Involvement of CYP2A6 in the Formation of a Novel Metabolite, 3-Hydroxypilocarpine, from Pilocarpine in Human Liver Microsomes

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ABSTRACT:

Pilocarpine is a cholinergic agonist that is metabolized to pilocarpic acid by serum esterase. In this study, we discovered a novel metabolite in human urine after the oral administration of pilocarpine. The structure of the metabolite was identified as 3-hydroxypilocarpine by liquid chromatography-tandem mass spectrometry and NMR analyses and by comparing to the authentic metabolite. To clarify the human cytochrome P450 responsible for the metabolite formation, in vitro experiments using P450 isoform-selective inhibitors, cDNA-expressed human P450s (Supersomes; CYP1A2, -2A6, -2B6, -2C9, -2C19, -2D6, -2E1, and -3A4), and liver microsomes from different donors were conducted. The formation of 3-hydroxypilocarpine in human liver microsomes was strongly inhibited (>90%) by 200 μM coumarin. Other selective inhibitors of CYP1A2 (furafylline and α-naphthoflavone), CYP2C9 (sulfaphenazole), CYP2C19 ([S]-methylenetetrazol, CYP2E1 (4-methylpyrazole), CYP2D6 (quinidine), and CYP3A4 (troleandomycin) had a weak inhibitory effect (<20%) on the formation. The highest formation activity was expressed by recombinant CYP2A6. The \( K_{in} \) value for recombinant CYP2A6 was 3.1 \( \mu \)M, and this value is comparable with that of human liver microsomes (1.5 \( \mu \)M). The pilocarpine 3-hydroxylation activity was correlated with coumarin 7-hydroxylation activity in 16 human liver microsomes \((r = 0.98)\). These data indicated that CYP2A6 is the main enzyme responsible for the 3-hydroxylation of pilocarpine. In conclusion, we identified a novel metabolite of pilocarpine, 3-hydroxypilocarpine, and we clarified the involvement of CYP2A6 in the formation of this molecule in human liver microsomes.
Materials and Methods

Materials. Pilocarpine hydrochloride and [imidazol-2-14C]pilocarpine hydrochloride ([14C]pilocarpine hydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO) and GE Healthcare (Little Chalfont, Buckinghamshire, UK), respectively. The radiochemical purity of [14C]pilocarpine hydrochloride was confirmed to exceed 98% (by HPLC). [4-(1-Imidazolyl)-phenyl]oxoacetic acid (internal standard) and 3-hydroxypilocarpine were synthesized by Kissei Pharmaceutical (Nagano, Japan). Coumarin, 7-hydroxycoumarin, treoleandomycin, quinidine sulfate dihydrate, sulfaphenazole, and 4-methylpyrazole hydrochloride, α-naphthoflavone, and furafylline were purchased from Sigma-Aldrich. Aldrich (St. Louis, MO) and GE Healthcare (Buckinghamshire, UK), respectively. The radiochemical purity of [14C]pilocarpine hydrochloride was achieved from its single crystal X-ray diffraction pattern (data not shown).

The isolated M-1 was characterized and identified by NMR spectroscopy. 1H NMR spectra in deuteromethanol (CD3OD) were recorded at 500 MHz using a Bruker DRX500 spectrometer (Bruker Instruments Inc., Billerica, MA). The chemical shifts are expressed in parts per million relative to tetramethylsilane or the residual methyl signal (3.30 ppm) of methanol as an internal standard.

Based on the mass spectral and NMR analyses, the proposed structure for M-1 was constructed as shown in Fig. 1. The synthesized (S)- and (R)-diastereomers, namely, 3-hydroxyisopilocarpine and 3-hydroxypilocarpine, were separated on a C18 column. The configuration of the authentic sample was confirmed from its single crystal X-ray diffraction pattern (data not shown).

