Timolol Metabolism in Human Liver Microsomes Is Mediated Principally by CYP2D6

Marjo Volotinen, Miia Turpeinen, Ari Tolonen, Jouko Uusitalo, Jukka Mäenpää, and Olavi Pelkonen

Santen Oy, Tampere, Finland (M.V., J.M.); University of Oulu, Departments of Pharmacology and Toxicology (M.T., O.P.) and Chemistry (A.T.), Oulu, Finland; and Novamass Analytical Ltd., Oulu, Finland (A.T., J.U.)

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ABSTRACT:
Timolol has mainly been used topically for the treatment of glaucoma. It has been suggested that the drug is metabolized by cytochrome P450 CYP2D6. The matter has not, however, been extensively studied. The aim here was to tentatively identify timolol metabolites and to determine the P450-associated metabolic and interaction properties of timolol in vitro. Four metabolites were identified, the most abundant being a hydroxy metabolite, M1. The \( K_{i} \) value for the formation of M1 was 23.8 \( \mu M \) in human liver microsomes. Metabolism of timolol with recombinant P450s and correlation analysis have confirmed the conception that the drug is metabolized principally by CYP2D6, CYP2C19 being only a minor contributor (<10%) to the intrinsic microsomal clearance. The CYP2D6 inhibitor quinidine proved a potent competitive inhibitor of timolol metabolism, with an in vitro \( K_{i} \) value of 0.08 \( \mu M \). Fluvoxamine, an inhibitor of CYP2C19, inhibited timolol metabolism to a lesser extent, confirming its minor contribution. Timolol itself did not inhibit CYP2D6-catalyzed dextromethorphan O-demethylation. Judging from the disappearance of timolol in human liver homogenate, the in vivo half-life was extrapolated to be about 3 h, an estimate close to the half-life of about 2 to 5 h observed in vivo. In conclusion, the inhibition of timolol metabolism by quinidine should be taken into account when patients are treated with timolol; however, when plasma timolol concentrations in patients remain low (<0.2 \( \mu g/l \)), it is suggested that such interaction is of minor clinical relevance.

Timolol is a nonselective \( \beta \)-adrenergic receptor blocking agent that has mainly been used topically for the treatment of glaucoma since 1978 (Brooks and Gillies, 1992). In addition, it has been used in the treatment of hypertension and prophylaxis of migraine (Dunn and Frohlich, 1981; Blumenfeld, 2005). Topically applied timolol is absorbed not only into the eye but also systemically. It has been reported that approximately 80% of a topically administered eye drop drains through the nasolacrimal duct and is systemically absorbed (Shell, 1982; Korte et al., 2002). Systemic effects reported after ophthalmic timolol administration include cardiac, pulmonary, central nervous system, dermatologic, and gastrointestinal reactions (Van Buskirk, 1980; Nelson et al., 1986). It is thus of importance to know the metabolic route of timolol.

The metabolism of timolol has not been extensively studied. Earlier investigators have suggested that in humans at least two major metabolites are formed from timolol by cleavage of the morpholine ring (Tocco et al., 1975, 1980). Timolol has mainly been used topically for the treatment of glaucoma since 1978 (Brooks and Gillies, 1992). In addition, it has been used in the treatment of hypertension and prophylaxis of migraine (Dunn and Frohlich, 1981; Blumenfeld, 2005). Topically applied timolol is absorbed not only into the eye but also systemically. It has been reported that approximately 80% of a topically administered eye drop drains through the nasolacrimal duct and is systemically absorbed (Shell, 1982; Korte et al., 2002). Systemic effects reported after ophthalmic timolol administration include cardiac, pulmonary, central nervous system, dermatologic, and gastrointestinal reactions (Van Buskirk, 1980; Nelson et al., 1986). It is thus of importance to know the metabolic route of timolol.

The metabolism of timolol has not been extensively studied. Earlier investigators have suggested that in humans at least two major metabolites are formed from timolol by cleavage of the morpholine ring (Tocco et al., 1975, 1980). Timolol has been suggested to be metabolized by cytochrome P450 enzyme CYP2D6 (Kaila et al., 1991; Edeki et al., 1995, Nieminen et al., 2005a). Due to the highly polymorphic nature of CYP2D6, individuals are classified into extensive (EMs) and poor metabolizers (PMs), the latter being unable to metabolize many clinically used drugs (Pelkonen et al., 1998; Zanger et al., 2004).

