Identification of human cytochrome P450 isozymes involved in diphenhydramine N-demethylation

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a) Running title: P450 isozymes involved in diphenhydramine N-demethylation

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c) The number of text pages: 26
The number of tables: 2
The number of figures: 5
The number of references: 36
The number of words in Abstract: 244
The number of words in Introduction: 542
The number of words in Discussion: 1165

d) Non standard abbreviations: P450, cytochrome P450; LC/MS, liquid chromatography-mass spectrometry; RAF, relative activity factor; G6P, Glucose 6-phosphate; G6PDH, glucose 6-phosphate dehydrogenase; NADP⁺, nicotinamide adenine dinucleotide phosphate.
Abstract

Diphenhydramine is widely used as an over-the-counter antihistamine. However, the specific human cytochrome P450 (P450) isozymes that mediate the metabolism of diphenhydramine in the range of clinically relevant concentrations (0.14 to 0.77 µM) remain unclear. Therefore, P450 isozymes involved in N-demethylation, a main metabolic pathway of diphenhydramine, were identified by a liquid chromatography-mass spectrometry method developed in our laboratory. Among 14 recombinant P450 isozymes, CYP2D6 showed the highest activity of diphenhydramine N-demethylation (0.69 pmol/min/pmol P450) at 0.5 µM. CYP2D6 catalyzed diphenhydramine N-demethylation as a high-affinity P450 isozyme, the $K_m$ value of which was 1.12 ± 0.21 µM. In addition, CYP1A2, CYP2C9 and CYP2C19 were identified as low-affinity components. In human liver microsomes, involvement of CYP2D6, CYP1A2, CYP2C9 and CYP2C19 in diphenhydramine N-demethylation was confirmed by using P450-isozyme specific inhibitors. In addition, contributions of these P450 isozymes estimated by the relative activity factor were in good agreement with the results of inhibition studies. Although an inhibitory effect of diphenhydramine on the metabolic activity of CYP2D6 has been reported previously, the results of the present study suggest that it is not only a potent inhibitor but also a high-affinity substrate of CYP2D6. Therefore, it is worthy to mention that the sedative effect of diphenhydramine might be caused by co-administration of CYP2D6 substrate(s)/inhibitor(s). In addition, large differences in the metabolic activities of CYP2D6, and those of CYP1A2, CYP2C9 and CYP2C19 could cause the individual differences in anti-allergic efficacy and sedative effect of diphenhydramine.
Introduction

Histamine H1 receptor antagonists (antihistamines) are useful in the treatment of various allergic diseases such as allergic rhinitis, conjunctivitis, atopic dermatitis, urticaria, asthma and anaphylaxis (Mizushima, 2006). Recently, there has been increasing interest in the metabolism and pharmacodynamic effects of classic antihistamines because of severe deleterious cytochrome P450 (P450)-based drug-drug interactions observed with second-generation antihistamines such as terfenadine (Paakkari, 2002; Monahan et al., 1990). Diphenhydramine is a first-generation antihistamine synthesized in the 1940’s (Loew et al., 1946) and has been widely used as an over-the-counter drug (Kay and Quig, 2001; Food and Drug Administration, 2002). Since this antihistamine is available without prescription, it is possible that it is taken with various drugs so that an interaction may occur when the same P450 isozyme(s) is involved in the major metabolic processes of diphenhydramine and concomitant drug(s).

Diphenhydramine is extensively metabolized by demethylation to N-demethyl diphenhydramine followed by rapid demethylation to N,N-didemethyl diphenhydramine. N,N-Didemethyl diphenhydramine is further metabolized by oxidative deamination to diphenylmethoxyacetic acid. These metabolic pathways are thought to be major pathways in humans (Chang et al., 1974; Blyden et al., 1986), although little is known about the enzymes involved in these metabolic pathways of diphenhydramine because of the limited requirements for pharmacokinetics/metabolism data prior to marketing classic antihistamines. However, the metabolism of classic antihistamines seems to be mediated by CYP2D6 because of the similar structural characteristics of many CYP2D6 substrates and inhibitors (de Groot et al., 1997; Koymans et al., 1992; Smith and Jones, 1992). In fact, several classic antihistamines such as chlorpheniramine, mequitazine and promethazine have been shown to be metabolized by CYP2D6 (Yasuda et al., 2001; Nakamura et al., 1998; Nakamura et al., 1996). In addition, previous studies revealed that diphenhydramine inhibited the metabolism of several CYP2D6
substrates *in vivo* and *in vitro* (Hamelin et al., 1998, 2000; He et al., 2002; Lessard et al., 2001). These findings suggest that the metabolism of diphenhydramine is also mediated by CYP2D6.

