DMD #12906

Timolol metabolism in human liver microsomes is mediated principally by CYP2D6*

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

Santen Oy, Tampere, Finland (M.V., J.M.)

University of Oulu, Department of Pharmacology and Toxicology, Oulu, Finland (M.T., O.P.)

Novamass Analytical Ltd, Oulu, Finland (A.T., J.U.)

University of Oulu, Department of Chemistry, Oulu, Finland (A.T.)

1

DMD #12906

RUNNING TITLE PAGE

a)   Running title: Metabolism of timolol by CYP2D6

b)   Corresponding author:

Olavi Pelkonen

Department of Pharmacology and Toxicology

P.O. Box 5000

90014 University of Oulu

Finland

Telephone: +358-8-5375230, Fax: +358-8-5375247

E-mail: olavi.pelkonen@oulu.fi

c)   Text pages: 32

Number of tables: 3

Number of figures: 4

Number of references: 28

Number of words in abstract: 235

Number of words in introduction: 527

Number of words in discussion: 1260

1)   Abbreviations used are: P450, cytochrome P450; LC/MS, liquid chromatography

hyphenated with mass spectrometry; HPLC, high-performance liquid chromatography

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 extensively studied. The aim here was to tentatively identify timolol metabolites and to determine the CYP-associated metabolic and interaction properties of timolol *in vitro*. Four metabolites were identified, the most abundant being a hydroxymetabolite M1. The K_m value for the formation of M1 was 23.8 µM in human liver microsomes. Metabolism of timolol with recombinant CYPs 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 µM. 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 hours, an estimate close to the half-life of about 2-5 hours 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 µg/L), it is suggested that such interaction is of minor clinical relevance.

INTRODUCTION

Timolol is a non-selective β-adrenergic receptor blocking agent which 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 man at least two major metabolites are formed from timolol by cleavage of the morpholine ring (Tocco *et al.* 1975; Tocco *et al.* 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 (EM) and poor metabolizers (PM), 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

DMD #12906

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_2-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 β-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 - compared to the very high concentrations observed in PMs. Attempts have been made to develop timolol-containing ophthalmic products which 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-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol) maleate was supplied from Santen Oy (Tampere, Finland). The metabolite standards dextrorphan, desethylamodiaquine, 6-hydroxychlorzoxazone, hydroxytolbutamide and 6β-hydroxytestosterone were purchased from BD Biosciences Discovery Labware (Bedford, MA). Hydroxybupropion was a generous donation from Glaxo SmithKline (Research Triangle, NC), 1-hydroxymidazolam from F. Hoffmann- La Roche (Basel, Switzerland), and omeprazole sulphone and 5-hydroxyomeprazole from Astra Zeneca (Mölndal, Sweden). Formic acid and LichroSolv GG acetonitrile were obtained from Merck KGaA (Darmstadt, Germany). All other chemicals were from Sigma Chemical Company (St. Louis, MO) and were of the highest purity available. Water was in-house freshly prepared 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 CYPs 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 40 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, Hamburg, Germany) at +37 ^{o}C and reaction 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 60min (without NADPH), respectively. Each reaction was terminated by adding 100 µl of ice-cold acetonitrile, with subsequent cooling in an icebath to precipitate the proteins. The tubes were stored at –18 ^{o}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 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

DMD #12906

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 (only solvent), 0.01, 0.1, 1, 10 and 100 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 CYPs (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5) co-expressing human P450 reductase (SUPERSOMES™) were purchased from BD Biosciences Discovery Labware (Bedford, MA) and used according to manufacturer's instructions. For evaluation of the contribution of different CYPs to timolol metabolism, a final concentration of 25 µM of timolol was employed. For kinetic studies with recombinant CYP2C19 and CYP2D6, final concentrations of 0.5, 2, 8, 32 and 128 µM of timolol were used.