Edeki et al. (1995) studied the effects of timolol on the exercise heart rate and plasma concentrations of timolol in both EMs and PMs. In addition, the interaction between topically administered timolol and orally administered quinidine was studied in EMs. The reduction in heart rate was significantly greater and plasma concentrations of timolol were higher in PMs compared with EMs. Quinidine caused a further significant decrease in exercise heart rate and an increase in plasma timolol concentration compared with timolol alone. Although only a small amount of timolol is administered topically, the amount systemically absorbed is high enough to exert cardiovascular effects and to interact with the CYP2D6 inhibitor quinidine. Similar findings have been reported after administration of ophthalmic timolol concomitantly with the histamine H\(_1\)-receptor antagonist cimetidine (Ishii et al., 2000). Administration of cimetidine with timolol ophthalmic solution resulted in additional reductions in resting heart rate and intraocular pressure in healthy young subjects. It was concluded that cimetidine increased the \( \beta \)-blocking effect of timolol.

Timolol plasma levels have been shown to correlate with adverse cardiovascular reactions after administration of ophthalmic timolol (Nieminen et al., 2005b). In some patients, plasma timolol levels are very low, perhaps by reason of ultrarapid metabolizer status, com-

ABBREVIATIONS: EM, extensive metabolizer; PM, poor metabolizer; HLM, human liver microsome; P450, cytochrome P450; LC/MS, liquid chromatography-mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass spectrometry; HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring; CL, clearance.
pared with the very high concentrations observed in PMs. Attempts have been made to develop timolol-containing ophthalmic products that will minimize systemic absorption.

To our knowledge, the metabolism of timolol has not been measured in human in vitro studies. Here we determined the metabolic properties of timolol in the presence of human liver preparations in vitro and tentatively identified timolol metabolites. The objective was to establish the extent to which in vitro timolol metabolism tallies with and is extrapolatable to in vivo timolol kinetics, and to study the inhibition of timolol metabolism by quinidine in vitro and make assumptions of its clinical consequences.

Materials and Methods

Chemicals. Timolol (S)-(–)-1-(tert-butylamino)-3-[4-(morpholinolino)-1,2,5-thiadiazol-3-yl-oxyl]-2-propanol maleate was supplied by Santen Oy (Tampere, Finland). The metabolite standards dextromethorphan, desethylamodiaquine, 6-hydroxychlorzoxazone, hydroxytolbutamide, and 6β-hydroxytestosterone were purchased from BD Biosciences Discovery Labware (Bedford, MA). Hydroxybupropion was a generous donation from GlaxoSmithKline (Research Triangle, NC), 1-hydroxymidazolam from F. Hoffman-La Roche (Basel, Switzerland), and omeprazole sulphone and 5-hydroxyomeprazole were from AstraZeneca (Mölndal, Sweden). Formic acid and Lichrosolv GC acetonitrile were obtained from Merck KGaA (Darmstadt, Germany). All other chemicals were from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available. Water was freshly prepared in-house with a Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and UP grade (ultra pure, 18.2 MΩ).

Human Liver Homogenates, Microsomes and cDNA-Expressed Human P450s. The human liver samples used in this study were obtained from the University Hospital of Oulu as surplus (livers for various reasons ineligible for liver transplantation) from kidney transplantation donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. A weight-balanced pool of ten liver microsomal preparations extensively characterized for use in primary screening (sufficient model activities, expected effects of model inhibitors, quantification of P450s by Western blotting) was employed. The characteristics of the liver samples and the preparation of microsomes are described in detail in an article by Turpeinen et al. (2004). Liver homogenates were prepared by homogenizing normal-looking tissue in four volumes of ice-cold 0.1 M phosphate buffer, pH 7.4, and the homogenate was used as such in the incubations.

Incubation of Timolol with Liver Homogenates. For initial screening of the disappearance of the parent drug and identification of the metabolites formed, reactions were performed with human liver homogenate. The incubation mixtures contained 10 μl of liver homogenate, 0.1 M phosphate buffer (pH 7.4), and 1 mM NADPH in a total volume of 200 μl. Timolol was diluted in water to achieve final concentrations of 1, 5, and 25 μM. Samples were preincubated for 2 min in a shaking incubator block (Eppendorf Thermomixer 5436; Eppendorf AG, Hamburg, Germany) at +37°C and reaction was started by addition of NADPH. Reactions with and without NADPH were set up and incubated for 20, 40, and 60 min with (NADPH) and 0 and 60 min (without NADPH), respectively. Each reaction was terminated by adding 100 μl of ice-cold acetonitrile, with subsequent cooling in an ice bath to precipitate the proteins. The tubes were stored at −18°C until analysis. All reactions were performed in duplicate.

