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

Takuro Endo, Masaaki Ban, Kazuma Hirata, Akitoshi Yamamoto, Yoshiki Hara, and Yasunori Momose

Pharmacokinetics Research (T.E., A.Y., Y.H., Y.M.), Process Chemistry (M.B., K.H.), Kissei Pharmaceutical Co., Ltd., Nagano, Japan

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. We investigated the metabolic enzyme responsible for the metabolite formation. 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 (P450) responsible for the metabolite formation, in vitro experiments using P450 isoform-selective inhibitors, cDNA-expressed human P450s (Supersomes; CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4), 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-mephénytoin), 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_{m} value for recombinant CYP2A6 was 3.1 μM, and this value is comparable with that of human liver microsomes (1.5 μM). The pilocarpic 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.

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. We investigated the metabolic enzyme responsible for the metabolite formation. 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 (P450) responsible for the metabolite formation, in vitro experiments using P450 isoform-selective inhibitors, cDNA-expressed human P450s (Supersomes; CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4), 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-mephénytoin), 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_{m} value for recombinant CYP2A6 was 3.1 μM, and this value is comparable with that of human liver microsomes (1.5 μM). The pilocarpic 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.

Pilocarpine is an alkaloid derived from the leaves of South American plants of the genus Pilocarpus. It is a muscarinic, cholinergic agonist, and for many years it has been widely used as eyedrops for the treatment of glaucoma (Hoyng and van Beek, 2000). Pilocarpine has the ability to stimulate salivary secretion; recently, it has been widely used as an oral medication for the treatment of Sjögren’s syndrome and xerostomia resulting from radiation therapy to the head and neck regions (Rhodus and Schuh, 1991; Johnson et al., 1993; Nyarady et al., 2006). In spite many years of clinical use for pilocarpine, its absorption, distribution, metabolism, and excretion are not well understood. Omori et al. (2004) reported on the absorption, distribution, and excretion properties in rats using 14C-labeled pilocarpine. Orally administered [14C]pilocarpine hydrochloride was rapidly and almost completely absorbed from the small intestine and widely distributed throughout the tissues in rats. Approximately 90% of the administered radioactivity was excreted into the urine within 24 h. In humans, the plasma concentration of pilocarpine reached a peak at approximately 1 h after oral administration and was then rapidly eliminated with a half-life of approximately 1 h (St. Peter et al., 2000). The pharmacokinetic parameters for the intravenous administration of pilocarpine in humans revealed a wide distribution (3 l/kg) and a relatively high plasma clearance (0.03 l/min/kg) (Tanzer et al., 1995). However, the metabolic fate of pilocarpine has not been studied. To date, pilocarpic acid is the only identified metabolite, and it is produced by the cleavage of the pilocarpine lactone ring. The enzyme responsible for the formation of pilocarpic acid has been characterized as a cation-dependent esterase present in serum and other organs (Ellis et al., 1972; Aromdee et al., 1996). Regarding other metabolites, Aromdee et al. (1999) reported that an unidentified metabolite of mol. wt. 224 (M-1) was detected in human urine after the oral administration of pilocarpine. The amount of M-1 excreted was approximately 35% of the dose. Even though a significant amount of M-1 was found in human urine, its structure has yet to be determined. To clarify the metabolic fate of pilocarpine, it is important to understand the pharmacokinetic properties of pilocarpine in humans.

In this study, we isolated a hydroxylated metabolite from human urine after the oral administration of pilocarpine and identified this by

ABBREVIATIONS: LC-MS/MS, liquid chromatography-tandem mass spectrometry; HPLC, high-performance liquid chromatography; SIM, selected ion monitoring; P450, cytochrome P450; SM-12502, (±)-cis-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride.
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]oxycetic acid (internal standard) and 3-hydroxypilocarpine were synthesized by Kissei Pharmaceutical (Nagano, Japan). Coumarin, 7-hydroxycoumarin, tolledamycin, quinidine sulfate salt dihydrate, sulfaphenalzole, 4-methylpyrazole hydrochloride, α-naphthoflavone, and furafylline were purchased from Sigma-Aldrich (St. Louis, MO) and GE Healthcare (Little Chalfont, Buckinghamshire, UK). (R)-(+)-Mephenytoin was purchased from Ultra-Spec Chemicals (Manchester, UK). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and NADP⁺ were obtained from Oriental Yeast (Tokyo, Japan). All other reagents were of the highest grade possible. Human liver microsomes were purchased from Xenotech (Lenexa, KS). Recombinant human cytochrome P450 450 isoforms (CYP1A2, -2A6, -2B6, -2C9, -2C19, -2D6, -2E1, and -3A4) expressed in baculovirus-infected insect cells (Supersomes) and control microsomes that had not been transfected (control Supersomes) were purchased from BD Gentest (Woburn, MA).

