JPET Fast Forward. Published on February 17, 2005 as DOI: 10.1124/jpet.104.081109

JPET #81109

MODULATION OF METOPROLOL PHARMACOKINETICS AND HEMODYNAMICS BY DIPHENHYDRAMINE CO-ADMINISTRATION DURING EXERCISE TESTING IN HEALTHY PREMENOPAUSAL WOMEN

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Running title: Metoprolol/diphenhydramine interaction - premenopausal women

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Number of text pages: 35
Number of tables: 4
Number of figures: 4
Number of references: 30
Number of words in the abstract: 248
Number of words in the introduction: 745
Number of words in the discussion: 1359

Abbreviations: EM, extensive metabolizer; PM, poor metabolizer; PCB, placebo; DPH, diphenhydramine; MET, metoprolol; C_{max}, peak plasma concentration; T_{max}, time to reach peak plasma concentration; $AUC_{0-\infty}$, area under the concentration time curve (0 to infinity); $t_{1/2}$, half-life; CL/F, total clearance; CL_R, renal clearance; CL_{NR}, non-renal clearance; $CL_{MET \rightarrow \alpha\text{-OH-MET}}$, partial metabolic clearance of metoprolol to α-hydroxymetoprolol; Emax, maximum effect; $EC_{50,}$ drug concentration at half of the maximum effect; AUEC, area under the effect curve.

Recommended section assignment : Absorption, distribution, metabolism and excretion.

ABSTRACT

Premenopausal women may be most vulnerable to acute coronary syndromes at a point in their menstrual cycle when their plasma estrogen levels are the lowest during and immediately after menstruation. Metoprolol is a first line drug in the management of patients with acute coronary syndrome, however, when metoprolol was marketed (1982), women were largely excluded from clinical trials. Further, the over-the-counter antihistamine diphenhydramine inhibited the metabolism of the CYP2D6 substrate metoprolol in healthy, young men with pharmacokinetic and pharmacodynamic consequences. The pharmacokinetics and pharmacodynamics of metoprolol and its interaction with diphenhydramine were investigated in a randomized, double-blind, cross-over, placebo-controlled fashion in healthy, premenopausal extensive (EM; n=16) and poor metabolizer (PM; n=4) women immediately after menstruation. During the placebo phase, EMs had between 5.2-8.4 fold higher total clearance (CL/F) of R- and S-metoprolol compared to PMs while the latter had a 35% greater area under the effect curve (AUEC) and 60% greater EC_{50} for heart rate reduction than EMs (all $p < 0.05$). Diphenhydramine coadmininstration caused a 2.2-3.2 fold decrease in CL/F of metoprolol enantiomers with a resulting 21% increase in AUEC and 29% increase in EC_{50} for heart rate reduction in EMs (all $p < 0.05$). This is the first study to report an in-depth elucidation of metoprolol's pharmacokinetics and hemodynamics in premenopausal EM and PM women at a point in their menstrual cycle when vulnerability for acute coronary events may be greatest. Caution is warranted when the over-the-counter antihistamine diphenhydramine is part of a chronic therapeutic regimen.

INTRODUCTION

Metoprolol is extensively metabolized in humans into 3 major metabolites: α-hydroxymetoprolol (around 10% of the administered dose), O-desmethylmetoprolol, and deaminated metoprolol (Lennard, 1985; Borg et al., 1975). O-Desmethylmetoprolol is further metabolized to form a carboxylic acid metabolite (metoprolol acid) with the latter accounting for approximately 65% of the administered dose. All these metabolites together account for around 85% of the administered dose (Godbillon and Duval, 1984). Both, α-hydroxymetoprolol and O-desmethylmetoprolol were found to have significant beta-blocking activity when tested in cats. However, their ED_{50} values were around 9-10 times (heart rate), 5-8 times (contractile force), and 2-7 times (vasodilatation) higher than those of metoprolol (Borg et al., 1975). The α-hydroxylation pathway is controlled predominantly by the cytochrome P450 isoform CYP2D6. This CYP isoform is subject to a genetic polymorphism with around 6-10% of the Caucasian population, the so-called PMs, lacking this enzyme due to the inheritance of 2 mutant CYP2D6 null alleles. The other 90% of Caucasians have been classified as EMs, although more recently gene multiplications were found to result in an ultrarapid metabolizer status (3-5% of Caucasians) (Johansson et al., 1993) and around 10-15% of Caucasians are now referred to as intermediate metabolizers due to the presence of certain rare CYP2D6 alleles that result in reduced CYP2D6 activity (Raimundo et al., 2004).

Metoprolol is administered as a racemic mixture of R-(+) and S-(-) metoprolol. S-metoprolol possesses 500 times greater β_1-adenoceptor affinity than R-metoprolol

(Wahlund et al., 1990). While S-metoprolol has 33-fold greater β_1 receptor blocking activity on rabbit heart compared to the R-enantiomer, the latter possesses 10 folds greater β_2 receptor blocking activity in the rabbit ciliary process (Nathanson, 1988).

Although a multitude of mechanistic and observational studies suggest a protective effect of estrogen substitution in postmenopausal women against heart disease, its use was recently discouraged when the popular equine estrogens were associated with an increase of venous thromboembolic events in a placebo controlled study (Hully et al., 1998). Nevertheless, epidemiological data have clearly shown that women in their reproductive years have a low incidence of heart disease and that the cardiovascular risk increases after menopause when endogenous hormone levels are naturally low (Lerner and Kannel, 1986). Our group has recently reported that premenopausal women with at least one known risk factor of coronary artery disease were most likely to suffer acute myocardial infarctions or unstable angina attacks during or immediately after menstruation suggesting that relatively low levels of circulating estrogen may contribute or act as a trigger for acute coronary events in this young, female population (Hamelin et al., 2003). While the treatment of women with heart disease are usually based on extrapolations of data obtained in men, sex specific differences in the activities of metabolic enzymes and the pharmacokinetics/pharmacodynamics of many drugs are known to exist (Labbé et al. 2000; Meibohm et al., 2002). Our group has recently reported that the activity of CYP2D6 as determined by the metabolic ratio of dextromethorphan:dextrorphan was significantly greater in 56 pre-menopausal female EMs compared to 86 male EMs. The β1-receptor antagonist metoprolol, a prominent CYP2D6 substrate, is a first line treatment choice in the

management patients presenting with acute coronary syndromes. As women were essentially excluded from clinical trials when metoprolol was brought to market in 1982, pharmacokinetic/ pharmacodynamic data on this drug in women are scarce. We were interested in studying the pharmacokinetics and hemodynamics of metoprolol in healthy premenopausal women as a representative group for all women with fluctuating endogenous hormone levels during the reproductive years. The study was performed as close to the menstruation as possible to standardize for the potential effects of fluctuations in endogenous hormones on metoprolol's disposition while approaching the period of increased risk for acute events in this population.