However, Lessard et al. (2001) reported that the clearance of diphenhydramine to its N-demethylated metabolite (2-benzhydroxy-N-methyl-ethanamine) is not different in extensive and poor metabolizer phenotypes of CYP2D6 and suggested that diphenhydramine is not extensively metabolized by this P450 isozyme. In addition, multiple P450 isozymes, CYP1A2, CYP2C18, CYP2C19, CYP2D6 and CYP2B6, have been reported to be involved in the N-demethylation of diphenhydramine at 20 µM (Hamelin et al., 2001, Sharma and Hamelin, 2003). Nonetheless, it remains unclear whether these P450 isozymes also catalyze the N-demethylation of diphenhydramine at clinically relevant concentrations, which are 37 to 83 ng/ml (0.14 to 0.33 µM) in plasma after oral administration and 99 to 196 ng/ml (0.38 to 0.77 µM) in plasma after intravenous injection of 50 mg of diphenhydramine hydrochloride (Carruthers et al., 1978; Blyden et al., 1986).

Therefore, the aim of this study was to identify the P450 isozymes involved in N-demethylation of diphenhydramine using recombinant P450 isozymes and human liver microsomes at a clinically relevant concentration (0.5 µM) of diphenhydramine by a liquid chromatography-mass spectrometry (LC/MS) method developed in our laboratory. Contributions of multiple P450 isozymes to human liver microsomal N-demethylation of diphenhydramine were also estimated by application of the relative activity factor (RAF) (Crespi et al., 1995) and were verified by the results of inhibition studies using P450 isozyme-specific inhibitors.
Materials and methods

Chemicals and reagents

Diphenhydramine hydrochloride, quinidine sulfate, furafylline, sulfaphenazole and omeprazole were purchased from SIGMA (Milwaukee, WI). Orphenadrine hydrochloride was purchased from MP Biomedicals (Aurora, OH). SKF-525A was purchased from Toronto Research Chemicals (North York, Canada). Glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH) and nicotinamide adenine dinucleotide phosphate (NADP⁺) were purchased from Oriental Yeast Co. (Tokyo, Japan). 2-Benzhydryloxy-N-methyl-ethanamine (N-demethyl diphenhydramine) was synthesized by WAKO Pure Chemical Industries (Osaka, Japan). Human liver microsomes (HG3, 6, 23, 30, 42, 43, 56, 66, 70, 89, 93, 112 and pooled) and microsomes prepared from baculovirus-infected insect cells expressing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP4A11 individually were obtained from BD Gentest (Woburn, MA). All recombinant P450s had been co-expressed with NADPH P450 oxidoreductase. Recombinant CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 were also co-expressed with cytochrome b₅. Microsomal protein expressing NADPH P450 oxidoreductase and cytochrome b₅ was used as a control. Other chemicals and reagents used in this study were research-grade purchased from WAKO.

Diphenhydramine N-demethylation assay

The basic incubation mixture contained recombinant P450 isozyme (5 pmol P450/ml) or human liver microsomes (0.1 mg/ml), 0.1 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4), and 0.5 µM diphenhydramine in a final incubation volume of 250 µl. The reaction was initiated by the addition of an NADPH-generating system (0.5 mM NADP⁺, 2.0 mM G6P, 1 U/ml G6PDH and 4 mM MgCl₂). Incubation was performed for 20 min for recombinant P450
isozyme or 60 min for human liver microsomes at 37°C and terminated by the addition of 250 µl of 0.1 M HCl. Orphenadrine was added as an internal standard at a final concentration of 0.2 µM. The reaction was performed in a linear range with respect to the protein concentration and the incubation time for each recombinant P450 isozyme and pooled human liver microsomes. In the incubation conditions of the present study, negligible metabolism of N-demethyl diphenhydramine to N,N-didemethyl diphenhydramine was confirmed by single ion monitoring at m/z 228, pseudomolecular ion of N,N-didemethyl diphenhydramine. For calibration standards, incubation was performed without diphenhydramine and terminated by the addition of 250 µl of 0.1 M HCl. Then N-demethyl diphenhydramine was added to the reaction mixture at final concentrations of 5 nM to 5 µM. The correlation coefficient (r) for the calibration curve calculated by the least-squares regression method was \( r > 0.99 \) at final concentrations of 5 nM to 5 µM. The coefficient of variation for the assay in the present study was 9.7% (n = 5). All procedures were performed in siliconized glass tubes to avoid adsorption of the microsomes, substrate and metabolites.

**Solid phase extraction**

Solid phase extraction was performed by the method of Miyaguchi et al. (2006) with slight modifications. The incubation mixture was applied to a mix-mode solid phase extraction cartridge (Oasis MCX cartridge, Waters, Milford, MA). The cartridge was conditioned with 1 ml each of acetonitrile and distilled water before use. After application of the reaction mixture, the cartridge was washed with 1 ml each of 0.1 M HCl and acetonitrile and then eluted with 1 ml of 5% ammonium hydroxide in acetonitrile. In this solid-phase extraction method, lipophilic basic compounds were selectively eluted. The eluate was dried under a gentle stream of nitrogen at 45°C and reconstituted by 100 µl of distilled water/acetonitrile (50/50, v/v). Ten µl of the reconstituted sample was applied to an LC/MS system. In this extraction procedure, the
recovery rates of N-demethyl diphenhydramine were calculated by three or four independent analyses to be 100.0 ± 2.2% at 50 nM and 95.0 ± 2.2% at 5 µM. Coefficients of variation of this solid-phase extraction method were 2.2% at 50 nM and 2.3% at 5 µM.