Isolation of M-1 from Human Urine. Human urine samples were collected from healthy volunteers who participated in a phase 1 trial of pilocarpine hydrochloride. This study was approved by the Institutional Review Board of the Kitasato Institute Bio-Iatric Center. All volunteers understood the procedures and agreed to participate in the study by giving written informed consent. A single oral dose of pilocarpine hydrochloride (10 mg) was administered to six male volunteers. Urine samples were collected at 0 to 4, 4 to 8, 8 to 12, and 12 to 24 h after the dose and stored at −20°C until analysis. Blank urine samples were collected from the same volunteers before drug administration. Three milliliters of the 0- to 4-h urine sample from each volunteer was pooled and used for the isolation of M-1. The pooled urine was diluted with a 2-fold volume of 10 mM ammonium acetate buffer, pH 5.0. The diluted urine was applied to a Bond-Elute C18 column (500 mg, 3 ml; Varian, Inc., Harbor City, CA), preconditioned by washing with 1.5 ml of methanol and 10 mM ammonium acetate buffer, pH 5.0, washing with 0.5 ml of 10 mM ammonium acetate buffer, pH 5.0, and then eluting with 0.5 ml of methanol/water (4:6, v/v). The combined eluent was subsequently applied to a Bond-Elute PRS column (100 mg, 10 ml; Varian, Inc.) preconditioned by washing with 1 ml of methanol/water (4:6, v/v). The column was washed with 1 ml of a mixture of methanol and 2 M HCl (98:2, v/v) and 1 ml of methanol. The fraction containing M-1 was eluted with 1 ml of a mixture of methanol and 25% NH₄OH (98:2, v/v). The eluent was then diluted under nitrogen stream and reconstituted in 10 mM ammonium acetate buffer, pH 5.0. The dissolved solution was used for the following isolation using HPLC. The HPLC system consisted of two L-7100 pumps, an L-7400 UV detector, an ERC degasser (ERC, Tokyo, Japan), and an ERC-3215a degasser (ERC, Tokyo, Japan). A Mightsil RP-18 GP column (150 × 4.6 mm i.d.; Kanto Chemicals, Tokyo, Japan) was used for the isolation of M-1. The column temperature was ambient, and the flow rate was 1 ml/min. The mobile phase was a mixture of 10 mM ammonium acetate, pH 5.0/acetonitrile (97.3:2.7, v/v). After the injection of the reconstituted sample, the eluate containing M-1 was monitored at 214 nm, and the fraction (retention time 6 min) was collected manually. The isolated fraction was evaporated to dryness and stored at −20°C until analysis.

LC-MS/MS and NMR Analyses. The pooled human urine was analyzed using an LC-MS/MS system to confirm the retention time of pilocarpic acid, the M-1 reported by Aromdee et al. (1999), and pilocarpine. A blank urine sample was also analyzed to control for interference arising from endogenous materials. The LC-MS/MS system consisted of an HP-1100 series HPLC system (Hewlett Packard, Palo Alto, CA) and a Finnigan TSQ7000 tandem mass spectrometer (Thermo Electron Corporation, Waltham, MA) fitted with an electrospray ionization interface. The voltage on the electrospray ionization interface was maintained at 4.5 kV in the positive-ion mode, and the capillary temperature was set at 250°C. Pilocarpic acid, M-1, and pilocarpine were detected by using the selected ion monitoring (SIM) mode, the monitor ions being set at m/z 227, 225, and 209, respectively. The separation conditions for the metabolites were the same as those described for the HPLC analysis, with the exception that the diameter of the column was 3.0 mm and the flow rate was 0.5 ml/min. Subsequently, to characterize M-1, LC-MS/MS analysis was performed to obtain a fragment ion spectrum at m/z 225. The MS/MS conditions were 1.7 mTorr argon collision gas with a −27-V collision potential.

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 possessed a hydroxyl group on carbon-3 with (R)-stereochemistry. To confirm this structure, an authentic sample was synthesized as shown in Fig. 1. The synthesized (R)- and (S)-diastereomers, namely, 3-hydroxypilocarpine and 3-hydroxyisopilocarpine, 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 stability of pilocarpine in the reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.4, and human liver microsomes was also investigated. The reaction was initiated by the addition of microsomes after a 3-min preincubation at 37°C. After a 60-min incubation, the reaction was stopped by the addition of 0.8 ml of ice-cold acetonitrile. After centrifugation (16,000g for 2 min at 4°C) of the mixture, the supernatant was evaporated under a stream of nitrogen gas at room temperature and reconstituted in 10 mM ammonium acetate buffer, pH 5.0. The metabolites were analyzed by HPLC as described under 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-
ence, Boston, MA). The detected metabolite was analyzed by LC-MS/MS as described above, with the modification that the precursor ion was m/z 227 (18°C) instead of m/z 225.