Our group has further demonstrated that the classic over-the-counter prototype antihistamine diphenhydramine (2-(diphenylmethoxy)-N,N-dimethylethylamine) inhibits the oral, nonrenal and partial metabolic clearances of racemic metoprolol to α-hydroxymetoprolol (and thus increasing metoprolol $AUC_{(0-\infty)}$) and prolonged the negative chronotropic and inotropic effects of the drug in EM but not PM men (Hamelin et al., 2000). However, the exact nature of the interaction (solely pharmacokinetic versus pharmacokinetic/pharmacodynamic) as well as the potential differential effects of diphenhydramine on the disposition of metoprolol's enantiomers were still unknown. Thus, the goals of the present study were to determine (1) the racemic and stereoselective disposition of metoprolol (2) the resultant hemodynamic effects of metoprolol and (3) the interaction between the CYP2D6 inhibitor diphenhydramine and metoprolol in healthy, premenopausal women immediately after menstruation and prior to ovulation.

METHODS

This study was approved by the Laval Hospital Ethics Committee and all volunteers provided written informed consent prior to participating in this study.

Volunteers

Twenty young, healthy, non-smoking Caucasian women (age range: 18 to 40 years; mean age: 26.8 ± 8 years; weight range: 49 to 100 Kg; mean weight: 60 ± 12 Kg) not consuming oral contraceptives, and having regular menstrual cycles were recruited from the Quebec City area. The participants were recruited according to their CYP2D6 activity and were either EMs (n = 16) or PMs (n = 4) as determined by phenotyping and genotyping (described below). The general health status of the participants was determined based on a general questionnaire and a physical examination including electrocardiogram and routine laboratory tests to determine renal and hepatic function. Volunteers followed their menstruation and ovulation schedule for 1-3 months prior to participation in the study. A Conceive LH® (Quidel Corp., San Diego, CA) kit that accurately predicts ovulation was provided during that period and an ovulation test was also done on the study day mornings to ensure that the subjects were not ovulating that day. During screening all volunteers also underwent two-dimensional echocardiography using SONOS 5500® echocardiograph (Philips Medical Systems, Bothell, WA) to rule out stenosis and in order to determine the left ventricular outflow tract (LVOT) area.

Phenotyping and genotyping for CYP2D6 activity

The CYP2D6 phenotype was determined by using dextromethorphan (3 methoxy-17-methylmorphinan monohydrate) as the probe drug as previously described (Labbé et al., 2000). Individuals with a dextromethorphan:dextrorphan metabolic ratio of >0.3 were considered poor metabolizers. A 10 mL blood sample was obtained for genotyping. DNA was extracted from peripheral blood lymphocytes and CYP2D6*1A, CYP2D6*3, CYP2D6*4, CYP2D6*5, CYP2D6*6, CYP2D6*7 and CYP2D6*8 alleles were determined using a classic multiplex-PCR (Stuven et al., 1996).

Study design

This study was conducted in a randomized, double-blind, cross-over, placebo-controlled fashion and was carried out after menstruation but prior to ovulation over a 2-months period (Figure 1). During each study arm, diphenhydramine (50mg) or placebo (lactose) was administered to the women three times daily for 5 days. A single oral dose of 100mg metoprolol tartrate was administered on the morning of the 3rd study day. The $t_{1/2}$ of diphenhydramine ranges between 4 to 9.2 hours (Spector et al., 1980; Blyden et al., 1986). Hence, at the time of metoprolol administration diphenhydramine plasma concentrations were at steady-state. Diphenhydramine or placebo administration was continued beyond metoprolol administration, until the 5th study day, i.e., end of the study arm. Randomization tables were prepared by the biostatistician of the research center while a hospital pharmacist dedicated to research protocols controlled the randomization schedule and dispensed crushed placebo tablets and diphenhydramine capsules hidden inside identical looking colored hard gelatin capsules.

Hemodynamic assessment

The individual workload necessary to obtain an exercise heart rate of 140 beats/min on a stationary upright bicycle was determined for each individual prior to metoprolol administration (time 0). This workload was applied at final 4-minutes of each 8-minute exercise test at 0.75, 1.5, 2.25, 3, 4, 8 and 12 hours following metoprolol administration. Heart rate (HR), blood pressure (BP) were obtained using an automated blood pressure monitor (Q410, Quinton, Bothel, Wash) and continuous-wave Doppler recordings of flow velocity were obtained from the suprasternal notch using a nonimaging transducer connected to a SONOS 5500® echocardiograph during rest and exercise at various time points. The nonimaging transducer was angulated to record the signal with maximal flow velocity in the ascending aorta. The Doppler velocity signals were analyzed to obtain the following parameters: aortic velocity time integral (VTI), acceleration time, and ejection time. The values of stroke volume (SV), stroke volume index (SVI), cardiac output (CO) and cardiac index (CI) and rate pressure product (RPP) were calculated as indicated below:

A.) SV = VTI x LVOT area

B.) SVI = SV/BSA

C.) CO = SV × HR

D.) CI = CO / BSA

E.) RPP = HR × systolic BP

Pharmacokinetic assessment

Serial blood samples were obtained from the subjects at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 3, 3.5, 4, 6, 8, 10, 12, 24, 28, 32, 36, and 48 hours following

metoprolol administration. Samples were obtained through an indwelling catheter up to 12 hours and by venous puncture thereafter. Blood samples were immediately spun down, plasma was harvested and frozen at –20ºC until analysis. Urine collection was done from 0-12, 12-24, and 24-48 hours following metoprolol dosing. Urine volume and pH were determined and aliquots were frozen at –20ºC until analysis. Subjects reported for the study after overnight fasting and were provided a light snack at 2 hours and lunch at 4 hours post-metoprolol. Subjects were instructed to abstain from alcohol, grapefruit juice, cruciferous vegetables, charbroiled food, any form of medication (including any over-the counter medications, drugs belonging to an alternate system of medicine, herbal supplements, vitamins and minerals etc) from at least 2 days prior to starting diphenhydramine/placebo administration until end of study arm.