Incubation of Timolol with Liver Microsomes. For determination of kinetic parameters and inhibition studies, incubations were carried out with human liver microsomes (HLMs). The incubation mixtures contained 0.5 mg of microsomal protein/ml, 0.1 M phosphate buffer (pH 7.4), and 1 mM NADPH in a total volume of 200 μl. Timolol was diluted in water to achieve final concentrations of 1, 2.5, 5, 10, and 25 μM. The incubation periods were 10 min; otherwise, samples were incubated as described above. All reactions were conducted in duplicate. Product formations were linear with respect to incubation time, protein content and substrate concentration under the assay conditions described.

For inhibition studies, quinidine (diluted in methanol) at concentrations of 0 (only solvent), 0.001, 0.01, 0.1, 1, and 10 μM, and fluvoxamine (diluted in water) at concentrations of 0 (solvent only), 0.01, 0.1, 1, and 10 μM were added to the incubation mixtures. The final proportion of primary solvents in the incubation mixture was under 1% (v/v).

Incubation of Timolol with cDNA-Expressed Human P450s. Baculovirus-expressed human P450s (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) coexpressing human P450 reductase (Supersomes) were purchased from BD Biosciences Discovery Labware (Bedford, MA) and used according to the manufacturer’s instructions. For evaluation of the contribution of different P450s to timolol metabolism, a final concentration of 25 μM timolol was used. For kinetic studies with recombinant CYP2C19 and CYP2D6, final concentrations of 0.5, 2, 8, 32, and 128 μM timolol were used.

Inhibition of Dextromethorphan O-Demethylation by Timolol and Quinidine. The samples were prepared as described above (reactions with microsomes), but with using 10 μM dextromethorphan as substrate. Samples containing 0.01, 0.1, 1, or 10 μM timolol or the CYP2D6 reference inhibitor quinidine were prepared. The samples were incubated for 10 min. All reactions were conducted in duplicate. Formation of dextromorphin in the presence of the inhibitors was compared with control reactions (samples containing solvent but no inhibitor).

Correlation Analysis of Timolol Metabolism versus P450-Isomeric Selective Activities. Microsomes from 15 different human livers were used to establish correlation of the formation of timolol metabolites in human liver microsomes to the measured P450 isomeric-selective activities in these livers. The incubation conditions and instrumentation used to assess the enzyme activities of CYP1A1/2 (ethoxyresorufin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2C9 (tolbutamide hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2E1 (chloroxazone 6-hydroxylation), and CYP3A4/5 (midazolam 1′-hydroxylation) have been described previously in detail in an article by Taavitsainen et al. (2001). The amodiaquine hydroxylation assay for CYP2B6 was slightly modified from that applied by Fuccette et al. (2000) and Hesse et al. (2000): incubation mixtures contained 2.0 mg of microsomal protein/ml and 50 μM bupropion, and incubation was for 15 min. The amodiaquine N-desethylase assay for CYP2C8 was a modification of that of Li et al. (2002): incubation mixtures contained 0.5 mg of protein/ml and 30 μM amodiaquine, and the incubation time was 20 min. The omeprazole 5-hydroxylation and sulfoxidation assays for CYP2C19 and CYP3A4, respectively, were adapted from those described by Åbèl et al. (2000): incubation mixtures contained 0.5 mg of microsomal protein/ml and 40 μM omeprazole, and the incubation time was 20 min. Otherwise the reactions contained buffer and NADPH and were performed as described above. Pearson’s correlation coefficients (r) were calculated and are given under Results. For statistical testing, an analysis of variance model for repeated measures and Student’s t test for post hoc analysis were used. The limit of statistical significance was set at P < 0.05.