Incubation of [14C]Pilocarpine in Human Microsomes. In vitro metabolism was investigated in human liver microsomes using 14C-labeled pilocarpine. The mixture of [14C]pilocarpine and nonlabeled pilocarpine (1:1; final 10 μM) was incubated in a reaction mixture made up of 50 mM potassium phosphate buffer, pH 7.4, 1 mg/ml human liver microsomes, an NADPH-generating system (0.8 mM NADP+, 8 mM glucose 6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, and 5 mM MgCl2), and 1 mM EDTA in a final volume of 200 μl. The reaction mixture was monitored by TLC and confirmed by thin layer chromatography (TLC). The metabolites were identified by co-chromatography on TLC plates with known standards. The identity of the metabolites was confirmed by LC-MS/MS and NMR analyses. The radioactivity in the column eluate was detected by the use of a radiochemical detector (Radiomatic 525TR; PerkinElmer Life and Analytical Sci-
Pilocarpine 3-Hydroxylation in Human Liver Microsomes. The generation of 3-hydroxypilocarpine (M-1) in human liver microsomes was conducted under the conditions described above. To establish the optimal reaction time and microsomal protein concentration, the reaction was investigated with the reaction times of 30, 60, 90, and 120 min and with protein concentrations of 0.5, 1.0, and 1.5 mg/ml at a pilocarpine concentration of 10 μM. The amount of production was observed to increase linearly with incubation time and protein concentration. Accordingly, the reaction time and protein concentration were set at 60 min and 1.0 mg/ml, respectively. The pilocarpine concentration was set at 2 μM, with the exception of the enzyme kinetics studies (0.125–25 μM). For the determination of the kinetic parameters (Km and Vmax), an Edadie-Hofstee plot was constructed. The values were used to estimate the apparent kinetic parameters by linear least-squares regression analysis.

Inhibition Study with P450 Isoform-Selective Inhibitors. The following P450 isoform-selective inhibitors were used at the designated concentrations for the inhibition study: α-Naphthoflavone (1 μM final concentration) and furafylline (20 μM) for CYP1A2, coumarin (200 μM) for CYP2A6, sulfaphenazole (20 μM) for CYP2C9, (S)-mephenytoin (250 μM) for CYP2C19, quinidine (5 μM) for CYP2D6, 4-methylpyrazole (500 μM) for CYP2E1, and troleandomycin (100 μM) for CYP3A4. Quinidine and 4-methylpyrazole were dissolved in distilled water; α-naphthoflavone, coumarin, sulfaphenazole, (S)-mephenytoin, and troleandomycin were dissolved in acetonitrile; and furafylline was dissolved in methanol. The final concentration of the organic solvents was 0.5%. For the competitive inhibition studies with α-naphthoflavone, coumarin, sulfaphenazole, (S)-mephenytoin, quinidine, and 4-methylpyrazole, the reaction was initiated by the addition of microsomes after a 3-min preincubation at 37°C. For the mechanism-based inhibition studies with furafylline and troleandomycin, the reaction was initiated by the addition of microsomes after a 3-min preincubation at 37°C. The formation activities in the presence of the inhibitors are expressed as a residual percentage of the corresponding control values, in the presence of solvent instead of the inhibitors.

Pilocarpine 3-Hydroxylation by Recombinant P450 Isosforms. To identify the P450 isoforms responsible for the formation of 3-hydroxypilocarpine, microsomes from baculovirus-infected insect cells expressing CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 (Supersomes) were used. All the recombinant P450 isoforms were coexpressed with NADPH-cytochrome P450 oxidoreductase; CYP2A6, -2B6, -2C9, -2C19, -2E1, and -3A4 were also coexpressed with cytochrome b5. The concentrations of pilocarpine and each recombinant P450 isoform in the incubation mixture were 2 μM and 50 pmol/ml, respectively. The other incubation conditions were the same as described above, with the exception that the incubation time was 30 min.

The kinetic study with recombinant CYP2A6 was performed as described above, with the modification that 50 mM Tris buffer, pH 7.4, was used instead of phosphate buffer. The amount of 3-hydroxypilocarpine produced increased linearly with incubation time up to 60 min and also increased linearly with P450 content up to 75 pmol/ml at a pilocarpine concentration of 2 μM.