HPLC analysis of metoprolol and α-hydroxymetoprolol in biological samples

Racemic metoprolol (1-(isopropylamino)-3-(4-(2-methoxyethyl)phenoxy)propan-2-ol) and its α-hydroxy metabolite (1-(4-(1-hydroxy-2-methoxyethyl)phenoxy)-3-isopropylamino)propan-2-ol) were determined in plasma and urine using a previously reported HPLC method from our laboratory (Hamelin et al., 2000). The method was found to be highly reproducible with inter- and intraday coefficients of variation below 5%.

R- and S-metoprolol were determined with modifications of a previously published method (Lanchote et al., 2000). The analysis was carried out using a Shimadzu HPLC system with fluorescence detection (λ_{exe} 229 and λ_{em} 298). The resolution was achieved on a chiralcel OD-H® analytical column (250 x 4.6 mm; Daicel chemical industries limited, Exton, PA) using a C-18 guard column (Waters Corporation, Milford, MA). The mobile

phase consisted of n-hexane/isopropanol/diethylamine/trifluoroacetic acid (92:8:0.15:0.025) which was recirculated in a closed-loop system to analyze up to 45 samples at a time. Following addition of alprenolol (internal standard; 400 ng; 1-((methylethyl)amino)-3-(2-(2-propenyl)phenoxy)-2-propanol), methanol (50 μl), saturated sodium carbonate solution (400 μl) and 0.5N sodium hydroxide (600 μl) to 1 mL plasma sample, liquid-liquid extraction was performed twice using 5 ml dichloromethane/diethyl ether (1:1). The limit of detection using this HPLC method was 0.016 nmol/mL for each enantiomer. Although this method was capable of separating O-desmethylmetoprolol enantiomers and α-hydroxymetoprolol diastereomers as well, no attempt was made to measure them because enantiomeric standards are not commercially available.

Pharmacokinetic data analysis

Metoprolol (racemic and enantiomeric) plasma concentration-time data was analyzed by noncompartmental pharmacokinetic analysis. Plasma concentration time data of the active enantiomer S-metoprolol was also analyzed by compartmental analysis to generate appropriate input variables for pharmacokinetic/pharmacodynamic modeling (see below). All pharmacokinetic analysis was done using Kinetica 2000® (Innaphase Corporation, Philadelphia, PA).

For noncompartmental analysis, the area under the concentration-time curve (AUC) was computed by mixed log linear rule up to the last point and was extrapolated to infinity as: C_{last}/β where C_{last} was the last measured concentration-time point and β was the terminal disposition rate constant. The terminal $t_{½}$ was calculated as 0.693/β. Metoprolol total clearance (CL/F) was calculated as dose/$AUC_{(0-\infty)}$, whereas the renal clearance (CL_R) was

determined as $A_{MET}/AUC_{(0-\infty)}$ where A_{MET} was the total unchanged metoprolol eliminated in the urine. Metoprolol nonrenal clearance (CL_{NR}) was computed as: $CL/F - CL_{R.}$ Metoprolol to α-hydroxymetoprolol partial clearance ($CL_{MET \rightarrow \alpha\text{-OH-MET}}$) was calculated as: $A_{OH\text{-}MET}/ AUC_{(0-\infty)}$ where $A_{OH\text{-}MET}$ was the total α-hydroxymetoprolol excreted in urine. Except for $CL_{MET \rightarrow \alpha\text{-OH-MET}}$, all above mentioned parameters were calculated for racemic as well as for R- and S-metoprolol. $CL_{MET \rightarrow \alpha\text{-OH-MET}}$ was measured only for racemic metoprolol because the quantities of α-hydroxymetoprolol diastereomers could not be determined.

For compartmental pharmacokinetic analysis, nonlinear regression with iteratively reweighed least square estimation was used to fit 1 (n=18)- or 2 (n=2)-compartment models to S-metoprolol concentration-time data according to the following criteria: value of objective function, Akaike and Schwartz criteria, correlation matrix, distribution of residuals, and visual fit. The fitting procedure was repeated by changing the start values for pharmacokinetic parameters to ensure that the nonlinear regression algorithm converged at the global rather than a local minimum. The results from compartmental analysis were used as input variables for S-metoprolol PK/PD analysis (see below).

Metoprolol pharmacodynamics

The total area under the response-time curve (AUEC) in EMs and PMs on metoprolol with or without diphenhydramine co-administration was calculated for three pharmacodynamic response markers during exercise, i.e., heart rate, blood pressure and rate-pressure product, by a calculation of the cumulative reduction from the baseline values over the 12-hour study duration. Baseline values were the maximum values of the three

response markers. The AUEC was estimated by the linear trapezoidal rule (Microsoft Excel, ON). The maximum effect compared to baseline, i.e., $E_{max,}$ was read directly from the response-time data.

S-Metoprolol pharmacokinetic/pharmacodynamic (PK/PD) modeling

Integrated PK/PD modeling was employed to relate S-metoprolol plasma concentrations to three pharmacodynamic response markers, i.e., heart rate, blood pressure and rate-pressure product. Pharmacodynamic parameters were modeled as changes relative to baseline values (highest values for any individual in this case).

The results of compartmental pharmacokinetic modeling (absorption rate constant, elimination rate constant, apparent volume of distribution and lag-time) of S-metoprolol along with S-metoprolol plasma concentrations were modeled relative to effect-time profiles for each individual. Based on objective function, residual distribution, visual goodness-of-fit and physiologic reality of the parameter estimates a direct link, direct response pharmacodynamic model using a sigmoid E_{max} relationship, with the effect compartment in the central compartment, was used (Holford and Sheiner, 1991; Meibohm and Derendorf, 1997).

$$E = (E_{max}*C^n)/(EC_{50}{}^n + C^n)$$

where n is the shape factor, E_{max} is the maximum hemodynamic effect, EC_{50} is the plasma concentration needed to achieve half of E_{max}, E and C refer to the observed response parameter and plasma concentration values. The values of E_{max} and EC_{50} were determined by the modeling procedure. All calculations were performed with Scientist® software (Micromath, Salt Lake City, UT).

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Statistical analysis

The analysis of Kolmogorov-Smirnov and Levine were used to test the normality and variance homogeneity of the data. Because all data were normally distributed and variances were equal, data were expressed as mean ± standard deviation in all statistical analysis. Pharmacokinetic parameters of racemic and enantiomeric metoprolol in the whole population (n=20) and in EMs (n=16) and PMs (n=4) were compared using a cross-nested design with two experimental factors (*metabolizer* and *medication*, i.e., administration of metoprolol with or without diphenhydramine) and medication randomly assigned to subjects as the nested factor. A mixed model analysis was also performed with an interaction term between the fixed factors (*metabolizer* and *medication*). Pharmacokinetic parameters of metoprolol enantiomers were analysed using the same statistical approach with a fourth factor added to the model to compare the R-enantiomer with the S-enantiomer.