LC/MS Conditions. All samples were thawed at room temperature, shaken, and centrifuged for 10 min at 13,400 rpm (Eppendorf Mini Spin) and transferred to Total Recovery vials (Waters Corporation, Milford, MA). A Waters Alliance 2695 high-performance liquid chromatographic (HPLC) system (Waters Corporation) was used in all analyses. In the analysis of timolol and its metabolites a Waters XTerra MS C18 column (2.1 × 50 mm, 3.5 μm particle size) together with a Phenomenex Luna C18 precolumn (4.0 × 20 mm, 3.0 μm; Phenomenex, Torrance, CA) was used. The eluents were A, 0.1% acetic acid in water (pH 3.2), and B, acetonitrile. A linear gradient elution from 4% B to 35% B in 8 min was applied followed by fast wash of the column with 70% B within 1 min and equilibration with initial conditions. For dextromethorphan and its metabolites, a Waters X Terra RP18 column (2.1 × 50 mm, 3.5 μm) and a Phenomenex Luna-C18 precolumn (4.0 × 20 mm, 3.0 μm) were used. The eluents were A, 0.1% formic acid (pH 2.7) in water and B, methanol. A linear gradient elution from 12% B to 75% B within 6 min was applied, followed by equilibrium to initial conditions. The eluent flow rate in both chromatographic methods was 0.3 ml/min and the column oven temperature was 30°C. The flow was split postcolumn with an Acurate Postcolumn Stream Splitter (LC Packings, Amsterdam, the Netherlands) with a ratio of 1:3 to MS and waste, respectively.

The LC/MS data in the screening of timolol metabolites were recorded with a Micromass LCT time-of-flight high resolution mass spectrometer (Waters) equipped with a LockSpray electrospray ionization source. The LockSpray feature was used with leucine enkephalin as a lock mass reference compound (M + H⁺ at m/z 556.2771). The ion count for leucine enkephalin was adjusted...
to about 160 ions/count. A cone voltage of 26 V was used. In hydrogen-deuterium exchange experiments the D₂O was delivered into the HPLC eluent flow postcolumn by a syringe pump (Harvard Apparatus, Holliston, MA) via a T-mode flow splitter with a flow rate of 100 μl/min. The number of labile protons in the metabolites was calculated according to the mass shift of deuterium-exchanged molecular ions compared with the normal LC/MS protons in the metabolites was calculated according to the mass shift of labile protons.

All LC/MS/MS data for interaction studies were recorded with a Micromass Quattro II triple quadrupole mass spectrometer (Waters) equipped with a T-mode flow splitter with a flow rate of 100 μl/min. The number of labile protons in the metabolites was calculated according to the mass shift of deuterium-exchanged molecular ions compared with the normal LC/MS protons in the metabolites was calculated according to the mass shift of labile protons.

All LC/MS/MS data for interaction studies were recorded with a Micromass Quattro II triple quadrupole mass spectrometer (Waters) equipped with a Z-Spray electrospray ionization source, using a multiple reaction monitoring approach on the basis of human liver homogenate incubation was adopted. The analyte to be directly comparable at different concentrations.

Quantification references, assuming the mass spectrometric response of the determinate the Kᵢ values and mode of inhibition, graphical analysis of the data was made according to Lineweaver-Burk, Dixon, Hofstee, and Hanes plots. The limit of statistical significance was set at P < 0.05.

Kinetic in Vitro/in Vivo Extrapolations. To roughly estimate the intrinsic hepatic clearance (CLᵢ) (whole liver) of timolol, a substrate depletion approach on the basis of human liver homogenate incubation was adopted. The first-order rate constant k (min⁻¹) can be calculated from the equation −ln ([S]₀)/(d[S]/dt₀) = k [S]₀, where the timolol concentration at t₀ is 1 μM and at t₀ 0.65 μM, account being taken of the volume of incubation (0.2 ml) and the amount of homogenate added per incubate (8 mg of liver homogenate, weight basis). Hepatic clearance (CLᵢ) was calculated using the equation CLᵢ = Q_HV/CLᵢ, where Q_HV = liver blood flow. The half-life (t₁/₂) of timolol was predicted using the equation t₁/₂ = 0.693/kᵢ, where kᵢ = CLᵢ/CLᵢ (Yang and Wyatt, 1991). Total (systemic) clearance (CLᵢ) is the sum of hepatic clearance (CL_HV) and renal clearance (CLᵢ).

The following assumptions were adhered to in the calculations: the mass of human liver is 1500 g, liver blood flow Q_HV = 1.45 l/min, renal clearance (assumption) CLᵢ = 0.18 l/min, mass of virtual human is 70 kg, and distribution volume Vᵢ = 2.5 l/kg body weight (Dollery et al., 1991; Davies and Morris, 1993).