**LC/MS conditions**

Determination of diphenhydramine and its N-demethylated metabolite was carried out using an LC/MS system. The LC system consisted of an Alliance 2695 separation module (Waters) and an XTerra MS C18 column (particle size of 3.5 µm, 2.1 mm in I.D. x 100 mm, Waters). The mobile phase consisted of 0.1% ammonia in water/acetonitrile (50/50, v/v) at a flow rate of 0.25 ml/min. The column temperature was maintained at 40˚C. This LC system was connected to an ion trap MS system (Micromass ZQ, Waters) with an electron spray ionization probe. The compounds were detected in the positive-ion electron spray ionization mode. For maximal sensitivity, the ion source temperature and cone voltage were optimized at 120˚C and 20V, respectively. Nitrogen was used for desolvation at a flow rate of 500 l/hr. The desolvation temperature was 350˚C. For quantitation, monitoring of single ions was performed at m/z of pseudomolecular ions of N-demethyl diphenhydramine (m/z 242), diphenhydramine (m/z 256) and orphenadrine (m/z 270). N-Demethyl diphenhydramine, diphenhydramine and orphenadrine were identified by matching the retention time and m/z of pseudomolecular ions with those of authentic materials. The retention times of N-demethyl diphenhydramine, diphenhydramine and orphenadrine in this analytical condition were 5.5 min, 6.6 min and 9.0 min, respectively. Limits of quantification in the assays with recombinant P450s and with human liver microsomes are final concentrations of 2.0 nM and 2.8 nM, respectively, calculated as signal-to-noise ratios of more than 10.

**Kinetics of diphenhydramine N-demethylation in pooled human liver microsomes**
Kinetics of diphenhydramine N-demethylation in pooled human liver microsomes were determined from formation rates of N-demethyl diphenhydramine at diphenhydramine concentrations ranging from 0.5 to 500 µM. Involvement of multiple enzymes was assessed by Eadie-Hofstee plots. When the Eadie-Hofstee plot was not linear, the kinetic parameters of high- and low-affinity components (\(K_{m1}\) and \(V_{max1}\) for the high-affinity component and \(K_{m2}\) and \(V_{max2}\) for the low-affinity component) were estimated by graphic analysis of the 2-component Michaelis-Menten model calculated by using DeltaGraph 4.5 (Nippon Polaroid, Tokyo, Japan).

**Correlation study**

Correlation coefficients between diphenhydramine N-demethylation activity at 0.5 µM and other P450 isozyme-specific activities were calculated in human liver microsomes from 12 individual donors. Data of activities of phenacetin \(O\)-deethylation for CYP1A2, coumarin 7-hydroxylation for CYP2A6, (S)-mephenytoin N-demethylation for CYP2B6, paclitaxel 6α-hydroxylation for CYP2C8, diclofenac 4’-hydroxylation for CYP2C9, (S)-mephenytoin 4’-hydroxylation for CYP2C19, bufuralol 1’-hydroxylation for CYP2D6, chlorzoxazone 6-hydroxylation for CYP2E1, testosterone 6β-hydroxylation for CYP3A4 and lauric acid 12-hydroxylation for CYP4A were provided by BD Gentest.

**Chemical inhibition study**

The effects of P450 isozyme-specific inhibitors on diphenhydramine N-demethylation activity were investigated in human liver microsomes. Human liver microsomal protein (0.1 mg/ml) was incubated with 0.5 µM diphenhydramine in the presence of 1 µM quinidine (a selective CYP2D6 inhibitor) at 37°C for 60 min. Correlation coefficients between the residual diphenhydramine N-demethylation activity in the presence of 1 µM quinidine and other P450
Isozyme-specific activities were calculated in human liver microsomes from 12 individual donors. Inhibition studies with 10 µM furafylline for CYP1A2, 10 µM sulfaphenazole for CYP2C9 and 10 µM omeprazole for CYP2C19 in individual human liver microsomes HG30, HG66, HG89 and HG112 and in pooled human liver microsomes were also performed. These human liver microsomes from individual donors were selected because they showed characteristic activities of CYP2D6, CYP1A2, CYP2C9 and CYP2C19 and comparable activities of diphenhydramine N-demethylation. The concentrations of specific inhibitors were verified by inhibition of the specific activities of corresponding P450 isozymes in human liver microsomes (Bourrie et al., 1996; Kobayashi et al., 1999; Giraud et al., 2000).