For comparison of pharmacodynamic parameters in the whole population and in EMs and PMs, a cross-nested design was used to analyse changes of mean cardiac index, blood pressure and rate pressure product data. This design was performed with two fixed factors (metabolizer and medication), one random factor (subject within groups) and a repeated factor (time) nested into the random factor subject. Different statistical models were tested and the final analysis was done with heterogeneity between metabolizers (covariance structures not similar). The multivariate normality was verified using Mardia tests (Mardia, 1974). The results were considered significant with p-values ≤ 0.05. All

analyses were conducted using the statistical package SAS (SAS Institute Inc, Cary, NC, U.S.A.).

RESULTS

Clinical study

Sixteen (n=16) EMs and 4 PMs were recruited for the study. All subjects completed the study and reported no significant adverse effects other than somnolence in some subjects (6 EMs and all 4 PMs) during the diphenhydramine co-administration arm of the study. Results of phenotyping using dextromethorphan were in line with those of genotyping. Seven out of 16 EMs were found to be homozygous for the wild type allele while 9 subjects were heterozygous with 1 wild type and 1 mutant allele (CYP2D6*3 in 1 subject, CYP2D6*4 in 7 subjects and CYP2D6*5 in 1 subject). Two of the 4 PMs were homozygous for CYP2D6*4 while 2 were heterozygous (CYP2D6*4/CYP2D6*5).

Pharmacokinetics

Influence of CYP2D6 phenotype on racemic metoprolol pharmacokinetics in women

The mean pharmacokinetic profile of racemic metoprolol in 16 EM and 4 PM women is presented in Figure 2 and calculated non-compartmental pharmacokinetic parameters are summarized in Table 1. The plasma concentration-time profiles of metoprolol were significantly different in EMs compared to PMs ($P < 0.05$). C_{max} and $AUC_{0-\infty}$ values were 2- and 4-fold higher, respectively, in PMs on placebo compared to EMs on placebo. This was the result of an approximately 7-fold lower CL/F, an 8-fold

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lower CL_{NR} and a 300-fold lower partial metabolic $CL_{MET \rightarrow \alpha\text{-OH-MET}}$ in PMs compared to EMs ($P < 0.05$). The $t_{1/2}$ was 2.5 fold longer in PMs compared to EMs ($P < 0.0001$). In contrast, T_{max} and CL_R values were similar among the phenotypes ($P > 0.05$; Table 1)

Influence of diphenhydramine co-administration on racemic metoprolol and α-hydroxymetoprolol pharmacokinetics in women

Diphenhydramine co-administration aligned the pharmacokinetic profile of racemic metoprolol in EMs towards that of PMs (Figure 2 and Table 1). Diphenhydramine co-administration resulted in a 30% increase in racemic metoprolol C_{max} and an almost 2 fold increase in $AUC_{(0-\infty)}$ ($P < 0.05$). This was related to a 2.5 fold decline in CL/F and CL_{NR} values and a 3-fold decline in partial $CL_{MET \rightarrow \alpha\text{-OH-MET}}$ consistent with inhibition of CYP2D6 by diphenhydramine. In contrast, T_{max} and CL_R were not influenced by the co-administration of the antihistamine. C_{max} and $AUC_{0-\infty}$ values of α-hydroxymetoprolol declined by around 42% and 17%, respectively, in EMs when receiving diphenhydramine, and there was an around 50% increase in α-hydroxymetoprolol Tmax (all Ps ≤ 0.004; Table 2). As expected, diphenhydramine did not alter the disposition of metoprolol or α-hydroxymetoprolol in PMs ($P > 0.05$).

Influence of CYP2D6 phenotype on metoprolol enantiomer pharmacokinetics in women

R-metoprolol mean C_{max} and $AUC_{(0-\infty)}$ were 1.5 fold lower and CL/F and CL_{NR} were 1.7 fold greater compared to S-metoprolol in EMs during the placebo arm (Table 3). In contrast, exposure to R- and S-metoprolol was similar in PMs during the placebo phase.

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The exposure to both enantiomers was significantly lower in EMs compared to PMs (Table 3; Figure 3). PMs had a 2-fold greater R- and S-metoprolol C_{max} ($P < 0.05$), a 5-fold greater R-metoprolol $AUC_{(0-\infty)}$ ($P < 0.05$), and a 4-fold greater S-metoprolol $AUC_{(0-\infty)}$ ($P < 0.05$). This was due to an 8-fold (R-metoprolol) and 5-fold (S-metoprolol) higher mean CL/F and a 9-fold (R-metoprolol) and 6-fold (S-metoprolol) higher CL_{NR} in EMs compared to PMs (all $P < 0.05$).

Influence of diphenhydramine co-administration on metoprolol enantiomer pharmacokinetics in women

Co-administration of diphenhydramine resulted in a 30 to 40% increase in R- and S-metoprolol C_{max} and $t_{½}$ and a 2-fold increase in R- and S-metoprolol $AUC_{(0-\infty)}$ in EMs (all $P < 0.05$). These changes were the result of a 2.6 and 2.2 fold decrease in CL/F and CL_{NR} for the R- and S-metoprolol enantiomers, respectively ($P < 0.05$). Inhibition of metoprolol hepatic elimination brought R- and S-metoprolol pharmacokinetic parameters closer to those of PMs, but inhibition was not complete (Table 3; Figure 3). Diphenhydramine did not significantly affect exposure to either metoprolol enantiomer in PMs (Table 3; $P > 0.05$).

