Involvement of CYP2A6 in the formation of a novel metabolite, 3-hydroxypilocarpine, from pilocarpine in human liver microsomes

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Abbreviations used are: P450, cytochrome P450; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; SIM, selected ion monitoring
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 hydrochloride and investigated the metabolic enzyme responsible for the metabolite formation. The structure of the metabolite was identified as 3-hydroxypilocarpine by LC-MS/MS and NMR analyses and by comparing to the authentic metabolite. In order 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, 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, α-naphthoflavone), CYP2C9 (sulfaphenazole), CYP2C19 (S-mephenytoin), 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 to that of human liver microsomes (1.5 µM). 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 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 eye drops for the treatment of glaucoma (Hoyng and van Beek, 2000). Pilocarpine has the ability to stimulate salivary secretion and 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 of pilocarpine’s many years of clinical use, 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 $^{14}$C-labeled pilocarpine. Orally administered $[^{14}\text{C}]$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 this 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 (Aromdee et al., 1996; Ellis et al., 1972). Regarding other metabolites, Aromdee (1999) reported that an unidentified metabolite of MW 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. In order 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 LC-MS/MS and NMR analyses. Moreover, we identified the human P450 responsible for the formation of the novel metabolite, 3-hydroxypilocarpine, in human liver microsomes.
Materials and Methods

Materials

Pilocarpine hydrochloride and [imidazol-2-\(^{14}\)C]pilocarpine hydrochloride (\[^{14}\text{C}\]pilocarpine hydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO) and Amersham plc. (Little Chalfont, UK), respectively. The radiochemical purity of \[^{14}\text{C}\]pilocarpine hydrochloride was confirmed to exceed 98% (by HPLC). [4-(1-Imidazolyl)phenyl]oxyacetic acid (internal standard) and 3-hydroxypilocarpine were synthesized by Kissei Pharmaceutical (Nagano, Japan). Coumarin, 7-hydroxycoumarin, troleandomycin, quinidine sulfate salt dihydrate, sulfaphenazole, 4-methylpyrazole hydrochloride, \(\alpha\)-naphthoflavone, and furafylline were purchased from Sigma-Aldrich. \((+)-\text{Mephenytoin was purchased from Ultrafine Chemicals (Manchester, UK).}\) 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 Xeno Tech (Lenexa, KS). Recombinant human cytochrome P450 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 I 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 0–4, 4–8, 8–12, and 12–24 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 0–4 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, 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 eluent was subsequently applied to a Bond-Elute PRS column (100 mg, LRC; Varian) 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 dried under a nitrogen stream and then 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, a D-7500 integrator (all from Hitachi, Tokyo, Japan), and an ERC-3215α degasser (ERC, Tokyo, Japan). A Mightysil RP-18 GP column (150 × 4.6 mm I.D.; Kanto Chemicals, Tokyo, Japan) was utilized for the isolation of M-1. The column temperature was ambient and the flow rate was 1.0 ml/min. The mobile phase was a mixture of 10 mM ammonium acetate (pH 5.0)/acetonitrile (97:3, 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 in order 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 in order 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 (ESI) interface. The voltage on the ESI 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, \( m/z \) 225, and \( m/z \) 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, in order 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. \(^1\)H-NMR spectra in deuteromethanol (CD\(_3\)OD) were recorded at 500 MHz using a Bruker DRX500 spectrometer (Bruker Instruments Inc., Billerica, MA). The chemical shifts are expressed in ppm relative to tetramethylsilane (TMS) 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. In order 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 (SXRD) (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 non-labeled pilocarpine (1:1, final 10 µM) was incubated in a reaction mixture comprising 50 mM potassium phosphate buffer (pH 7.4), 1 mg/ml of human liver microsomes, an NADPH-generating system (0.8 mM NADP+, 8 mM glucose-6-phosphate, 1 unit/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 (16,000 g 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 in *LC-MS/MS and NMR analyses*. The radioactivity in the column eluate was detected by the use of a radiochemical detector (Radiomatic 52STR; Perkin-Elmer Life Sciences, Boston, MS.). The detected metabolite was analyzed by LC-MS/MS as described above,
with the modification that the precursor ion was $m/z$ 227 ($^{14}$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. In order 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 ($K_m$ and $V_{max}$), 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: 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 pilocarpine after a 15-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 isoforms**