**Kinetics of diphenhydramine N-demethylation in recombinant P450 isozymes**

Kinetics of diphenhydramine N-demethylation in recombinant CYP1A2, CYP2C9, CYP2C19 and CYP2D6 were determined from formation rates of N-demethyl diphenhydramine at diphenhydramine concentrations ranging from 0.05 to 20 µM for CYP2D6 and from 0.5 to 500 µM for CYP1A2, CYP2C9 and CYP2C19. The kinetic parameters ($K_m$, $V_{max}$ and $V_{max}/K_m$) were estimated by graphic analysis of the 1-component Michaelis-Menten model calculated by using DeltaGraph 4.5.

**Contribution of each P450 isozyme to diphenhydramine N-demethylation in human liver microsomes**

The percentage contribution of each P450 isozyme to diphenhydramine N-demethylation in human liver microsomes was estimated by application of the RAF (Crespi et al., 1995). The RAF for CYP1A2 was determined as the ratio of the activity of phenacetin O-deethylation, a specific metabolic reaction of CYP1A2, in human liver microsomes to that of recombinant CYP1A2. Similarly, diclofenac 4’-hydroxylation, (S)-mephenytoin 4’-hydroxylation and
bufuralol 1'-hydroxylation were used for calculations of the RAFs of CYP2C9, CYP2C19 and CYP2D6, respectively. The clearance of each P450 isozyme in human liver microsomes is represented by the clearance of each recombinant P450 isozyme multiplied by the RAF. Clearances of human liver microsomes are shown as the sum of the clearances of P450 isozymes in human liver microsomes. The contribution of each P450 isozyme to diphenhydramine N-demethylation in human liver microsomes is represented by the percentage of clearance of human liver microsomes. The percentages of contribution of P450 isozymes to diphenhydramine N-demethylation in human liver microsomes estimated by application of the RAF were compared to the percentages of inhibition by P450 isozyme-specific inhibitors.
Results

*Diphenhydramine N-demethylation activity in recombinant P450 isozymes*

Diphenhydramine N-demethylation activities at 0.5 µM in microsomes of baculovirus-infected insect cells expressing 14 individual P450 isozymes were determined (Fig. 2). CYP2D6 showed the highest activity of diphenhydramine N-demethylation (0.69 pmol/min/pmol P450). The activity of recombinant CYP2C19 was 0.071 pmol/min/pmol P450, the second highest. Recombinant CYP1A2, CYP2B6 and CYP2C18 also showed diphenhydramine N-demethylation activities (0.043, 0.023 and 0.036 pmol/min/pmol P450, respectively), while CYP1A1, CYP2C9 and CYP3A4 showed detectable but low activity (0.010, 0.0082 and 0.0084 pmol/min/pmol P450, respectively).

*Kinetics of diphenhydramine N-demethylation activity in pooled human liver microsomes*

The Eadie-Hofstee plot for diphenhydramine N-demethylation activity in pooled human liver microsomes showed a biphasic pattern (data not shown). $V_{max1}$ and $K_{m1}$, kinetic parameters for the high-affinity component, were calculated to be 30.2 pmol/min/mg protein and 13.3 µM, respectively. $V_{max2}$ and $K_{m2}$, kinetic parameters for the low-affinity component, were calculated to be 551 pmol/min/mg protein and 401 µM, respectively. Each Parameter was calculated by the mean of three independent analyses.

*Diphenhydramine N-demethylation activity in individual human liver microsomes*

As shown in Fig. 3, diphenhydramine N-demethylation activities at 0.5 µM varied 4.5 fold (0.87 to 3.89 pmol/min/mg protein) among 12 human liver microsomes tested. Diphenhydramine N-demethylation activity in human liver microsomes was completely inhibited by SKF-525A (1 mM), a typical P450 inhibitor, which was incubated concomitantly with diphenhydramine, and was not detected in the absence of the NADPH generating system.
Correlations between diphenhydramine N-demethylation activity in the presence or absence of quinidine and specific activities of other P450 isozymes in human liver microsomes

As shown in Table 1, correlation coefficients between diphenhydramine N-demethylation activities in the presence or absence of 1 µM quinidine and specific activities of other P450 isozymes were calculated in human liver microsomes from 12 individual donors. In the absence of quinidine, diphenhydramine N-demethylation activity was significantly correlated with bufuralol 1’-hydroxylation activity \( (r = 0.672, p < 0.05) \) and lauric acid 12-hydroxylation activity \( (r = 0.616, p < 0.05) \). On the other hand, when CYP2D6 activities were inhibited by quinidine, phenacetin \( O \)-deethylation activity \( (r = 0.721, p < 0.01) \) and diclofenac 4’-hydroxylation activity \( (r = 0.822, p < 0.01) \) were significantly correlated with residual diphenhydramine N-demethylation activity. Percentages of inhibition of diphenhydramine N-demethylation by quinidine in human liver microsomes from 12 individual donors were 4.8 to 76.1%.