Determination of 3-Hydroxypilocarpine Using LC-MS/MS. The concentration of 3-hydroxypilocarpine was determined by LC-MS/MS following acetonitrile precipitation of the incubation mixture. Each of 500-μl aliquots was mixed with 100 μl of 10 ng/ml internal standard (IS) [4-(1-imidazolyl) phenyl] oxycacetic acid. The mixture was evaporated under a stream of nitrogen gas at room temperature and reconstituted in 100 μl of 10 mM ammonium acetate buffer, pH 5.0, containing 2% (v/v) acetonitrile. A 20-μl aliquot was run on a Shimadzu LC-10ADVP system (Shimadzu, Kyoto, Japan). 3-Hydroxypilocarpine and the IS were separated on a CAPCELL PAK C18 MG column (3 μm, 2 × 35 mm; Shiseido, Tokyo, Japan). The column temperature was set at 40°C. The mobile phase consisted of 10 mM ammonium acetate buffer, pH 5.0, containing 2% (v/v) acetonitrile (A) and acetonitrile (B). The initial concentration of B was set at 0%, increased linearly to 70% over 4 min, held for 1 min, and then returned to the initial condition in 0.1 min. The flow rate was maintained at 0.2 ml/min. The HPLC was interfaced with a PE Sciex API-3000 tandem mass spectrometer (Applied Biosystems, Foster City, CA) operated in the positive ionization mode using a turbo ion spray ionization source. The heated nebulizer probe temperature was 550°C, and the turbo ion spray voltage was set at 5000 V. Multiple ion monitoring of the following precursor-product ion combinations was used for the detection of analytes: 3-hydroxypilocarpine, m/z 225 → 123; and IS, m/z 219 → 160.

Determination of Coumarin 7-Hydroxylation Activity. Coumarin 7-hydroxylation activity was determined as described previously (Greelee and Poland, 1978; Pearce et al., 1992).

Statistical Analysis. The correlations between pilocarpine 3-hydroxylase activities and coumarin 7-hydroxylase activities in human liver microsomes were determined by the Spearman rank correlation test.

Results

Identification of the Metabolite. In this study, an unidentified metabolite of pilocarpine (M-1) was isolated by HPLC and identified by LC-MS/MS and NMR analyses.

The SIM chromatograms of pilocarpic acid, M-1, and pilocarpine from human urine samples (B) and from the blank urine samples (A) are shown in Fig. 2. Two peaks at m/z 227 with retention times of 1.8 and 3.0 min, corresponding to the pilocarpic acid protonated molec-
When the incubation was conducted with an NADPH-generating system, only a single metabolite with a retention time of 6 min was detected; this retention time was similar to that of 3-hydroxy pilocarpine. Based on this observation, the generated metabolite was thought to be 3-hydroxy pilocarpine. This supposition was confirmed by the liquid chromatography-mass spectrometry and LC-MS/MS analyses. From the liquid chromatography-mass spectrometry analysis, it was demonstrated that the metabolite peak exhibits a protonated molecule [M + H]+ at m/z 225 and 227 (14C). Further fragmentation of the precursor ion m/z 227 (14C) resulted in a major fragment ion at m/z 125 (14C); this fragmentation pattern was identical to that of 3-hydroxy pilocarpine (data not shown).

The generation of 3-hydroxy pilocarpine was observed in human liver microsomes with an NADPH-generating system, and this fact suggested that P450 is involved in the generation. Pilocarpic acid and other metabolites were not detected in human liver microsomes regardless of the presence or absence of an NADPH-generating system.

**Kinetics of 3-Hydroxy Pilocarpine Formation in Human Liver Microsomes.** Figure 6 shows an Eadie-Hofstee plot for the 3-hydroxylation of pilocarpine in pooled human liver microsomes. The plot was almost monophasic and indicated that a single enzyme was responsible for the formation of 3-hydroxy pilocarpine from pilocarpine. The apparent Km and Vmax values were 1.5 μM and 8.3 pmol/min/mg, respectively.