R-metoprolol /S-metoprolol ratios

For EMs in the placebo phase, CL/F and CL_{NR} of R-metoprolol were 1.7 times faster than that of S-metoprolol ($P < 0.05$), but there was no significant difference in the renal clearance of either enantiomer (CL_R R-/S-metoprolol: 1.05; $P > 0.05$). As a consequence, R-/S-metoprolol $AUC_{(0-\infty)}$ was 0.67 ($P < 0.05$). Interestingly, diphenhydramine co-

administration reduced the differences in disposition between the two enantiomers. Diphenhydramine decreased CL/F and CL_{NR} approximately 2.6-fold for the R- compared to approximately 2.2-fold for the S- enantiomer resulting in a R-/S-metoprolol ratio of the clearances of approximately 1.4 ($P > 0.05$ for difference in CL/F and CL_{NR} between enantiomers). In contrast, following diphenhydramine, CL_R values of the two enantiomers were similar to each other (CL_R R-/S-metoprolol: 1.02; $P > 0.05$) and to those values observed during the placebo phase. Following the changes in clearances caused by diphenhydramine administration, $AUC_{(0-\infty)}$ R-metoprolol increased 2.1-fold compared to 1.8-fold for S-metoprolol, resulting in a R-/S-metoprolol ratio of $AUC_{(0-\infty)}$ of 0.76 ($P > 0.05$ for differences in $AUC_{(0-\infty)}$ between enantiomers). In PMs, the $AUC_{(0-\infty)}$ R-/S-metoprolol ratio was found to be 0.98 without and 1.00 with diphenhydramine co-administration showing no stereoselectivity. Correspondingly, there was no significant difference ($P > 0.05$) in the CL/F, CL_{NR} and CL_R of the two enantiomers in the placebo and diphenhydramine phase. The R-/S-metoprolol ratios for $AUC_{(0-\infty)}$,CL/F, CL_{NR} and CL_R were significantly different between EMs and PMs ($P < 0.0001$)

Pharmacodynamics

Changes in four hemodynamic response markers, i.e., heart rate, rate-pressure product, stroke volume index and cardiac index, in response to the administration of metoprolol in the presence or absence of diphenhydramine during exercise are shown in Figure 4. Metoprolol administration also affected these parameters while obtained at rest. However, changes at rest were small and hence are not reported herein.

Exercise heart rate

Metoprolol administration resulted in a significant change in mean exercise heart rate over 12 hours for PMs and EMs (P = 0.001; Figure 4a). PMs and EMs on placebo followed a significantly different heart rate profile over time (P < 0.05 from 1.5 - 12 hours). The mean heart rate was reduced by 31% and 24% compared with baseline values at 1.5 hours post metoprolol in PMs and EMs (P < 0.05). Compared with baseline values, the heart rate was still reduced by 26% (placebo) and 28% (diphenhydramine) in PMs but only by 8% in EMs (placebo) at 12 hours post-metoprolol (PMs P < 0.05 and EMs P > 0.05). However, EMs on diphenhydramine had heart rate reduced by 17% compared to baseline values, at 12-hours post metoprolol (P < 0.05). Diphenhydramine co-administration had no significant (P > 0.05) influence on the heart rate profile of PMs. Starting at 4 hours, the heart rate profile of EMs on placebo evolved distinctly from the heart rate profile of EMs on diphenhydramine and was significantly different (P < 0.05) at 4, 6 (P = 0.06), 8 and 12 hours.

Exercise rate-pressure product

In all 20 volunteers, rate-pressure product was significantly affected (P = 0.0001; Figure 4b) by metoprolol administration regardless of placebo or diphenhydramine co-treatment. The rate-pressure product profile of PMs was significantly different from that of EMs (P = 0.0007 from 1.5 – 12 hours). Initially, mean rate-pressure product had maximal decreases of 46% and 35% compared with baseline values at 1.5 hours post-metoprolol in PMs and EMs on placebo, respectively (PMs *vs* EMs P = 0.0004). Compared with baseline values, the rate-pressure product was still reduced by about 27% (placebo and

diphenhydramine phase) in PMs but only by around 8% in EMs (placebo) at 12 hours post-metoprolol (PMs $P = 0.0005$ and EMs $P = 0.02$ compared to baseline). However, the rate-pressure product of EMs on diphenhydramine was reduced by 14% compared to baseline values at 12 hours post metoprolol ($P = 0.004$). Diphenhydramine co-administration significantly ($P < 0.05$) altered the rate pressure profile of EMs shifting it towards the profile of PMs (Figure 4b). However, diphenhydramine co-administration had no significant effect ($P > 0.05$) on the rate pressure product profile of PMs.

Exercise stroke volume index

Stroke volume index values changed significantly over time in all 20 volunteers ($P = 0.0001$; Figure 4c). There was no significant difference between the profile of PMs and EMs ($P > 0.05$). However, diphenhydramine co-administration significantly affected the stroke volume index profile of EMs compared to placebo ($P < 0.05$ at 0.75 – 4, 8 and 12 hours). The profile of EMs on diphenhydramine dropped lower than the profile of EMs on placebo and followed closely the profile of PMs (regardless of the treatment) at most times. Concomitant diphenhydramine did not significantly alter the stroke volume index profile of PMs.

Exercise cardiac index

Cardiac index changed significantly in all 20 subjects over the 12-hour study period ($P = 0.0001$; Figure 4d). In the placebo phase, PMs followed a significantly different cardiac index profile over time compared to the EMs ($P = 0.009$). Initially, mean cardiac index had a maximal decrease of around 27% and 17% compared with baseline in PMs and

JPET Fast Forward. Published on February 17, 2005 as DOI: 10.1124/jpet.104.081109
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EMs on placebo, respectively (PMs *vs.* EMs; $P < 0.05$). Co-administration of diphenhydramine did not significantly affect the maximum effects on cardiac index in PMs and EMs (27% and 19% decrease for PMs and EMs, respectively; $P > 0.05$ for the treatment effect). However, diphenhydramine co-administration significantly influenced the cardiac index profile of EMs ($P < 0.05$ at 3, 4, 8 and 12 hours compared to EMs on placebo) with the profile becoming similar to that of PMs receiving either co-treatment (PMs *vs.* EMs on diphenhydramine $P > 0.05$ from 3-12 hours). Diphenhydramine co-administration had no significant effect on this response marker in PMs. ($P > 0.05$).

Metoprolol Pharmacodynamics and S-metoprolol PK/PD modeling

The results of the pharmacokinetic/pharmacodynamic modeling are summarized in Table 4. PMs had a significantly greater $AUEC_{(heart\ rate)}$ and $AUEC_{(rate\text{-}pressure\ product)}$ compared to EMs on placebo (irrespective of the co-treatment; $P \leq 0.006$). Concomitant administration of diphenhydramine resulted in a significant increase in AUECs for heart rate and rate-pressure product values in EMs (P = 0.01 compared to placebo co-administration) but not in PMs. In contrast, AUEC for systolic blood pressure was neither influenced by the phenotype nor the co-treatment (P > 0.05). Emax for heart rate, blood pressure and rate-pressure product values were not significantly different between EMs and PMs on either co-treatment. $EC_{50(heart\ rate)}$ ($P = 0.009$), $EC_{50(systolic\ blood\ pressure)}$ ($P = 0.03$), and $EC_{50(rate\text{-}pressure\ product)}$ ($P = 0.01$) were significantly higher in PMs compared to EMs, While diphenhydramine co-administration did not significantly affect $EC_{50(systolic\ blood\ pressure)}$ or $EC_{50(rate\text{-}pressure\ product)}$ (P>0.05), it resulted in a significant ($P = 0.03$) increase of $EC_{50(heart\ rate)}$ in EMs.