Inhibition studies with furafylline, sulfaphenazole and omeprazole in individual and pooled human liver microsomes

Diphenhydramine N-demethylation activities in the presence of furafylline, sulfaphenazole or omeprazole (each 10 µM) were determined in four individual samples and one pooled sample of human liver microsomes. The inhibitory effects of these inhibitors and quinidine on diphenhydramine N-demethylation activity are shown in Fig. 4. As shown in Fig. 4B, diphenhydramine N-demethylation of HG66, having a high bufuralol 1’-hydroxylation activity, was hardly inhibited by furafylline (5.9%), sulfaphenazole (5.8%) or omeprazole (6.2%). In HG30, having no detectable bufuralol 1’-hydroxylation activity and high phenacetin activity...
O-deethylation and diclofenac 4’-hydroxylation activity, diphenhydramine N-demethylation was inhibited by furafylline (53.1%), sulfaphenazole (39.2%) and omeprazole (46.7%) (Fig. 4A). In HG89, having a low bufuralol 1’-hydroxylation activity and medium phenacetin O-deethylation, diclofenac 4’-hydroxylation and (S)-mephenytoin 4’-hydroxylation activity, diphenhydramine N-demethylation was inhibited by furafylline (56.0%), sulfaphenazole (25.8%) and omeprazole (37.0%) (Fig. 4C). In HG112, having low bufuralol 1’-hydroxylation and phenacetin O-deethylation activity and high diclofenac 4’-hydroxylation and (S)-mephenytoin 4’-hydroxylation activity, diphenhydramine N-demethylation was inhibited by furafylline (11.8%), sulfaphenazole (34.8%) and omeprazole (54.1%) (Fig. 4D). In pooled human liver microsomes, diphenhydramine N-demethylation was inhibited by furafylline (23.0%), sulfaphenazole (19.7%) and omeprazole (11.2%) (Fig. 4E).

**Kinetics of diphenhydramine N-demethylation in recombinant CYP1A2, CYP2C9, CYP2C19 and CYP2D6**

Concentration-activity relationships for diphenhydramine N-demethylation activities of recombinant CYP1A2, CYP2C9, CYP2C19 and CYP2D6 were able to be fitted by a Michaelis-Menten equation. The kinetic parameters in recombinant CYP1A2, CYP2C9, CYP2C19 and CYP2D6 are listed in Table 2. \( V_{\text{max}} \) value of recombinant CYP2D6 was 2.38 ± 0.24 pmol/min/pmol P450. Recombinant CYP2D6 showed the lowest \( K_m \) value (1.12 ± 0.21 µM). \( V_{\text{max}} \) values of CYP1A2, CYP2C9 and CYP2C19 were 14.0 ± 2.3, 4.43 ± 0.28 and 11.0 ± 2.6 pmol/min/pmol P450, respectively. \( K_m \) values of CYP1A2, CYP2C9 and CYP2C19 were 295 ± 46, 134 ± 32 and 55.7 ± 6.5 µM, respectively.

**Contributions of CYP1A2, CYP2C9, CYP2C19 and CYP2D6 to diphenhydramine N-demethylation in human liver microsomes**

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Contributions of CYP2D6, CYP1A2, CYP2C9 and CYP2C19 to diphenhydramine N-demethylation in four individual samples and one pooled sample of human liver microsomes estimated by the application of RAFs were shown in Fig. 5A. In HG66, which showed a high level of bufuralol 1’-hydroxylation activity, the contribution of CYP2D6 to diphenhydramine N-demethylation was estimated to be up to 80%, whereas the contributions of CYP1A2, CYP2C9 and CYP2C19 were low. In HG30, HG89 and HG112, which showed no detectable or low levels of bufuralol 1’-hydroxylation activity, the contribution of CYP2D6 to diphenhydramine N-demethylation was estimated to be negligible or low (0 to 12.1%). On the other hand, contributions of CYP1A2, CYP2C9 and CYP2C19 in these microsomes were estimated to be relatively high (around 30%) for each isozyme except for CYP1A2 in HG112 (4.2%), which showed a low phenacetin O-deethylolation activity. In pooled human liver microsomes, the contribution of CYP2D6 was estimated to be half of the total activity of diphenhydramine N-demethylation.