In order 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 (OR); CYP2A6, 2B6, 2C9, 2C19, 2E1, and 3A4 were also coexpressed with cytochrome b$_5$. 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] oxyacetic 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 Corp., 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 acetonitrile (B) was set at 0%, increased linearly to 70% over 4 min, held for 1 min, 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 \textit{m/z} 227 with retention times of 1.8 and 3.0 min, corresponding to the pilocarpic acid protonated molecular ion, were detected. The former peak was also detected in the chromatograms of blank urine, suggesting that this peak was derived from an endogenous material. There were two peaks in the SIM chromatogram at \textit{m/z} 225 corresponding to the M-1 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 \textit{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 6.0 min was considered to be M-1 and this peak was isolated for characterization. Since the peak with a retention time of \textit{ca.} 8 min comprised less than 10\% of M-1, no further investigations on this peak were carried out. A pilocarpine peak (\textit{m/z} 209) was detected in the SIM chromatogram with a retention time of 13.9 min and in the HPLC chromatogram with a retention time of 13.5 min.

Figures 4A and 4B 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 (NOE) analysis was performed in order to confirm the configuration of the site of hydroxylation. As a result of the irradiation of “b,” an NOE 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 believed to be 3-hydroxypilocarpine. This supposition was confirmed by the LC-MS and LC-MS/MS analyses. From the LC-MS 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-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 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-mephenytoin (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 pilocarpine in human liver microsomes.

**Pilocarpine 3-hydroxylation by recombinant P450 isoforms**

In order 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
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exhibited the highest pilocarpine 3-hydroxylation activity (102.0 fmol/min/pmol P450). 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-hydroxypilocarpine (<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 $K_m$ and $V_{max}$ values obtained were 3.1 µM and 99.5 fmol/min/pmol P450, respectively. The $K_m$ value was comparable to 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 different human livers (Fig. 10). The activity correlated well ($r = 0.96$, $P < 0.01$) 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 $^1$H-NMR analyses. Based on the HPLC analysis with UV detection, 3-hydroxypilocarpine appears 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 believed to be a metabolite of pilocarpine. The amount of this metabolite was less than 10% of 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 appears 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 epimerisation 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. Carboxyesterase 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 carboxyesterase 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-chloromercuribenzoic acid (Lavallee and Rosenkrantz, 1965). Recently, Li et al. (2006) reported that butyrylcholinesterase (EC 3.1.1.8), paraoxonase (EC 3.1.8.1), and albumin esterase (EC 3.1.1.7), but not carboxylesterase (EC 3.1.1.1), 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 to 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, $K_i = 1–4 \, \mu M$) (Bourrié et al., 1996; Kimonen et al., 1995; 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. In fact, 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 acts as a substrate for CYP2A6.

The pilocarpine Cmax value (normalized to 5 mg doses) after a single oral administration of pilocarpine to subjects was approximately 20–30 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 $[14\text{C}]$pilocarpine to rats (Omori et al., 2004) and the protein binding of pilocarpine in human plasma appears 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 \textit{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 ($t_{\text{max}}$), volume of distribution ($V_d/F$), clearance ($CL/F$), or area under the curve (AUC), whereas Garg et al. (1996) reported the AUC and CL were significantly changed in the subjects with hepatic impairment as 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. Since 3-hydroxypilocarpine is the main metabolite accounting for at least one-third of the administered pilocarpine in humans, and is produced by first-pass metabolism in the liver, the 3-hydroxylation of
pilocarpine by CYP2A6 is believed 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 CL/F (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.
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Footnotes

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Legends for figures

FIG. 1. Synthesis of 3-hydroxypilocarpine from pilocarpine.