**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 Eadie-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: 1-Naphthoflavone (1 μM final concentration) and furafylline (20 μM) for CYP1A2, coumarine (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, coumarine, 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, coumarine, 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 pilocarpine after a 15-min preincubation. 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 Isomers.** 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] oxacyclic 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 (Greenlee 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-hydroxypilocarpine. Based on this observation, the generated metabolite was thought to be 3-hydroxypilocarpine. 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 molecular ion with retention times of 5.9 and 7.8 min; these peaks were absent from the blank urine. Two peaks with retention times of 6.0 and ca. 8 min were also detected in the HPLC chromatograms derived from the UV analysis of the samples extracted from pilocarpine-administered human urine (Fig. 3). The major peak with a retention time of 6.0 min was considered to be M-1, and this peak was isolated for characterization. Because the peak with a retention time of ca. 8 min made up less than 10% of M-1, no further investigations on this peak were carried out. A pilocarpine peak (m/z 209) was detected in the SIM chromatogram with a retention time of 13.9 min and in the HPLC chromatogram at 13.5 min.

Figure 4, A and B, show the NMR spectra of pilocarpine and the isolated M-1, respectively. The results demonstrated that peak c (2.8 ppm) assigned as the proton “c” of pilocarpine in Fig. 4A disappeared in the NMR chart of M-1 (Fig. 4B). Based on the above-mentioned results, M-1 was conjectured to be the structure in which the site indicated by c had been hydroxylated. In addition, nuclear Overhauser effect analysis was performed to confirm the configuration of the site of hydroxylation. As a result of the irradiation of “b,” a nuclear Overhauser effect was observed at the “a,” “f,” and “d (β)” protons (data not shown). Based on these results, it was revealed that the carbon atoms at positions b and f take the cis-configuration in M-1 as well as in pilocarpine. Moreover, the result of the NMR analysis of the isolated M-1 was consistent with that of the authentic sample (data not shown). Based on the above-mentioned results, the M-1 found in human urine was identified as a novel metabolite, 3-hydroxypilocarpine.

In Vitro Metabolism of [14C]Pilocarpine in Human Liver Microsomes. The radiochromatograms obtained after the incubation of [14C]pilocarpine with human liver microsomes are shown in Fig. 5. 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-hydroxypilocarpine. Based on this observation, the generated metabolite was thought to be 3-hydroxypilocarpine. 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 molecular ion [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-hydroxypilocarpine (data not shown).

The generation of 3-hydroxypilocarpine 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-Hydroxypilocarpine 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-hydroxypilocarpine from pilocarpine. The apparent K_m and V_max values obtained 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-hydroxypilocarpine at 2 μM pilocarpine in pooled human liver microsomes is shown in Fig. 7. Only coumarin (CYP2A6) strongly inhibited the formation of 3-hydroxypilocarpine (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-hydroxypilocarpine. These data suggested that CYP2A6 accounted for the majority of the 3-hydroxylation of human liver microsomes.

Pilocarpine 3-Hydroxylation by Recombinant P450 Isoforms. To identify the human P450 involved in the formation of 3-hydroxypilocarpine, 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) in pooled human liver microsomes, while the activity was very low compared with that of CYP2A6. No detectable levels of 3-hydroxypilocarpine (<3.3 fmol/min/mg) 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 K_m and V_max values obtained were 3.1 μM and 99.5 fmol/min/mg, respectively. The K_m value was comparable with that obtained from human liver microsomes.

Correlation Analysis of 3-Hydroxypilocarpine Formation. The formation of 3-hydroxypilocarpine 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-hydroxypilocarpine.

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

![Fig. 4. 1H NMR spectra of pilocarpine (A) and isolated metabolite (M-1) (B) from pilocarpine-administered human urine produced by HPLC fractionation.](image-url)
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 \(^{[14]C}\)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 carboester, 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 \(p\)-
chloromercuibenzoic 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 $C_{\text{max}}$ value (normalized to 5-mg 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-hydroxypropionylpilocarpine 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-hydroxypropionylpilocarpine 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](image8.png) 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).

![Fig. 9](image9.png) 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.

![Fig. 10](image10.png) Correlation of 3-hydroxypilocarpine formation and 7-hydroxycoumarin formation in microsomes from 16 human livers. Each data point represents the mean of duplicate determinations.
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


Prof. Takuro Endo, Pharmacokinetics Research Center, Kanazawa, Japan for help and advice in preparing this article.

Address correspondence to: Prof. Takuro Endo, Pharmacokinetics Research Center, Kissei Pharmaceutical Co., Ltd., 19-48 Yoshino Matsumoto-city, Nagano 399-8710, Japan. E-mail: takuro.endo@pharm.kissei.co.jp

Downloaded from findfulltext.aspetjournals.org at ASPETh on April 18, 2017