Contributions of CYP1A2, CYP2C9, CYP2C19 and CYP2D6 to diphenhydramine N-demethylation estimated by the application of RAFs and by the results of inhibition studies were compared in Fig. 5A and 5B. Contributions of these P450 isoymes to diphenhydramine N-demethylation estimated by the application of RAFs (Fig. 5A) were in good agreement with those estimated by inhibition studies using P450 isozyme-specific inhibitors in four individual samples and one pooled sample of human liver microsomes (Fig. 5B).
Discussion

In the present study, the highest level of activity of diphenhydramine N-demethylation was found in recombinant CYP2D6 at a clinically relevant concentration (0.5 µM) by an LC/MS method developed in our laboratory (Fig. 2). A significant correlation was also found between diphenhydramine N-demethylation activity and bufuralol 1’-hydroxylation activity (r = 0.672, p < 0.05) in human liver microsomes from 12 individual donors (Table 1). Moreover, diphenhydramine N-demethylation was strongly inhibited by quinidine in human liver microsomes having a high level of CYP2D6 activity (HG66, Fig. 4B). Furthermore, the Km value for diphenhydramine N-demethylation of recombinant CYP2D6 is the lowest (1.12 ± 0.21 µM) among P450 isozymes tested (Table 2). These results indicate that N-demethylation of diphenhydramine is mainly catalyzed by CYP2D6 as a high-affinity P450 isozyme in vitro.

In a previous case report, the half-life of diphenhydramine was 2-fold greater than the usual value in an elderly woman who had a prolonged half-life of imipramine (Glassman et al., 1985), which is mainly metabolized by CYP2D6 (Koyama et al., 1997), suggesting that diphenhydramine is a substrate of CYP2D6 in vivo. In addition, diphenhydramine is known to inhibit the metabolism of several other CYP2D6 substrates in vitro and in vivo (Hamelin et al., 1998; He et al., 2002; Hamelin et al., 2000; Lessard et al., 2001). These findings coupled with the results of the present study suggest that diphenhydramine is not only a potent inhibitor of CYP2D6 but also a high-affinity substrate of CYP2D6 in vitro and in vivo.

Since an Eadie-Hofstee plot in pooled human liver microsomes showed a biphasic pattern, multiple P450 isozymes are thought to be involved in N-demethylation of diphenhydramine in addition to CYP2D6. In the present study, we obtained several results supporting the involvement of CYP1A2, CYP2C9 and CYP2C19 in N-demethylation of diphenhydramine as low-affinity components. In previous studies (Hamelin et al., 2001; Sharma and Hamelin 2003), N-demethylation of diphenhydramine was reported to be mediated by multiple P450 isozymes,
CYP1A2, CYP2C18, CYP2C19, CYP2D6 and CYP2B6, at 20 µM. This finding is consistent with the results of the present study showing that CYP1A2 and CYP2C19 are involved in diphenhydramine N-demethylation as low-affinity components, whereas it is inconsistent with the finding in the present study that CYP2C9 is involved as one of the low-affinity enzymes in diphenhydramine N-demethylation. However, the results of the present study showed that diclofenac 4’-hydroxylation was significantly correlated with diphenhydramine N-demethylation activity ($r = 0.822$, $p < 0.01$) in human liver microsomes from 12 individual donors when CYP2D6 activity was inhibited by quinidine (Table 1). Furthermore, an inhibitory effect of sulfaphenazole on N-demethylation of diphenhydramine was seen in human liver microsomes having low levels of or no detectable activity of CYP2D6 (Fig. 4) and was in good agreement with the contribution of CYP2C9 estimated by application of the RAF (Fig. 5). Therefore, diphenhydramine N-demethylation seems to be mediated by CYP2C9 in addition to CYP1A2 and CYP2C19 as low-affinity components in human liver microsomes. In the present study, although a significant correlation was also found between lauric acid 12-hydroxylation and diphenhydramine N-demethylation activity (Table 1), involvement of CYP4A was not examined because a correlation was not found when CYP2D6 was inhibited by quinidine (Table 1) and diphenhydramine N-demethylation activity was not detectable in recombinant CYP4A11 (Fig. 2). In addition, involvement of CYP2C18 and CYP2B6 was not examined in the present study because the results of inhibition studies using P450 isozyme-specific inhibitors in individual and pooled human liver microsomes showed that low-affinity components involved in N-demethylation of diphenhydramine are almost completely explained by CYP1A2, CYP2C9 and CYP2C19 (Fig. 4).

The main adverse effects of classic antihistamines are effects on the central nervous system such as sedation, impairment of cognitive function and psychomotor performance (Hindmarch and Shamsi, 1999; Welch and Meltzer, 2002). The sedative effect of
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diphenhydramine has been reported to be correlated with its plasma concentration (Carruthers et al., 1978). Therefore, when the major metabolic process of diphenhydramine and a concomitant drug(s) is mediated by the same P450 isozyme(s), a sedative effect may be induced by an increase in the plasma concentration of diphenhydramine. The results of the present study indicate that diphenhydramine is mainly metabolized by CYP2D6, which is known to catalyze the oxidative metabolism of various clinically important drugs (Zanger et al., 2004). In addition, since diphenhydramine is available without prescription (Food and Drug Administrations, 2002), it is possible that it is taken with various drugs. Therefore, it should be emphasized that the plasma concentration of diphenhydramine may be increased and a sedative effect may be induced by co-administration of CYP2D6 substrates/inhibitors. Besides, in the case of poor metabolizer phenotypes of CYP2D6, drug-drug interaction may occur between diphenhydramine and substrates/inhibitors/inducers of CYP1A2, CYP2C9 and CYP2C19, because N-demethylation of diphenhydramine seems to be mediated by these P450 isozymes as low-affinity components.