FIG. 2. SIM chromatograms of pilocarpic acid, M-1, and pilocarpine from blank urine (A) and from pilocarpine-administered human urine (B).

Pilocarpic acid, M-1, and pilocarpine were detected as their protonated molecule ions at $m/z$ 227, $m/z$ 225, and $m/z$ 209, respectively.

FIG. 3. HPLC chromatogram of pilocarpine and M-1 extracted from pilocarpine-administered human urine. M-1, retention time 5.97 min; pilocarpine, retention time 13.53 min.

FIG. 4. $^1$H-NMR spectra of pilocarpine (A) and isolated metabolite (M-1) (B) from pilocarpine-administered human urine produced by HPLC fractionation.

FIG. 5. Radiochromatograms of the metabolites generated during a 60-min incubation of 10 µM $[^{14}$C]$]pilocarpine with human liver microsomes, in the presence (A) or absence (B) of an NADPH-generating system.

FIG. 6. An Eadie–Hofstee plot for 3-hydroxypilocarpine formation from pilocarpine in human liver microsomes.
Pooled human liver microsomes were incubated with 0.125 to 25 µM pilocarpine at 37°C for 60 min. Each data point represents the mean of duplicate determinations.

FIG. 7. Effect of P450 isoform-selective inhibitors on 3-hydroxypilocarpine formation from pilocarpine in human liver microsomes.

Microsomes were incubated at 37°C for 60 min with 2 µM pilocarpine in the absence (control) or presence of P450 isoform-selective inhibitors: 1 µM α-naphthoflavone, 20 µM furafylline, 200 µM coumarin, 20 µM sulfaphenazole, 250 µM S-mephenytoin, 5 µM quinidine, 500 µM 4-methylpyrazole, and 100 µM troleandomycin. Control activities of 3-hydroxypilocarpine formation in the presence of distilled water, methanol, or acetonitrile instead of inhibitors were 3.6, 3.6, and 2.2 pmol/min/mg protein, respectively. Each column represents the mean of duplicate determinations.

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 isoforms (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).


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. Correlation of 3-hydroxypilocarpine formation and 7-hydroxycoumarin formation in microsomes from 16 human livers. Each data point represents the mean of duplicate determinations.
Figure 1

Pilocarpine hydrochloride

1) aq. NaHCO₃
2) LHMDS*¹, THF, -78°C
3) MoOPH*², THF, -78→0°C
4) Column Separation

3-hydroxypilocarpine
((3R)-3-hydroxypilocarpine)

R/S = ca. 1/2 mol ratio

3-hydroxyisopilocarpine
((3S)-3-hydroxypilocarpine)

*¹: Lithium hexamethyldisilazide
*²: Oxodiperoxyxymolybdenum (pyridine) (hexamethylphosphoric triamide)
Figure 2

A

Relative Abundance

Time (min)

m/z 227

1.81

3.95E5

m/z 225

132

5.75E3

m/z 209

6.53

1.09E3

B

Relative Abundance

Time (min)

m/z 227

2.32E5

m/z 225

2.85

8.45E4

m/z 209

5.90 7.79

3.55E4
Figure 3
Figure 4

A

B
Figure 5

A

B
Figure 6
Figure 7

![Graph showing residual activity (% of control) for various compounds.

- NADPH(-)
- α-Naphthoflavone (1A2)
- Furafylline (1A2)
- Coumarin (2A6)
- Sulfaphenazole (2C9)
- S-Mephenytoin (2C19)
- Quinidine (2D6)
- 4-Methylpyrazole (2E1)
- Troleandomycin (3A4)
Figure 8

![Graph showing 3-hydroxypilocarpine formation for different CYP enzymes]

- Control: ND
- CYP1A2: ND
- CYP2A6: 102.0
- CYP2B6: ND
- CYP2C9: ND
- CYP2C19: ND
- CYP2D6: ND
- CYP2E1: ND
- CYP3A4: 8.7

3-hydroxypilocarpine formation (fmol/min/pmol P450)
Figure 9