Results

The Disappearance of Timolol in Human Liver Homogenates. The substrate disappearance of timolol at different initial substrate concentrations (1, 5, and 25 μM) was greatest in incubation with the 1 μM initial concentration, 65% remaining after 20 min incubation, i.e., 35% disappeared. Roughly 20% of timolol disappeared during a 20-min incubation with 5 and 25 μM initial substrate concentrations (data not illustrated). Substrate disappearances decreased considerably from 20 min to 60 min. In the incubations without the addition of NADPH, substrate losses were roughly 10% over the 60-min incubation. Such a clear difference between incubations with or without NADPH indicates that timolol disappearance is predominantly NADPH-dependent. On the basis of this experiment, a 10-min incubation time was selected, since substrate consumption remains at about 15% even at a timolol concentration of 1 μM.

Calculation of kinetic parameters on the basis of substrate depletion in the above-mentioned homogenate experiment gave the following values: the intrinsic clearance for the whole liver was 825 ml/min, the organ clearance (CLᵢ) was approximately 32 l/h, total body clearance (CLᵢ) renal clearance taken into consideration) was 42 l/h, the elimination constant (Kᵢ) was 0.24/h, and half-life (t₁/₂) was 2.9 h.

Identification of the Timolol Metabolites. Four different metabolites were produced by human liver homogenate. The retention times were 6.4 min for timolol and 5.4, 5.5, 5.8, and 7.1 min for M1, M2, M3, and M4, respectively. All of these were tentatively identified on the basis of in-source fragmentation data, accurate mass measurement of molecular ions and fragment ions, and hydrogen-deuterium exchange experiments (Table 1).

Detection of molecular ions and fragment ions, and hydrogen-deuterium exchange experiments (Table 1). The suggested structures are shown in Fig. 1. M1 was ascertained to be formed via hydroxylation into the morpholino ring. M2 was left with several possible structures involv—
ing addition of one oxygen and two hydrogen atoms into the substrate structure, either in the morpholine or the sulfur-containing ring. This structure may thus be attributable to the addition of water to break the morpholino ring and form two ethyl alcohol side chains, or hydroxylation followed by reduction of the double bond. The M3 structure has two additional oxygen atoms compared with the substrate, both of them being located in the morpholine ring area. The metabolite may thus either be di-hydroxylated timolol or, alternatively, formed via hydroxylation and oxidation to ketone followed by ring opening by the addition of water molecule. The latter structure, containing two C2-chains, of which the one ends with a hydroxyl group and the other with carboxylic acid, is more probable, as has previously been identified in an in vivo study (Tocco et al., 1980). The same in vivo study also reported the identification of the metabolite M1. M4 has a structure similar to the substrate with the exception of a double bond in the morpholine ring. This may be formed by direct cleavage of H2 or, alternatively, by cleavage of water from M1.

The results obtained from the LC/MS hydrogen-deuterium exchange experiments also support the above tentative structures in confirming that M1 has three, M3 four, and M4 two labile protons (= combined number of hydroxyl and amine protons). In the case of M2, the result of the hydrogen-deuterium exchange experiment was somewhat unclear, so that this metabolite may have either three or four labile protons, which gave no additional information as to the structure. These data for timolol correctly suggested the presence of two labile protons.

In terms of expressing metabolites as “timolol equivalents,” the hydroxylation product M1 proved to be the major metabolite in human liver homogenates and microsomes. The relative amount of the metabolite M2 increased at a higher timolol concentration (25 μM). The minor metabolites M3 and M4 were detected only at concentrations higher than 5 μM (data not shown).

**Enzyme Kinetics of Timolol Metabolism in HLMs.** The $K_m$ values for the timolol metabolites M1 and M2 in HLMs derived from Lineweaver-Burk plots were 23.8 μM and 78.1 μM, respectively (Table 2). Assuming the metabolites to possess mass spectrometric responses similar to those of the parent, approximate $V_{max}$ values for M1 and M2 were 91.2 and 7.7 pmol/min/mg protein, respectively. Since M3 and M4 are most probably secondary metabolites derived from M1, estimation of their kinetic properties (or assignment of metabolizing enzymes) is not possible without the parent(s).

**Metabolism and Kinetics of Timolol in cDNA-Expressed P450s.** The primary timolol metabolites M1 and M2 were produced predominantly by CYP2D6, although CYP2C19 was also moderately active (Table 2). The $K_m$ values for M1 formation by CYP2D6 and CYP2C19 were 7 and 50 μM, respectively, and those for M2 formation by CYP2D6 and CYP2C19 were 4.7 and 33 μM, respectively. A small contribution from CYP3A4 was seen in the production of M2 (data not illustrated). CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP2E1 made a negligible contribution to timolol metabolism and only M3 was observed with these enzymes, probably because the primary M1 was produced in such small measure that it was completely converted to M3 (data not illustrated). CYP2D6 demonstrated the highest rate of formation for all timolol metabolites. For M1 the rates of formation were approximately 8.4 and 3.2 pmol/min/pmol for P450s CYP2D6 and CYP2C19, respectively (Table 2).