In the present study, diphenhydramine N-demethylation activities at 0.5 µM varied 4.5 fold (0.87 to 3.89 pmol/min/mg protein) among human liver microsomes tested (Fig. 5). In addition, the results of the present study indicate that diphenhydramine N-demethylation is mainly catalyzed by CYP2D6. Therefore, the plasma concentration of diphenhydramine is thought to be influenced by the metabolic activity of CYP2D6, which varies extensively among individuals who can be categorized on the basis of their CYP2D6 activity as ultrarapid, extensive, intermediate, and poor metabolizers, which are reflected by CYP2D6 genetic polymorphism (Zanger et al., 2004). However, a previous study showed that the clearance of diphenhydramine to its N-demethylated metabolite was not different in extensive and poor metabolizer phenotypes of CYP2D6 (Lessard et al., 2001). Although the reason for this discrepancy is unclear, in the case of poor metabolizer phenotypes of CYP2D6, individual
differences in plasma concentration of diphenhydramine could be caused by large differences in metabolic activities of CYP1A2, CYP2C9 and CYP2C19 (Chiba, 1998; Kirchheiner and Brockmoller, 2005; Furuta et al., 2005). Furthermore, CYP1A2 is known to be a P450 isozyme inducible by environmental factors such as polycyclic aromatic hydrocarbons (Pelkonen et al., 1998; Sherson et al., 1992). Therefore, the effects of genetic polymorphisms of CYP2D6 on the disposition of diphenhydramine may be masked by the interindividual differences in the metabolic capacity of these P450 isozymes. Further studies are needed to clarify the involvement of CYP2D6 in the metabolism of diphenhydramine N-demethylation as a major enzyme in vivo. Such study is now underway in our laboratory.

In conclusion, N-demethylation of diphenhydramine is mainly catalyzed by CYP2D6 as a high-affinity P450 isozyme at a clinically relevant concentration. Therefore, it seems that diphenhydramine is not only a potent inhibitor of CYP2D6 but also a high-affinity substrate of CYP2D6. It should be noted that the sedative effect of diphenhydramine might be caused by an increase in the plasma concentration of diphenhydramine by concomitant use of CYP2D6 substrates/inhibitors. In addition, CYP1A2, CYP2C9 and CYP2C19 are involved in the metabolism of diphenhydramine as low-affinity P450 isozymes. Therefore, it should also be noted that diphenhydramine might interact with substrates/inhibitors/inducers of these P450 isozymes in the case of poor or intermediate metabolizer phenotype of CYP2D6.
References


of CYP2E1 as a low-affinity enzyme in phenacetin O-deethylation in human liver microsomes. Drug Metab Dispos 27: 860-865.


Footnotes

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Figure legends

Fig. 1. Mass chromatograms of 2-benzhydryloxy-N-methyl-etanamine (N-demethyl diphenhydramine) (A), diphenhydramine (B) and orphenadrine derived from an incubation mixture of human liver microsomes (C).

Pseudo-molecular ions of N-demethyl diphenhydramine, diphenhydramine and orphenadrine were monitored at m/z 242, 256 and 270, respectively. The retention times of N-demethyl diphenhydramine, diphenhydramine and orphenadrine in this analytical condition were 5.5, 6.6 and 9.0 min, respectively.

Fig. 2. Diphenhydramine N-demethylation activity at 0.5 µM in microsomes of baculovirus-infected insect cells expressing 14 different P450 isozymes individually.

Five pmol/ml of recombinant P450 isozyme was incubated with 0.5 µM diphenhydramine at 37˚C for 20 min. The incubation was performed in the presence of NADPH. Recombinant P450 isozymes used in the present study were co-expressed with cytochrome P450 oxidoreductase and/or cytochrome b5. The amounts of cytochrome P450 oxidoreductase and cytochrome b5 of the recombinant P450 isozymes used in the present study were 250 – 4900 nmol/min/mg protein and 200 – 1000 pmol/mg protein, respectively. Each column represents the mean of two independent analyses.

b5, cytochrome b5; OR, cytochrome P450 oxidoreductase.

Fig. 3. Diphenhydramine N-demethylation activity at 0.5 µM in human liver microsomes from 12 individual donors.