**Inhibition Study with P450 Isoform-Selective Inhibitors.** The effects of P450 isoform-selective inhibitors on the formation of 3-hydroxy pilocarpine at 2 μM pilocarpine in pooled human liver microsomes is shown in Fig. 7. Only coumarin (CYP2A6) strongly inhibited the formation of 3-hydroxy pilocarpine (9% of the control value). Troleandomycin (CYP3A4) and 4-methylpyrazole (CYP2E1) slightly inhibited the formation (84 and 81% of the control value, respectively). α-Naphthoflavone and furafylline (CYP1A2), sulfaphenazole (CYP2C9), (S)-mephentoin (CYP2C19), and quinidine (CYP2D6) had no inhibitory effect on the formation of 3-hydroxy pilocarpine. These data suggested that CYP2A6 accounted for the majority of the 3-hydroxylation of pilocarpine in human liver microsomes.

**Pilocarpine 3-Hydroxylation by Recombinant P450 Isoforms.** To identify the human P450 involved in the formation of 3-hydroxy pilocarpine, the capability of pilocarpine 3-hydroxylation by recombinant P450 isoforms expressed in baculovirus-infected insect cells (Supersomes) was investigated. As shown in Fig. 8, CYP2A6 exhibited the highest pilocarpine 3-hydroxylation activity (102.0 fmol/min/mg). CYP3A4 also expressed activity (8.7 fmol/min/pmol P450), although the activity was very low compared with that of CYP2A6. No detectable levels of 3-hydroxy pilocarpine (≤3.3 fmol/min/pmol P450) were observed in the presence of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, or CYP2E1.

An Eadie-Hofstee plot of the 3-hydroxylation of pilocarpine by recombinant CYP2A6 is presented in Fig. 9. The plot is monophasic, and the Km and Vmax values obtained were 3.1 μM and 99.5 fmol/min/pmol P450, respectively. The Km value was comparable with that obtained from human liver microsomes.

**Correlation Analysis of 3-Hydroxy Pilocarpine Formation.** The formation of 3-hydroxy pilocarpine was examined in human liver microsomes prepared from 16 human livers (Fig. 10). The activity correlated well (r = 0.98; P < 0.01) with the activity of CYP2A6-selective coumarin 7-hydroxylation. This finding strongly supports the supposition that CYP2A6 is involved in the formation of 3-hydroxy pilocarpine.

**Discussion**

Pilocarpine is used primarily in the treatment of glaucoma and is now increasingly being used to treat xerostomia caused either by a decrease in saliva production following radiation treatment for head...
and neck cancers or due to Sjögren's syndrome. Even though pilocarpine has a long history clinical use, information on its metabolic fate is limited. The aims of this study were to identify an unidentified metabolite of pilocarpine (M-1) and to identify the metabolic enzyme responsible for M-1 formation.

In human urine samples after the oral administration of pilocarpine, a significant amount of unidentified metabolite was detected on the SIM chromatogram at m/z 225 (Fig. 2). This metabolite was identified as 3-hydroxypilocarpine by both LC-MS/MS and 1H NMR analyses. Based on the HPLC analysis with UV detection, 3-hydroxypilocarpine seems to be one of the major metabolites of pilocarpine. A minor peak, also detected on the SIM chromatogram at m/z 225 and the HPLC-UV chromatogram with a retention time approximately 8 min, is also thought to be a metabolite of pilocarpine. The amount of this metabolite was less than 10% 3-hydroxypilocarpine, and the effect on the metabolic clearance of pilocarpine is considered to be insignificant. Since the diastereomer of 3-hydroxypilocarpine (i.e., 3-hydroxyisopilocarpine) was separated at an earlier time than 3-hydroxypilocarpine under the separation conditions used (data not shown), the minor metabolite seems to be a novel metabolite hydroxylated at other carbon positions. These findings indicated that pilocarpine is metabolized to 3-hydroxypilocarpine (3R-configuration) but not to 3-hydroxyisopilocarpine (3S-configuration); therefore, the hydroxylation was demonstrated to be highly stereoselective. The degradation of pilocarpine by epimerization to isopilocarpine and hydrolysis to isopilocarpic acid and pilocarpic acid in ophthalmic solutions has been
reported (Kreienbaum and Page, 1986); however, isopilocarpine and isopilocarpic acid were not detected on the SIM chromatograms. This result suggests that epimerization to isopilocarpine and its subsequent hydrolysis do not occur in vivo. Therefore, the degradation and/or metabolism to isopilocarpine and isopilocarpic acid are not the key to understanding the high-plasma clearance of pilocarpine.