The contributions of CYP2D6 and CYP2C19 to the microsomal metabolism of timolol were calculated on the basis of the average
microsomal concentrations of these two enzymes, 8 and 14 pmol/mg microsomal protein (Rowland-Yeo et al., 2004). Calculation of the relative intrinsic clearances for these two enzymes indicated that CYP2D6 is responsible for >90% of the formation of M1 and M2, whereas the contribution of CYP2C19 is <10%. The contribution of CYP2C19 increases with higher substrate concentrations.

**Correlation Analysis.** The rates of formation of all the timolol metabolites, M1, M2, M3, and M4, correlated significantly with CYP2D6 activity (dextromethorphan O-demethylation) (M1: \( r = 0.77, P = 0.001 \); M2: \( r = 0.79, P < 0.0001 \); M3: \( r = 0.56, P = 0.03 \); M4: \( r = 0.84, P < 0.0001 \)). The formation rate of M3 also correlated \( (r = 0.64, P = 0.01) \) with CYP2C9 activity [tolbutamide (methyl)-hydroxylation] (Table 3).

**Inhibition of Timolol Metabolism by the Selective CYP2D6 Inhibitor Quinidine and the CYP2C19 Inhibitor Fluvoxamine.** The effects of quinidine and fluvoxamine on timolol metabolism are shown in Fig. 2. Quinidine potently inhibited the formation of all metabolites with \( IC_{50} \) values of 0.035 \( \mu \)M for M1 and 0.05 \( \mu \)M for M2, M3, and M4. At the highest quinidine concentrations (10 \( \mu \)M), the metabolite counts were generally below 15% of those detected in incubations without quinidine. Fluvoxamine inhibited formation of M1, M2, M3, and M4 with \( IC_{50} \) values of 10 \( \mu \)M, 6.2 \( \mu \)M, 6.8 \( \mu \)M, and 10 \( \mu \)M, respectively. Lineweaver-Burk, Eadie-Hofstee, and Hanes analyses suggested that the mode of quinidine inhibition was competitive rather than noncompetitive. Dixon analysis of quinidine inhibition of M1 formation (Fig. 3) provides a \( K_I \) value of 0.08 \( \mu \)M, which is in agreement with some earlier in vitro studies (see Pelkonen et al., 1998).

**Formation of Dextrorphan in the Presence of Timolol or Quinidine.** The formation of the dextromethorphan O-demethylated metabolite dextrophan in incubations with different concentrations of timolol and quinidine is shown in Fig. 4. Addition of timolol up to a maximum concentration of 10 \( \mu \)M in the dextromethorphan assay had no effect on the formation of dextrophan, whereas quinidine clearly inhibited its formation with an \( IC_{50} \) value of 0.08 \( \mu \)M.

**Discussion**

Although timolol has been in use for decades, only fragmentary information is available on its metabolic characteristics. The aim of this study was to identify timolol metabolites and to elucidate the metabolism of timolol in vitro. Its metabolism was studied in the presence of human liver homogenate and microsomes and recombinant enzymes. Using a simple standard one-compartment pharmacokinetic model and some basic assumptions, the in vitro homogenate data allowed a quantitative prediction to be made concerning the metabolic elimination of timolol. On the basis of the first-order substrate depletion of timolol in human liver homogenate incubations, and adopting literature values for the renal clearance and volume of distribution of timolol, the half-life was calculated to be 2.9 h. This extrapolated half-life does not differ from those measured in actual in vivo studies; Dollery et al. (1991) report the half-life of timolol to be 2 to 5 h.

Four timolol metabolites were identified, the hydroxy metabolite M1 being the most abundant. In human liver homogenate incubations, metabolites M1 and M2 were formed in all substrate concentrations used, whereas the formation of metabolites M3 and M4 was much lower, if formed at all. It is suggested that metabolites M3 and M4 are formed from metabolite M1. A weakness of this study was that we were unable to obtain metabolite standards, all activities of metabolite formation being consequently calculated as timolol equivalents, i.e., assuming that the metabolites give the same response in the LC-MC analysis as the parent timolol. There are reasons to doubt this assumption: in vitro-in vivo extrapolation of hepatic clearance based on the activities of M1 and M2 formation by human liver microsomes provided clearance estimates that were about 5-fold lower than the estimate based on first-order substrate depletion (see above). This bias is nonetheless not expected to invalidate our data on the enzyme assignments or relative contributions of different P450 enzymes to timolol metabolism.