Human liver microsomal protein (0.1 mg/ml) was incubated with 0.5 µM diphenhydramine at 37˚C for 60 min. The incubation was performed in the presence of NADPH. The amounts of cytochrome P450 oxidoreductase and cytochrome b5 of the human liver
microsomes used in the present study were 241 – 415 nmol/min/mg protein and 445 – 754 pmol/mg protein, respectively. Each column represents the mean ± S.D. of five independent analyses.

Fig. 4. Effects of furafylline, sulfaphenazole, omeprazole and quinidine on diphenhydramine N-demethylation in individual and pooled human liver microsomes.

Individual human liver microsomes (HG33, 66, 89 and 112) and pooled human liver microsomes were incubated with 0.5 µM diphenhydramine and P450 isozyme-specific inhibitors (10 µM of furafylline, sulfaphenazole or omeprazole or 1 µM quinidine) at 37˚C for 60 min. Each value is the percentage of residual activity compared to the control sample (containing no inhibitor). The control activities of diphenhydramine N-demethylation of individual (HG30, 66, 89 and 112) and pooled human liver microsomes were 3.34 ± 0.44, 2.24 ± 0.19, 1.87 ± 0.15, 2.17 ± 0.39 and 1.41 ± 0.13 pmol/min/mg protein, respectively. Each column expresses the mean ± S.D. of three or four independent analyses. CTL, control; FFL, furafylline; SPZ, sulfaphenazole; OPZ, omeprazole; QND, quinidine.

Fig. 5. Comparisons of the contributions of CYP1A2, CYP2C9, CYP2C19 and CYP2D6 to diphenhydramine N-demethylation estimated by the application of RAFs and by the results of inhibition studies.

Contributions of P450 isozymes to diphenhydramine N-demethylation estimated by the application of RAFs (A) and by the results of inhibition studies (B). Data represent predicted contribution of each P450 isozyme to the total activity of each individual or pooled human liver microsomes. Contributions of CYP1A2, CYP2C9, CYP2C19 and CYP2D6 estimated by the application of RAFs in individual and pooled human liver microsomes are as follows; 31.6, 45.9, 22.5 and 0%, respectively, in HG30; 4.1, 13.4, 1.2 and 81.3%, respectively, in HG66; 36.0,
32.1, 27.4 and 4.6%, respectively, in HG89; 4.2, 35.8, 47.9 and 12.1%, respectively, in HG112; and 14.2, 27.5, 9.9 and 48.4%, respectively, in pooled human liver microsomes.
Table 1. Correlations of diphenhydramine N-demethylation activity in the presence or absence of quinidine with P450 isozyme-specific activities in human liver microsomes from 12 individual donors

<table>
<thead>
<tr>
<th>P450 isozymes</th>
<th>Specific activities</th>
<th>Without quinidine</th>
<th>With quinidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>0.468</td>
<td>0.721**</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>0.127</td>
<td>0.177</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>(S)-Mephenytoin N-demethylation</td>
<td>0.099</td>
<td>0.345</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Paclitaxel 6α-hydroxylation</td>
<td>0.143</td>
<td>0.468</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Dicrofenac 4’-hydroxylation</td>
<td>0.346</td>
<td>0.822**</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin 4’-hydroxylation</td>
<td>0.027</td>
<td>0.542</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Bufuralol 1’-hydroxylation</td>
<td>0.672*</td>
<td>-</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>0.310</td>
<td>0.050</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone 6β-hydroxylation</td>
<td>0.072</td>
<td>0.546</td>
</tr>
<tr>
<td>CYP4A</td>
<td>Lauric acid 12-hydroxylation</td>
<td>0.616*</td>
<td>0.404</td>
</tr>
</tbody>
</table>

The correlation coefficients (r) were calculated by the least-squares regression method.

*p < 0.05, **p < 0.01 (statistical significance for r).
Table 2. Michaelis-Menten kinetic parameters of diphenhydramine N-demethylation in baculovirus infected-insect cells expressing CYP1A2, CYP2C9, CYP2C19 or CYP2D6

<table>
<thead>
<tr>
<th>P450 Isozymes</th>
<th>Vmax (pmol/min/pmol P450)</th>
<th>Km (µM)</th>
<th>Vmax/Km (µl/min/pmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>14.0±2.3</td>
<td>295±46</td>
<td>0.047</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>4.43±0.28</td>
<td>134±32</td>
<td>0.033</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>11.0±2.6</td>
<td>55.7±6.5</td>
<td>0.198</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>2.38±0.24</td>
<td>1.12±0.21</td>
<td>2.13</td>
</tr>
</tbody>
</table>

Data represent mean ± S.D. of three independent analyses.
Fig. 1

(A) 2-Benzhydryloxy-N-methyl-ethanam
    (N-Demethyl diphenhydramine)
    (m/z 242)

(B) Diphenhydramine
    (m/z 256)

(C) Orphenadrine
    (m/z 270)
Fig. 5

(A) RAF

Human liver microsomes

(B) Inhibition study

Human liver microsomes