DISCUSSION

This is the first study to report an extensive assessment of pharmacokinetics and hemodynamics of metoprolol in young healthy premenopausal women (EMs and PMs) with and without the administration of a moderate inhibitor of CYP2D6, namely the over-the-counter antihistamine diphenhydramine in a randomized, double-blind, and placebo controlled fashion. The study was conducted under controlled conditions at a point in the menstrual cycle when circulating estrogens in the body are in the low range, i.e., after menstruation and prior to ovulation. Conducting the study at this point in the menstrual cycle is very pertinent considering the increased cardiovascular vulnerability of premenopausal women during and right after the menstruation (Hamelin et al., 2003). Further, conducting the study at a precise time period during the menstrual cycle is important for minimising any potential inter-individual variability in drug metabolism caused by cyclic hormonal changes (Benton et al., 2000; Walle et al., 1996). The study is also highly relevant considering the prescription-free availability of many classic antihistamines which carries the risk of interacting with a co-administered CYP2D6 substrate such as metoprolol in this age group and in older women.

The pharmacokinetic profiles of racemic metoprolol as well as of R- and S-metoprolol observed in PMs and EMs in our study are similar to those reported in the past (Lennard et al., 1983). In our study, the average $AUC_{(0-\infty)}$ S-/R-metoprolol ratio was 1.0 in PMs and 1.5 in EMs which is similar to literature values (Lennard et al., 1983). Of interest, the total clearance of R-metoprolol was significantly greater than that of S-metoprolol in EMs on placebo and coadministration of diphenhydramine decreased the clearance and

eliminated the difference between the enantiomers. This suggests that diphenhydramine has a greater inhibitory effect on the metabolism of R-metoprolol. Since others have shown that O-demethylation was significantly stereoselective for R-metoprolol (Murthy et al., 1990; Mautz et al., 1995; Kim et al., 1993) one may speculate that diphenhydramine inhibits O-demethylation of metoprolol to a greater extent.

Our group has previously demonstrated that, *in vitro*, diphenhydramine can competitively inhibit metoprolol metabolism with a Ki of 2 μmol/L (Hamelin et al., 2000). This *in vitro* inhibition persisted *in vivo* in healthy, young EM men, resulting in significantly decreased metoprolol clearance and thus more pronounced and significantly prolonged negative chronotropic and inotropic effects of metoprolol. In the present study we demonstrated that diphenhydramine administration to steady-state modulated the pharmacokinetics and hemodynamics of metoprolol in healthy young premenopausal EM women to a similar extent as previously found in men. Diphenhydramine co-administration shifted the heart rate profile of EMs towards that of PMs receiving either co-treatment thus demonstrating a prolongation of the negative chronotropic effect of metoprolol in EMs. Similarly, the stroke volume index profile of EMs during diphenhydramine coadministration was lower than their profile during placebo coadministration and followed the profile of PMs, even though the heart rate profiles of EMs on concomitant diphenhydramine and that of PMs was lower than that of EMs on concomitant placebo. This indicates a greater negative inotropic effect in PMs (on either co-treatments) and in EMs on diphenhydramine compared to EMs on placebo.

In the present study, the heart rate and rate-pressure product AUEC values increased significantly from EMs on placebo to EMs on diphenhydramine to PMs on either co-treatment (Table 4). This increase corresponds to a significant increase in the plasma $AUC_{(0-\infty)}$ of the drug from EMs on placebo to EMs on diphenhydramine to PMs on either co-treatment (Tables 1 and 3). No significant increase in systolic blood pressure AUEC values, similar to the increases in heart rate and rate-pressure product, were observed. However, this is not entirely unexpected. Although a linear relationship between log of plasma levels and exercise heart rate reduction has been described in the literature, no such association exists for antihypertensive activity of metoprolol (Bengtsson et al., 1975). It is also possible that the potential differences cannot be identified because blood pressure is a more complex regulated parameter than heart rate where numerous competing and compensatory mechanisms are interacting simultaneously. No significant differences in the E_{max} - heart rate, blood pressure and rate-pressure product were observed between EMs and PMs (regardless of the co-treatment) despite the fact that PMs (regardless of the co-treatment) and EMs on diphenhydramine had a higher $AUC_{(0-\infty)}$ and C_{max} of metoprolol and a higher AUEC. This could be explained by considering that these subjects, despite their phenotype and co-treatment, are reaching the observed E_{max} for the tested response markers at a certain time point with the administered dose. Hence, any increase in the concentration of S-metoprolol in the plasma (whether due to concomitant diphenhydramine as for EMs or due to phenotype) has no significant additional effect on this parameter. The EC_{50} – heart rate, systolic blood pressure and rate-pressure product values were significantly higher in PMs (placebo) than EMs (placebo) indicating that EMs had a higher sensitivity towards S-metoprolol than PMs. Diphenhydramine co-administration was found to increase the EC_{50}

– heart rate and rate pressure parameter in EMs but not in PMs. One may speculate that diphenhydramine or metoprolol related metabolites or a combination of both contribute to these observations regarding the EC50. Diphenhydramine or one of its metabolites might change the concentration-effect relationship by counteracting the effects of metoprolol on heart rate. On the other hand, the amounts of α-hydroxymetoprolol and /or O-demethylmetoprolol and their contribution to the hemodynamic effects may play a role. Even though their in vitro activity is 2-10 times lesser than that of metoprolol, both α-hydroxymetoprolol and metoprolol acid were reported to reach higher concentrations than S-metoprolol in plasma after a single dose (Mistry et al., 2002). In fact, the $AUC_{(0-\infty)}$ of racemic α-hydroxymetoprolol was 21% higher than that of S-metoprolol in our study. Hence, the concentration and activity would be high enough to actually contribute to the observed heart rate response to S-metoprolol. Since PMs had less α-hydroxymetoprolol (AUC 0.06 μmol·h/L for PMs on placebo compared to AUC 3.04 μmol·h/L for EMs on placebo) they require higher S-metoprolol concentrations to reach the same effect, i.e. EC_{50} was higher. Similarly, diphenhydramine co-administration decreased the formation of α-hydroxymetoprolol by 15.5% and possibly that of other metoprolol metabolites thereby requiring more S-metoprolol, i.e., higher EC50 values for the same heart rate response.

