regulation like the liver microsomal reaction. However, there is the possibility that environmental conditions surrounding the FMO enzymes are different in brain microsomal membranes between male and female rats, resulting in no sex difference in Ravindranath’s group under the conditions using detergents.

The addition of activator and detergent to microsomal fractions may drastically increase the activity, but there is no reliable information on to what extent the increased activity by the detergent and activator reflects a real contribution of \( N \)-oxidation by FMO to the overall metabolism of IMI \( in \) vivo. It is feasible that in some cases, moderate conditions without detergent or activator may reflect the real metabolism by FMO \( in \) vivo. In addition, the optimal pH for brain and liver microsomal IMI \( N \)-oxidation was 9.0 so that we employed this pH for in vitro studies, however, the reaction may occur at lower pH conditions \( in \) vivo. IMI \( N \)-oxide formed can be reduced to the parent compound under reductive conditions. Further characterization of FMO and its reactions under physiological conditions may uncover the real contribution of FMO to the pharmacokinetics of substrates, including IMI.

In summary, the present study demonstrated that IMI \( N \)-oxidation activity in rat brain microsomes was detectable by HPLC under conditions without any detergent or activator, although the activity was much lower than that of liver microsomes. IMI \( N \)-oxidation in brain and liver microsomes displayed biphasic kinetics that associated a low \( K_m \)-low \( V_{max} \) element with a high \( K_m \)-high \( V_{max} \) component. Furthermore, a sex difference (male>female) was observed in \( V_{max} \) values for the two phases in brain and liver microsomes, whereas \( K_m \) values were similar for the two phases of both genders.

**Acknowledgment** We thank Ciba Geigy Co., Ltd. (Basel, Switzerland) very much for the generous gift of IMI \( N \)-oxide, 2-hydroxyimipramine and 10-hydroxydesipramine.

**REFERENCES**