Inhibition of dextromethorphan O-demethylation by timolol and quinidine

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

Correlation analysis of timolol metabolism versus CYP-isoform 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 CYP-isoform 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 (chlorzoxazone 6-hydroxylation) and CYP3A4/5 (midazolam 1´-hydroxylation) are described previously in an article detail by Taavitsainen *et al*. (2001). The bupropion hydroxylation assay for CYP2B6 was slightly modified from that applied by Faucette *et al.* (2000) and Hesse *et al*. (2000): incubation mixtures contained 2.0 mg microsomal protein/mL and 50 µM bupropion and incubation was for 15 min. The amodiaquine *N*-desethylation assay for CYP2C8 was a modification from that of Li *et al*. (2002): incubation mixtures contained 0.5 mg protein/mL and 30 µM amodiaquine, the incubation time was 20 min. The omeprazole 5-hydroxylation and sulphoxidation assays for CYP2C19 and CYP3A4, respectively were adapted from those described by Äbelö *et al.* (2000): incubation mixtures contained 0.5 mg microsomal protein /mL and 40 µM omeprazole and incubation was for 20 min. Otherwise the reactions contained buffer and NADPH and were performed as described above. Pearson's correlation coefficients (r) were calculated and given in the Results section. For statistical testing an ANOVA model for repeated measures and Student's t-test for post hoc analysis were used. The limit of statistical significance was set at $P < .05$.

LC/MS conditions

All samples were thawed at room temperature (RT), shaken and centrifuged for 10 min at 13400 rpm (Eppendorf Mini Spin, Eppendorf AG, Hamburg, Germany) and transferred to

Total Recovery vials (Waters Corporation, Milford, MA). A Waters Alliance 2695 high-performance liquid chromatographic (HPLC) system (Waters Corporation, Milford, MA) 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 (4.0×2.0 mm, 3.0 µm, Phenomenex, Torrance, CA) were 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 minutes was applied followed by fast wash of the column with 70% B within one minute and equilibration with initial conditions. For dextromethorphan and its metabolites, a Waters XTerra RP18 column (2.1×50 mm, 3.5µm) and a Phenomenex Luna-C18 precolumn (4.0×2.0 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 six minutes was applied, followed by equilibration to initial conditions. The eluent flow rate in both chromatographic methods was 0.3 mL/min and the column oven temperature was 30 ℃. The flow was split post-column with Acurate Post-Column 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 (TOF) high resolution mass spectrometer (Micromass Ltd., Manchester, UK) equipped with a LockSpray electrospray ionization (ESI) 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 H/D (hydrogen-deuterium) exchange experiments the D_2O was delivered into the HPLC eluent flow post-column by a syringe pump (Harvard Apparatus, Holliston, MA) via a T-mode flow splitter with a flow rate of 100 µL/mL. The number of labile protons in the metabolites was calculated according the mass shift of deuterium-

exchanged molecular ions compared to the normal LC/MS molecular ions (Tolonen *et al*.

2005). The mass spectrometer and HPLC system were operated under Micromass MassLynx

4.0 software.

All LC/MS/MS data for interaction studies were recorded with a Micromass Quattro II triple

quadrupole mass spectrometer (Micromass Ltd.) equipped with a Z-Spray electrospray

ionization source, using a multiple reaction monitoring (MRM) mode. For timolol and its

metabolites the cone voltage was 28 V and collision energy 18 eV. The monitored MRM

transitions were m/z 317 > 261 for timolol, m/z 333 > 259 and 333 > 186 for M1, m/z 335 >

279 and m/z 335 > 206 for M2, m/z 349 > 293 and m/z 349 > 220 for M3, and m/z 315 > 259

and m/z 315 > 186 for M4. For dextromethorphan and dextrorphan, cone voltages of 40 V and

45 V were used, respectively, while the collision energies were 25 eV and 28 eV,

respectively. The monitored MRM transitions were m/z 272 > 213 and 272 > 147 for

dextromethorphan and m/z 258 > 199 and 258 > 133 for dextrorphan. In all LC/MSMS

experiments argon was used as collision gas at 1.6×10^{-3} mbar pressure.