On the basis of the present findings, timolol is metabolized mainly by CYP2D6, as has previously been suggested (Edeki et al., 1995; Nieminen et al., 2005a). Timolol was also found to be metabolized by the recombinant CYP2C19. All the metabolites, M1, M2, M3, and M4, were found, however, to be formed to a considerably lesser degree by CYP2C19 than by CYP2D6. M1 was the main metabolite produced by CYP2C19. On the basis of recombinant P450 experiments (Table 2), estimates of the contributions of CYP2D6 and CYP2C19 to timolol metabolism were >90% and <10%, respectively. It is also of note that fluvoxamine, a CYP2C19-selective inhibitor, was a relatively modest inhibitor of timolol metabolism, providing further confirmation of the relatively small contribution of CYP2C19.

The metabolic route has been reported to be the predominant route of timolol clearance (Dollery et al., 1991). Consequently, at least in theory, both the CYP2D6 poor metabolizer phenotype and inhibition of CYP2D6 would be expected to lead to relatively large increases in plasma concentrations of timolol. However, the contribution of renal clearance, estimated to be normally 20% (Dollery et al., 1991), may be expected to attenuate any deficiency or decrease in metabolic clearance. In the absence of direct studies, the magnitude of this attenuation is difficult to estimate. It is known that poor metabolizers have about twice the plasma timolol concentration of extensive metabolizers after single oral doses (Lennard et al., 1986). Renal clearance would thus appear to be capable of compensating at least partially any deficiency or inhibition of hepatic clearance. In addition, according to the present results, it is suggested that CYP2C19 has no clinically significant role in attenuating timolol metabolism in CYP2D6 poor metabolizers.

Quinidine was seen here to have a clear effect on the metabolism of timolol, which further indicates that CYP2D6 has a significant role in the process. The mode whereby quinidine inhibits timolol metabolism was found to be competitive, and the \( K_I \) value in vitro was 0.08 \( \mu \)M. For the calculation of inhibition in vivo, the inhibition constant or \( K_I \), mode of inhibition, and concentration of an inhibitor at the enzyme active site should be known. Furthermore, it is of importance to know whether the in vivo concentration of a drug is close to its \( K_I \) value or far below it. Assuming competitive inhibition and the substrate concentration to be far below its \( K_m \) value (i.e., \( [S] \ll K_m \)), the percentage inhibition can be simply calculated according to the equation \( I = \left( 1 - \frac{[S]}{K_m} \right) \times 100 \). It has to be stressed that the figure obtained is a very crude estimate and depends on a number of factors (see Pelkonen et al., 1998). For quinidine, the percentage of inhibition would be around

| Table 2: The kinetics of timolol (25 \( \mu \)M) metabolism M1 and M2 formation in a pool of human liver microsomes (HLM) and in cDNA-expressed CYP2C19 and CYP2D6 |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | \( V_{max} \)   | \( K_m \)       | \( V_{max} \)   | \( K_m \)       |
| HLMs            | 91.2            | 23.8            | 7.7             | 78.1            |
| CYP2C19         | 3.2             | 49.9            | 0.02            | 33.3            |
| CYP2D6          | 6.4             | 6.95            | 0.03            | 4.7             |

\( V_{max} \) in units of pmol/min/mg protein for HLMs and of pmol/min/pmol P450 for recombinant enzymes.

\( K_m \) in units of \( \mu \)M.
93%, i.e., practically complete inhibition of CYP2D6, assuming that the average quinidine concentration over a few hours is 1 μM. Timolol metabolism may be taken to be more or less completely inhibited as long as plasma concentrations of quinidine are greater than 0.08 μM. The impact of an increased concentration of timolol in plasma is dependent on alternative routes of clearance. As stated above, it would appear that CYP2C19 and renal clearance are capable of partially compensating the decrease in metabolic clearance. However, the in vivo contribution of this compensatory metabolism has not been properly established.