In the study of in vitro metabolism in human liver microsomes using [14C]pilocarpine, the only metabolite generated was 3-hydroxypilocarpine. The metabolism required NADPH, and this suggests that P450 is involved in the hydroxylation. Pilocarpic acid and other metabolites were not detected in human liver microsomes, regardless of the presence or absence of an NADPH-generating system. Carboxylesterase is present in human liver microsomal fractions, and it is known to hydrolyze carboxester, thioester, and the amide bonds of endogenous and exogenous compounds (Hosokawa et al., 1995). Our results suggest that P450 and carboxylesterase in human liver microsomes are not involved in the hydrolysis of pilocarpine. It was reported that pilocarpine is hydrolyzed in the serum and aqueous humor of rabbits and humans (Ellis et al., 1972; Aromdee et al., 1996). The hydrolysis in serum was strongly inhibited by EDTA and p-
Pilocarpine (2 μM) was incubated at 37°C for 30 min with each recombinant P450 isoform (50 pmol/ml) expressed in baculovirus-infected insect cells (Supersomes). Each column represents the mean of duplicate determinations. ND, below the lower limit of quantitation (3.3 fmol/min/pmol P450).

The lower limit of quantitation (3.3 fmol/min/pmol P450) was used as a threshold for detection. Each column represents the mean of duplicate determinations. ND, below the lower limit of quantitation (3.3 fmol/min/pmol P450).

Chloromercuribenzoic acid (Lavallee and Rosenkrantz, 1965). Recently, Li et al. (2005) reported that butyrylcholinesterase (EC3.1.1.8), paraoxonase (EC3.1.8.1), and albumin esterase (EC3.1.1.7), but not carboxylesterase (EC3.1.1.1), are present in human plasma. Paraoxonase hydrolyzes many lactone compounds, including lactone-containing drugs, such as simvastatin, lovastatin, and spironolactone (Billecke et al., 2000; Khersonsky and Tawfik, 2005). The hydrolytic activity was strongly inhibited by EDTA (Gan et al., 1991; Kuo and La Du, 1995). These properties are similar to those of pilocarpine esterase and suggest that pilocarpine esterase is synonymous with paraoxonase.

An Eadie-Hofstee plot for 3-hydroxypilocarpine formation in human liver microsomes revealed that a single enzyme is predominantly involved. The K_m value was comparable with that of recombinant CYP2A6. In the inhibition study using P450 isoform-selective inhibitors, only coumarin strongly inhibited 3-hydroxypilocarpine formation. In addition, a strong correlation was observed between pilocarpine 3-hydroxylation activity and coumarin 7-hydroxylation activity, the latter of which is an in vitro probe for CYP2A6 activity. These data strongly support the assumption that CYP2A6 is the main enzyme responsible for the formation of 3-hydroxypilocarpine from pilocarpine in human liver microsomes. It is established that pilocarpine is an inhibitor of CYP2A6 (i.e., coumarin 7-hydroxylase activity; K_i = 1–4 μM) (Kinonen et al., 1995; Bourrie et al., 1996; Li et al., 1997); however, although pilocarpine competitively inhibits coumarin-7-hydroxylase activity, it was previously believed that pilocarpine is not a substrate for CYP2A6. Moreover, the only metabolite identified is pilocarpic acid generated by hydrolysis. In the present study, however, we revealed that pilocarpine is not only an inhibitor of CYP2A6 but also a substrate for CYP2A6.