The disappearance of timolol was determined semiquantitatively from LC/MS runs with the

TOF instrument by comparing the peak area in an appropriate 0 min sample to that of the

corresponding metabolized sample (incubated with NADPH for 20, 40 or 60 min). The effects

of the inhibitors on the formation of timolol metabolites and dextrorphan were determined

from LC/MS/MS runs with a triple quadrupole instrument. The LC/MS/MS peak areas of the

metabolites were monitored and compared between incubations with and without the

interacting drug. Thus in both cases the determinations were carried out without standard

samples with known concentrations as quantification references, assuming the mass

spectrometric response of the analyte to be directly comparable at different concentrations.

Data analysis

The IC_{50} values for inhibitors were determined graphically by linear regression analysis of the

plot of the logarithm of the inhibitor concentration versus the percentage of activity remaining

after inhibition using a Microcal Origin, version 6.0 (Microcal Software, Inc., Northampton,

MA, USA). To determinine the K_i 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 $P < .05$.

Kinetic in vitro – in vivo extrapolations

To roughly estimate the intrinsic hepatic clearance (CL_{int} (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 $-\ln ([S]_{20}/[S]_0)/(t_{20} - t_0)$,

where the timolol concentration at t_0 is 1 μM and at t_{20} 0.65 μM, account being taken of the

volume of incubation (0.2 ml) and the amount of homogenate added per incubate (8 mg liver

homogenate, weight basis). Hepatic clearance CL_H was calculated using the equation $CL_H =$

$Q_H * CL_{int}/ (Q_H + CL_{int}))$, where Q_H = liver blood flow. The half-life ($t_{1/2}$) of timolol was

predicted using the equation $t_{1/2} = 0.693 / K_{el}$, where $K_{el}, = CL_{total}/ V_D$. Total (systemic)

clearance (CL_{total}) is the sum of hepatic clerance (CL_H) and renal clearance (CL_R). The

following assumptions were adhered to in the calculations: the mass of human liver is 1500 g,

liver blood flow Q_H = 1.45 L/min, renal clearance (assumption) CL_R = 0.18 L/min, mass of

virtual human 70 kg and distribution volume V_D = 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-minute 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 minutes' incubation time was selected, as 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 (CL_H) about 32 L/hr, total body clearance (CL_{total}, renal clearance taken into consideration) 42 L/hr; the elimination constant (K_{el}) was 0.24/h and half-life ($t_{1/2}$) 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). The suggested structures are shown in Figure 1. M1 was ascertained to be formed via hydroxylation into the morpholino ring. M2 was left with several possible structures involving addition of one oxygen and two hydrogen atoms into the substrate structure, either in the morpholine or the sulphur-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 to the substrate, both of them being located in the morpholine ring area. The metabolite may thus either be di-hydroxylated timolol or alternatively be 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 H_2 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 H/D 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 of 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 CYPs

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 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 CYPs 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 per mg of 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 Figure 2. Quinidine potently inhibited the formation of all metabolites with IC_{50} values of 0.035 µM for M1 and 0.05 µM for M2, M3 and M4. At the highest quinidine concentrations (10 µM), 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 µM, 6.2 µM, 6.8 µM and 10 µM, respectively. Lineweaver-Burk, Eadie-Hofstee and Hanes analyses suggested that the mode of quinidine inhibition was competitive rather than non-competitive. Dixon analysis of quinidine inhibition of M1 formation (Figure 3) provides a K_i

DMD #12906

value of 0.08μM, 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 dextrorphan in incubations with different concentrations of timolol and quinidine is shown in Figure 4. Addition of timolol up to a maximum concentration of 10 μM in the dextromethorphan assay had no effect on the formation of dextrorphan, whereas quinidine clearly inhibited its formation with an IC_{50} value of 0.08 μM.

17

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. Based on 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 hours. 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 - 5 hours.

Four timolol metabolites were identified, the hydroxymetabolite M1 being the most abundant. In human liver homogenate incubations metabolites M1 and M2 were formed in all substrate concentrations used, while 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 which 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

DMD #12906

the enzyme assignments or relative contributions of different CYP enzymes to timolol metabolism.