Cardiac effects of timolol become rather pronounced at plasma concentrations over 0.7 μg/l (Nieminen et al., 2005b). This could serve as a cutoff value in defining plasma concentrations likely to cause cardiac effects in a large majority of patients, although Nieminen et al. (2005b) have shown a correlation between timolol plasma concentration and β-blockade. Whether concentrations likely to cause cardiac effects are reached in connection with inhibition by quinidine or in poor metabolizers is largely dependent on the concentrations reached without these interfering factors. With conventional aqueous solutions containing 0.5% timolol, systemic absorption is fairly rapid and the maximum concentration reaches a mean value of 1.7 μg/l (Uusitalo et al., 2005). Timolol products based on a hydrophilic gel matrix with higher ocular bioavailability and low systemic exposure have been developed. Systemic absorption is stunted after application of 0.1% timolol hydrogel, so that the maximum concentration is of the order of 0.2 μg/l and the variability is much smaller than that after

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**TABLE 3**

Correlation analysis, Pearson’s correlation coefficients (r), for formation rates for timolol (25 μM) metabolites M1-M4 in incubations with a pool of human liver microsomes

<table>
<thead>
<tr>
<th>Assay</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2 Ethoxyresorufin O-deethylation</td>
<td>0.35</td>
<td>0.33</td>
<td>-0.22</td>
<td>0.21</td>
</tr>
<tr>
<td>CYP2A6 Coumarin 7-hydroxylation</td>
<td>-0.01</td>
<td>0.06</td>
<td>0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>CYP2B6 Bupropion hydroxylation</td>
<td>-0.52</td>
<td>-0.20</td>
<td>0.09</td>
<td>-0.10</td>
</tr>
<tr>
<td>CYP2C8 Amodiaquine dehydroxylation</td>
<td>-0.21</td>
<td>0.09</td>
<td>-0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>CYP2C9 Tolbutamide (methyl)hydroxylation</td>
<td>0.25</td>
<td>0.43</td>
<td>0.64*</td>
<td>0.23</td>
</tr>
<tr>
<td>CYP2C19 Omeprazole 5-hydroxylation</td>
<td>0.26</td>
<td>0.40</td>
<td>0.08</td>
<td>0.36</td>
</tr>
<tr>
<td>CYP2D6 Dextromethorphan O-demethylation</td>
<td>0.77*</td>
<td>0.79*</td>
<td>0.56*</td>
<td>0.84*</td>
</tr>
<tr>
<td>CYP2E1 Chlorzoxazone 6-hydroxylation</td>
<td>-0.20</td>
<td>-0.34</td>
<td>-0.34</td>
<td>-0.30</td>
</tr>
<tr>
<td>CYP3A4 Midazolam 1'-hydroxylation</td>
<td>0.19</td>
<td>0.35</td>
<td>0.1</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>0.26</td>
<td>0.1</td>
<td>0.29</td>
</tr>
</tbody>
</table>

* P < 0.05; Student’s t test.
application of aqueous solution. If timolol plasma concentrations remain low, it is probable that they will not be increased clinically significantly by quinidine. For example, if the maximum concentration attained after 0.1% timolol hydrogel is about 0.2 μg/l, the involvement of quinidine or PM phenotype is expected to increase this concentration to only about 0.4 μg/l, on the basis of an earlier study (Lennard et al., 1986). This is still well below the cutoff concentration of 0.7 μg/l. Since quinidine is one of the most potent inhibitors of CYP2D6, it seems fairly likely that concurrent administration of 0.1% timolol hydrogel and CYP2D6 inhibitors will not increase the plasma levels of timolol above the cutoff concentration.

In conclusion, the results of this study confirmed the suspicion that timolol is metabolized by CYP2D6 in vitro. The main metabolite was found to be the hydroxy metabolite M1. The CYP2D6 inhibitor quinidine clearly inhibited the metabolism of timolol, a factor to be taken into account when patients are treated with both systemically and topically applied timolol products. The CYP2D6 PM phenotype as well as concomitantly administered CYP2D6 inhibitor drugs may increase the systemic timolol concentration in patients. However, when plasma timolol concentrations in patients remain low (<0.2 μg/l), it is suggested that this type of interaction is of only minor clinical relevance. To avoid systemic effects caused by increased systemic timolol concentrations, it is important to minimize systemic absorption of timolol with topical products in the treatment of glaucoma.

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References


Address correspondence to: Olavi Pelkonen, Department of Pharmacology and Toxicology, P.O. Box 5000, 90014 University of Oulu, Finland. E-mail: olavi.pelkonen@oulu.fi