Interestingly, although the EM women in this study had a 300-fold higher $CL_{MET\rightarrow\alpha\text{-}OH\text{-}MET}$ (racemic metoprolol), a 6-fold higher CL_{NR} (S-metoprolol) and a 3.6-fold lower $AUC_{(0-\infty)}$ (S-metoprolol) compared to their PM counterparts during the placebo phase, PMs had merely between 1.3 to 1.5-fold higher AUEC and between 1.5 to 2.5-fold higher EC_{50}. These differences in pharmacokinetics and pharmacodynamics could possibly be explained

by significant synergistic cardiovascular effects of the metabolites in EMs. In contrast, a mere 3-fold decrease in $CL_{MET \rightarrow \alpha\text{-OH-MET}}$ (racemic metoprolol), a 2.3-fold decrease in CL_{NR} (S-metoprolol) and a 1.8-fold increase in $AUC_{(0\text{-}\infty)}$ (S-metoprolol) in EMs, caused by the coadministration of diphenhydramine instead of placebo, resulted in an around 1.3-fold increase in AUEC and about 1.5-fold increase in EC_{50}. This implies that the small changes in metoprolol disposition in EMs produced by diphenhydramine coadministration resulted in nearly as much pharmacodynamic effects as seen in PMs. This pharmacodynamic modulation could also be related to the cardiovascular effects of diphenhydramine (Zareba et al, 1997 and Khalifa et al., 1999).

A potential weakness of this study is that the pharmacodynamic parameters were measured only for a period of 12 hours post metoprolol which might have caused an underestimation of pharmacodynamic parameters in some volunteers. However, given the length of the protocol, it would have been beyond the physical capacity of the volunteers to extend the study longer.

The current study reports an extensive assessment of metoprolol pharmacokinetics (racemic and enantiomeric) and hemodynamics in young healthy premenopausal EM and PM women at the time of the menstrual cycle when they may be most predisposed to acute coronary syndromes (i.e., after menstruation and before ovulation). Significant differences in the PK/PD relationships for EM and PM women were observed and need to be taken into consideration in clinical practice. Further, caution is warranted when the over-the-counter antihistamine diphenhydramine is part of a chronic therapeutic regimen, especially

because relatively small, although significant effects on metoprolol's disposition result in relatively large pharmacodynamic effects.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Serge Simard (biostatistician, Laval Hospital) for statistical analysis, Ms. Brigitte Fortin (Laval Hospital) for nursing support and Ms. Joceyline Simard for technical help. We are grateful to Innaphase Corporation, USA for providing us with a complementary copy of the Kinetica 2000® software and to Astra-Zeneca Inc., Molndal, Sweden for providing the samples of racemic metoprolol, α-hydroxymetoprolol and R- and S-metoprolol.

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FOOTNOTES

a.) Supported by grants from Canadian Institutes of Health Research and American College of Clinical Pharmacy (Wyeth-Ayerst Women's Health Care Research Award to BAH). AS received a joint scholarship from the Canada's Research Based Pharmaceutical Companies and Canadian Institutes of Health Research. PP is the recipient of Canada Research Chair. The information contained in this article was part of the Ph.D. thesis work of AS. This work was presented in part at the American College of Clinical Pharmacy annual meeting in Los Angeles, California, 5-8 November 2000 and at the American Society of Clinical Pharmacology and Therapeutics annual meeting in Orlando, Florida, 8-10 March 2001.

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LEGENDS FOR FIGURES

Figure 1. Study design

Figure 2. Pharmacokinetic profile of racemic metoprolol in 16 extensive and 4 poor metabolizer women following the administration of a single oral dose of 100 mg metoprolol tartrate with or without concomitant administration of diphenhydramine or placebo to steady state. Results presented as mean ± SD; EM: extensive metabolizers; PM: poor metabolizers; MET: metoprolol; PCB: placebo; DPH: diphenhydramine.

Figure 3. Pharmacokinetic profile of metoprolol enantiomers in 16 extensive metabolizer (A) and 4 poor metabolizer (B) women after single oral dose of 100 mg metoprolol tartrate with and without concomitant administration of diphenhydramine to steady state. Results presented as mean ± SD; EM: extensive metabolizers; PM: poor metabolizers; MET: metoprolol; PCB: placebo; DPH: diphenhydramine.

Figure 4. Changes in (A) mean exercise heart rate (B) mean exercise rate-pressure product (C) mean exercise stroke volume index (D) mean exercise cardiac index in 16 extensive and 4 poor metabolizer women following a single oral dose of 100 mg metoprolol tartrate with or without concomitant administration of diphenhydramine or placebo. EM: extensive metabolizers; PM: poor metabolizers; MET: metoprolol; PCB: placebo; DPH : diphenhydramine. *Significantly different between EM – MET/PCB and PM – MET/PCB. #Significantly different between EM – MET/PCB and EM – MET/DPH.

JPET Fast Forward. Published on February 17, 2005 as DOI: 10.1124/jpet.104.081109
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Table 1. Noncompartmental pharmacokinetics of racemic metoprolol in 20 healthy young women (16 extensive and 4 poor metabolizers) following the administration of 100 mg metoprolol tartrate in the absence or presence of diphenhydramine dosed to steady-state.

	EM (n= 16)		PM (n=4)	
Parameters	MET/PCB	MET/DPH	MET/PCB	MET/DPH
C_{max} (μmol/L)	0.89 ± 0.42	1.18 ± 0.34[a]	1.74 ± 0.31[b]	1.50 ± 0.38[b]
T_{max} (h)	1.69 ± 0.63	1.97 ± 0.70	1.75 ± 0.20	2.06 ± 0.72
$t_{1/2}$ (h)	2.88 ± 0.80	3.95 ± 0.81[a]	7.44 ± 0.8[b]	7.08 ± 0.80[b]
$AUC_{0-\infty}$ (μmol•h/L)	4.05 ± 2.15	7.76 ± 3.34[a]	17.18 ± 2.48[b]	14.08 ± 3[b]
CL/F (L/h)	111 ± 90	47 ± 26[a]	17 ± 2.5[b]	21 ± 4[b]
CL_R (L/h)	3.98 ± 2.14	3.81 ± 1.39	3.64 ± 0.48	4.71 ± 1.99
CL_{NR} (L/h)	107.23 ± 89.12	42.99 ± 24.95[a]	13.61 ± 2.29[b]	16.71 ± 4.10[b]
$CL_{MET\rightarrow\alpha\text{-OH-MET}}$ (L/h)	11.97 ± 12.70	3.91 ± 3.21[a]	0.04 ± 0.01[b]	0.04 ± 0.01[b]

C_{max} : peak plasma concentration, T_{max}: time to reach peak plasma concentration, $t_{1/2}$: half-life, $AUC_{0-\infty}$: area under the concentration time curve (0 to infinity), Cl/F: total clearance, CL_R: renal clearance, CL_{NR}: nonrenal clearance, $CL_{MET\rightarrow\alpha\text{-OH-MET}}$: partial metabolic clearance of metoprolol to α-hydroxymetoprolol, [a]$P < 0.05$ EMs placebo vs. EMs diphenhydramine, [b]$P < 0.05$ significantly different between EMs and PMs in placebo as well as diphenhydramine week. Results presented as mean ± standard deviation.