Based on 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 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 CYP 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 µM. For the calculation of inhibition *in vivo*, the inhibition constant or K_i, mode of inhibition and the 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_m value or far below it. Assuming competitive inhibition and the substrate concentration to be far below its K_m value (i.e. $[S] << K_m$), the percentage inhibition can be simply calculated according to the equation $I/(I+K_i)$ x 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 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 over 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 cut-off 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

20

these interfering factors. With conventional aqueous solutions containing 0.5% of 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 after 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 cut-off 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 cut-off concentration.

In conclusion, the results of this study confirmed the suggestion that timolol is metabolized by CYP2D6 *in vitro*. The main metabolite was found to be the hydroxymetabolite 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 ($\leq$ 0.2 µg/L), it is suggested that this type of interaction is of only minor clinical relevance. In order to avoid systemic effects

21

DMD #12906

caused by increased systemic timolol concentrations it is important to minimize systemic

absorption of timolol with topical products in the treatment of glaucoma.

ACKNOWLEDGMENTS

The excellent technical assistance of Mrs. Ritva Tauriainen is gratefully acknowledged.

DMD #12906

REFERENCES

Abelö A, Andersson TB, Antonsson M, Naudot AK, Skånberg I and Weidolf L (2000) Stereoselective metabolism of omeprazole by human cytochrome P450 enzymes. *Drug Metab Dispos* 28: 966-72.

Blumenfeld A (2005) Clinical approaches to migraine prophylaxis. *Am J Manag Care* 11:55-61.

Brooks AMV and Gillies WE (1992) Ocular β-blockers in glaucoma management, clinical pharmacological aspects. *Drugs & Aging* 2:208-21.

Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharm Res* 10:1093-5.

Dollery C, Boobis AR, Burley D, Davies DS, Harrison PI, Orme ML, Park BK and Goldberg LI (1991) Timolol (maleate), in *Therapeutic Drugs (vol 2)* pp.T77-T80.

Dunn FG and Frohlich FD (1981) Pharmacokinetics, mechanisms of action, indications, and adverse effects of timolol maleate, a nonselective beta-adrenoreceptor blocking agent. *Pharmacotherapy* 1:188-200.

Edeki TI, He H and Wood AJJ (1995) Pharmacogenetic explanation for excessive β-blockade following timolol eye drops. Potential for oral-ophthalmic drug interaction. *JAMA* 274:1611-3.

Faucette SR, Hawke RL, Lecluyse EL, Shord SS, Yan B, Laethem RM and Lindley CM (2000) Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab Dispos* 28:1222-30.

Hesse LM, Venkatakrishnan K, Court MH, von Moltke LL, Duan SX, Shader RI and Greenblatt DJ (2000) CYP2B6 mediates the *in vitro* hydroxylation of bupropion: potential drug interactions with other antidepressants. *Drug Metab Dispos* 28:1176-83.

Ishii Y, Nakamura K, Tsutsumi K, Kategawa T, Nakano S and Nakatsuka K (2000) Drug interaction between cimetidine and timolol ophthalmic solution: effect on heart rate and intraocular pressure in healthy Japanese volunteers. *J Clin Pharmacol* 40:193-9.

Kaila T, Huupponen R, Karhuvaara S, Havula P, Scheinin M, Iisalo E and Salminen L (1991) Beta-blocking effects of timolol at low plasma concentrations. *Clin Pharmacol Ther* 49:53-8.

Korte JM, Kaila T and Saari KM (2002) Systemic bioavailability and cardiopulmonary effects of 0.5% timolol eye drops. *Graefes Arch Clin Exp Ophthalmol* 240:430-5.

Lennard MS, Tucker GT and Woods HF (1986) The polymorphic oxidation of adrenoceptor antagonists. Clinical pharmacokinetic considerations. *Clin Pharmacokin* 11:1-17.

Li XQ, Bjorkman A, Andersson TB, Ridderstrom M and Masimirembwa CM (2002) Amodiaquine clearance and its metabolism to N-desethylamodiaquine is mediated by CYP2C8: a new high affinity and turnover enzyme-specific probe substrate. *J Pharmacol Exp Ther* 300:399-407.