The pilocarpine C_max value (normalized to 5-μg doses) after a single oral administration of pilocarpine to subjects was approximately 20 to 30 ng/ml (0.08–0.12 μM) in plasma (St. Peter et al., 2000). The liver concentration of radioactivity was 10 times higher than that of plasma after a single oral administration of [14C]pilocarpine to rats (Omori et al., 2004), and the protein binding of pilocarpine in human plasma seems to be less than 5% (van de Merbel et al., 1998). The pilocarpine concentration in the human liver was estimated under the assumption that its liver/plasma ratio in humans is similar to that in rats, and the value was close to the K_m value for the 3-hydroxylation of pilocarpine. This suggests that CYP2A6 is involved in the metabolism of pilocarpine in vivo. In the human liver, CYP2A6 comprises 4% of the total P450 (Shimada et al., 1994), whereas it is not detectable in the human intestine (Paine et al., 2006). Orally administered pilocarpine is assumed to be primarily metabolized to 3-hydroxypilocarpine by CYP2A6 in the liver. The unchanged pilocarpine delivered to the systemic circulation would then be metabolized to pilocarpic acid by esterase. These metabolites are assumed to be excreted into the urine because of their relatively low molecular weight and low protein binding. It has been reported that approximately 35% of dosed 3-hydroxypilocarpine and 20% of dosed pilocarpine were excreted into urine (Aromdee et al., 1999), and approximately equal amounts of pilocarpic acid and pilocarpine were detected in urine (van de Merbel et al., 1998). These data are consistent with our results obtained from the SIM and HPLC chromatograms of urine samples.

St. Peter et al. (2000) reported that no significant regression relationships were noted between creatinine clearance and the pilocarpine elimination rate constant, time of maximum concentration, volume of distribution, clearance, or area under the curve, whereas Garg et al. (1996) reported that the area under the curve and clearance were significantly changed in the subjects with hepatic impairment compared with normal subjects. This suggests that the contribution of metabolic clearance in the liver to the clearance of pilocarpine is more significant than that of renal clearance. Because 3-hydroxypilocarpine is the main metabolite accounting for at least one third of the admin-

![Fig. 8. Pilocarpine 3-hydroxylation activities of recombinant human P450 isoforms. Pilocarpine (2 μM) was incubated at 37°C for 30 min with each recombinant P450 isoform (50 pmol/ml) expressed in baculovirus-infected insect cells (Supersomes). Each column represents the mean of duplicate determinations. ND, below the lower limit of quantitation (3.3 fmol/min/pmol P450).](image)

![Fig. 9. Kinetic analyses of pilocarpine 3-hydroxylation by CYP2A6 Supersomes. CYP2A6 Supersomes were incubated with 0.125 to 25 μM pilocarpine at 37°C for 30 min. Each data point represents the mean of duplicate determinations.](image)

![Fig. 10. Correlation of 3-hydroxypilocarpine formation and 7-hydroxycoumarin formation in microsomes from 16 human livers. Each data point represents the mean of duplicate determinations.](image)
istered pilocarpine in humans and it is produced by first-pass metabolism in the liver, the 3-hydroxylation of pilocarpine by CYP2A6 is thought to play a major role in pilocarpine clearance.

It has been demonstrated that CYP2A6 is involved in the metabolism of coumarin (Miles et al., 1990), nicotine (Nakajima et al., 1996), tegafur (Komatsu et al., 2000), SM-12502 (Nunoya et al., 1996), and caffeine (Kimura et al., 2005). In humans, a large individual variation has been demonstrated for CYP2A6 activity (Rautio et al., 1992), and it has been revealed that CYP2A6 gene polymorphism is involved in this variation (Nakajima et al., 2001). After the oral administration of pilocarpine to subjects, relatively large individual variations of clearance (5.5-fold difference) were observed (St. Peter et al., 2000). Based on the results in the present study, CYP2A6 is suggested to be significantly involved in the clearance of pilocarpine, and the pilocarpine clearance might be affected by the genetic polymorphism of the CYP2A6 gene.

Acknowledgments. We thank Drs. Tsuyoshi Yokoi and Miki Nakajima (Kanazawa University, Kanazawa, Japan) for help and advice in preparing this article.

References