DMD #12906

Nelson WL, Fraunfelder FT, Sills JM, Arrowsmith JB and Kuritsky JN (1986) Adverse respiratory and cardiovascular events attributed to timolol ophthalmic solution, 1978-1985. *Am J Ophthalmol* 102:606-11.

Nieminen T, Uusitalo H, Mäenpää J, Turjanmaa V, Rane A, Lundgren S, Ropo A, Rontu R, Lehtimäki T and Kähönen M (2005a) Polymorphisms of genes CYP2D6, ADRB1 and GNAS1 in pharmacokinetics and systemic effects of ophthalmic timolol. *Eur J Clin Pharmacol*. 61:811-9.

Nieminen T, Uusitalo H, Turjanmaa V, Bjärnhall G, Hedenström H, Mäenpää J, Ropo A, Heikkilä P and Kähönen M (2005b) Association between low plasma levels of ophthalmic timolol and haemodynamics in glaucoma patients. *Eur J Clin Pharmacol*. 61:369-74.

Pelkonen O, Mäenpää J, Taavitsainen P, Rautio A and Raunio H (1998) Inhibition and induction of human P450 (CYP) enzymes. *Xenobiotica* 28:1203-53.

Rowland-Yeo K, Rostami-Hodjegan A and Tucker GT (2004) Abundance of cytochromes P450 in human liver: a meta-analysis. *Br J Clin Pharmacol* 57:687–8.

Shell JW (1982) Pharmacokinetics of topically applied ophthalmic drugs. *Surv Ophthalmol* 26:207-18.

Taavitsainen P, Juvonen R and Pelkonen O (2001) *In vitro* inhibition of cytochrome P450 enzymes in human liver microsomes by a potent CYP2A6 inhibitor, trans-2-phenylcyclopropylamine (tranylcypromine), and its nonamine analog, cyclopropylbenzene. *Drug Metab Dispos* 29:1-6.

Tocco DJ, Duncan AEW, DeLune FA, Hucker HB, Gruber VF and Vandenheuvel WJA (1975) Physiological disposition and metabolism of timolol in man and laboratory animals. *Drug Metab Dispos* 3:361-70.

Tocco DJ, Duncan AEW, DeLuna FA, Smith JL, Walker RW and Vandenheuvel WJA. (1980) Timolol metabolism in man and laboratory animals. *Drug Metab Dispos* 8: 236-40.

Tolonen A, Turpeinen M, Uusitalo J and Pelkonen O. (2005) A simple method for differentiation of monoisotopic drug metabolites with hydrogen – deuterium exchange liquid chromatography/electrospray mass spectrometry. *Eur J Pharm Sci* 25: 155-62.

Turpeinen M, Nieminen R, Juntunen T, Taavitsainen P, Raunio H and Pelkonen O. (2004) Selective inhibition of CYP2B6-catalyzed bupropion hydroxylation in human liver microsomes *in vitro*. *Drug Metab Dispos* 32:626-31.

Uusitalo H, Niño J, Tahvanainen K, Turjanmaa V, Ropo A, Tuominen J and Kähönen M. (2005) Efficacy and systemic side-effects of topical 0.5% timolol aqueous solution and 0.1% timolol hydrogel. *Acta Ophthalmol Scand* 83:723-8.

DMD #12906

Van Buskirk EM (1980) Adverse reactions from timolol administration. *Ophthalmology* 87:447-50.

Zanger UM, Raimundo S and Eichelbaum M (2004) Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. *Naunyn Schmiedeberg's Arch Pharmacol* 369:23-37.

27

DMD #12906

FOOTNOTE PAGE

LEGEND FOR FIGURES

Figure 1. Structure of timolol and proposed structures for metabolites M1-M4 according to the mass spectrometric data acquired (Table 1). Addition of water to M2 may also occur as separate hydroxylation and reduction of double bond or ring opening, and M2 being thus probably formed via M1.

Figure 2. Effects of the CYP2D6 inhibitor quinidine (closed symbols) and the CYP2C19 inhibitor fluvoxamine (open symbols) on the formations of timolol metabolites M1 (circles), M2 (squares), M3 (triangles), and M4 (inverted triangles) in the incubations with a pool of HLMs. Timolol concentration 25 µM.

Figure 3. Dixon analysis of quinidine inhibition of the formation of M1 (hydroxyl)metabolite of timolol.

Figure 4. Inhibition of the CYP2D6-mediated dextromethorphan *O*-demethylation to dextrorphan by timolol (circles) and the CYP2D6 reference inhibitor quinidine (squares).

TABLES

Table 1. Mass spectrometric data obtained on timolol and timolol metabolites M1-M4.

Compound	*m/z*	Identification	Measured accurate mass (Da)	Calculated accurate mass (Da)	Labile protons
Timolol	317.15	$[M+H]^+ = [C_{13}H_{24}O_3N_4S+H]^+$	317.1647	317.1652	2
	261.1	$[M+H-C_4H_8]^+$	261.1014	261.1021	
	244.1	$[M+H-C_4H_{11}N]^+$	244.0775	244.0756	
	188.1	$[M+H-C_7H_{14}NOH]^+$	188.0527	188.0493	
M1	333.15	$[M+H]^+ = [C_{13}H_{24}O_4N_4S+H]^+$	333.1596	333.1596	3
	315.13	$[M+H-H_2O]^+$	*		
	259.1	$[M+H-H_2O-C_4H_8]^+$	259.0867	259.0865	
	242.1	$[M+H-H_2O-C_4H_{11}N]^+$	*		
	186.0	$[M+H-H_2O-C_7H_{14}NOH]^+$	186.0371	186.0337	
M2	335.15	$[M+H]^+ = [C_{13}H_{26}O_4N_4S+H]^+$	335.1741	335.1753	3 or 4
	279.1	$[M+H-C_4H_8]^+$	279.1132	279.1127	
	206.1	$[M+H-C_7H_{14}NOH]^+$	206.0637	206.0599	
M3	349.15	$[M+H]^+ = [C_{13}H_{24}O_5N_4S+H]^+$	349.1566	349.1545	4
	293.1	$[M+H-C_4H_8]^+$	*		
	220.1	$[M+H-C_7H_{14}NOH]^+$	220.0437	220.0392	
M4	315.15	$[M+H]^+ = [C_{13}H_{22}O_3N_4S+H]^+$	315.1450	315.1491	2
	259.1	$[M+H-C_4H_8]^+$	*		
	186.0	$[M+H-C_7H_{14}NOH]^+$	*		

* too low a count for exact mass measurement

DMD #12906

Table 2. The kinetics of timolol (25 µM) metabolite M1 and M2 formation in a pool of

human liver microsomes (HLM) and in cDNA-expressed CYP2C19 and CYP2D6.

	M1		**M2**	
	V_{max} [a]	K_m [b]	V_{max} [a]	K_m [b]
HLM	91.2	23.8	7.7	78.1
CYP2C19	3.2	49.9	0.02	33.3
CYP2D6	8.4	6.95	0.03	4.7

[a] V_{max} in units of pmol/min/mg protein for HLM and of pmol/min/pmol of CYP for

recombinant enzymes

[b] K_m in units of µM

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.

CYP	Assay	M1	M2	M3	M4
1A2	Ethoxyresorufin *O*-deethylation	0.35	0.33	-0.22	0.21
2A6	Coumarin 7-hydroxylation	-0.01	0.06	0.19	0.04
2B6	Bupropion hydroxylation	-0.52	-0.20	0.09	-0.10
2C8	Amodiaquine de-ethylation	-0.21	0.09	-0.05	0.03
2C9	Tolbutamide (methyl)hydroxylation	0.25	0.43	0.64*	0.23
2C19	Omeprazole 5-hydroxylation	0.26	0.40	0.08	0.36
2D6	Dextromethorphan *O*-demethylation	0.77*	0.79*	0.56*	0.84*
2E1	Chlorzoxazone 6-hydroxylation	-0.20	-0.34	-0.34	-0.30
3A4	Midazolam 1'-hydroxylation Omeprazole sulphoxidation	0.19 0.07	0.35 0.26	0.1 0.1	0.30 0.29

* $P < .05$. Student's t-test.

Figure 1
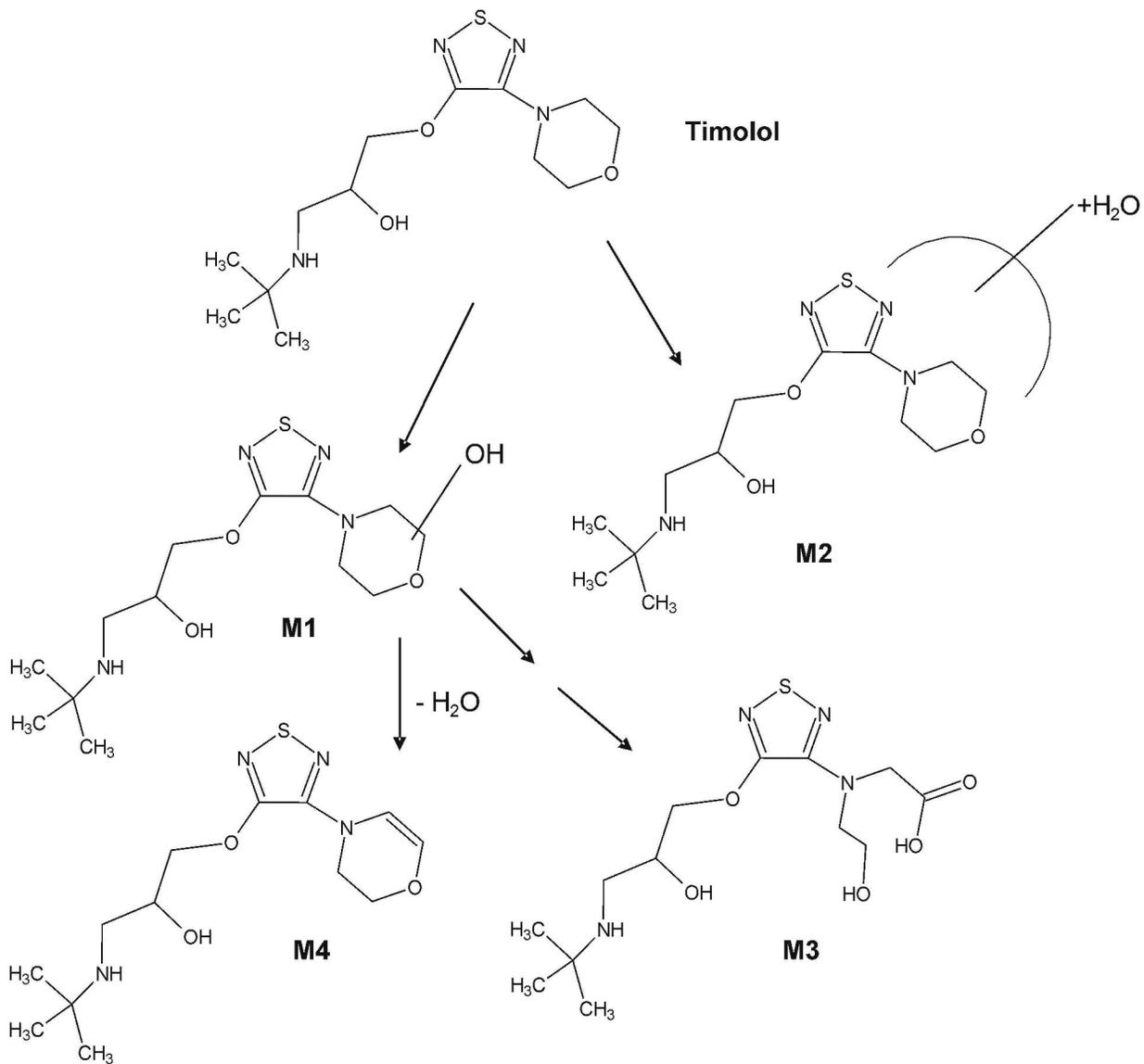
Figure 2

Figure 3

Figure 4

Timolol, $IC_{50} > 10$ µM
Quinidine, $IC_{50} = 0.08$ µM