JPET Fast Forward. Published on February 17, 2005 as DOI: 10.1124/jpet.104.081109
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Table 2. Noncompartmental pharmacokinetics of α-OH-metoprolol in 20 healthy, young women (16 extensive and 4 poor metabolizers) following the administration of 100 mg metoprolol tartrate in the absence or presence of diphenhydramine dosed to steady-state.

	EM-PCB	**EM-DPH**
C_{max} (μmol/L)	0.28 ± 0.11[a]	0.16 ± 0.05
T_{max} (h)	1.69 ± 0.66[a]	3.34 ± 2.05
$AUC_{0-\infty}$ (μmol•h/L)	3.04 ± 0.43[a]	2.53 ± 0.45

C_{max} : peak plasma concentration, T_{max}: time to reach peak plasma concentration, $AUC_{0-\infty}$: area under the concentration time curve (0 to infinity), [a]$P \leq 0.004$ EM-PCB vs. EM-DPH; Results presented as mean ± SD

Table 3. Results of noncomparmental pharmacokinetic analysis of metoprolol enantiomers in 20 healthy young women (16 extensive and 4 poor metabolizers) following the administration of 100 mg metoprolol tartrate in the absence or presence of diphenhydramine dosed to steady state.

	EM (n = 16)				PM (n = 4)			
Parameters	R-MET-PCB	S-MET-PCB	R-MET-DPH	S-MET-DPH	R-MET-PCB	S-MET-PCB	R-MET-DPH	S-MET-DPH
C_{max} (μmol/L)	0.38 ± 0.20[a]	0.51 ± 0.23[b]	0.53 ± 0.17[c]	0.65 ± 0.18[d]	0.84 ± 0.15	0.90 ± 0.15	0.72 ± 0.17	0.79 ± 0.21
T_{max} (h)	1.69 ± 0.63	1.72 ± 0.68	1.97 ± 0.7	2.02 ± 0.71	1.75 ± 0.20	1.75 ± 0.20	2.06 ± 0.72	2.06 ± 0.72
$t_{1/2}$ (h)	2.74 ± 0.95[a]	3.02 ± 0.90[b]	3.98 ± 0.93[c]	4.24 ± 0.93[d]	7.4 ± 0.38	7.11 ± 0.95	7.38 ± 0.98	6.79 ± 0.63
$AUC_{0\text{-}\infty}$ (μmol•h/L)	1.63 ± 0.92[a]	2.44 ± 1.25[b]	3.39 ± 1.56[c]	4.49 ± 1.84[d]	8.5 ± 1.3	8.67 ± 1.3	7.01 ± 1.81	7.04 ± 1.24
CL/F (L/h)	147 ± 124[a]	88.7 ± 68.4[b]	55.6 ± 33.3[c]	39.8 ± 21[d]	17.5 ± 2.6	17.1 ± 2.52	21.8 ± 5.2	21.2 ± 3.28
CL_R (L/h)	4.10 ± 2.18	3.86 ± 2.01	3.86 + 1.46	3.73 ± 1.35	3.98 ± 0.82	3.41 ± 0.79	5.33 ± 2.85	4.74 ± 2.32
CL_{NR} (L/h)	143.4 ± 123[a]	85.66 ± 67.1[b]	52.4 ± 32.9[c]	36.7 ± 20.5[d]	13.8 ± 1.48	13.9 ± 1.5	17.45 ± 2.60	16.41 ± 1.29

EM: extensive metabolizers; PM: poor metabolizers; PCB: Placebo; DPH: diphenhydramine; C_{max}: peak plasma concentration; $t_{1/2}$: half-life; $AUC_{0\rightarrow\infty}$: area under the concentration time curve (0 to infinity); Cl/F: total clearance; CL_r: renal clearance; CL_{nr}: nonrenal clearance; [f]$P <$ 0.05 Men vs Women. Results presented as mean ± standard deviation; [a]$P < 0.05$ EM-R-MET/PCB vs PM-R-MET/PCB; [b]$P < 0.05$ EM-S-MET/PCB vs PM-S-MET/PCB; [c]$P < 0.05$ EM-R-MET/PCB vs EM-R-MET/DPH; [d]$P < 0.05$ EM-S-MET/PCB vs EM-S-MET/DPH;

JPET Fast Forward. Published on February 17, 2005 as DOI: 10.1124/jpet.104.081109
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Table 4. Results of pharmacokinetic/pharmacodynamic modeling of S-metoprolol pharmacokinetic data with various pharmacodynamic response markers in 20 healthy young women (16 extensive and 4 poor metabolizers) in absence or presence of diphenhydramine dosed to steady state.

	Exercise Heart Rate			
	EM - PCB	**EM - DPH**	**PM – PCB**	**PM - DPH**
Observed Emax ± SD (beats/min)	37 ± 5	42 ± 8	46 ± 11	45 ± 8
EC_{50} ± SD (μmoles/L)	0.10 ± 0.09[bc]	0.14 ± 0.08	0.25 ± 0.08	0.17 ± 0.08
AUEC ± SD (beats•h/min)	275 ± 63[bc]	350 ± 88	423 ± 85	408 ± 78
	Exercise Systolic Blood Pressure			
	EM – PCB	**EM – DPH**	**PM – PCB**	**PM – DPH**
Observed Emax ± SD (mmHg)	31 ± 10	36 ± 13	43 ± 6	37 ± 12
EC_{50} + SD (μmoles/L)	0.13 ± 0.09[a]	0.21 ± 0.15[a]	0.24 ± 0.07	0.31 ± 0.07
AUEC ± SD (mmHg•h)	215 ± 95	245 ± 73	278 ± 80	231 ± 141
	Exercise Rate Pressure Product			
	EM – PCB	**EM – DPH**	**PM – PCB**	**PM – DPH**
Observed Emax ± SD (beats•mmHg/min)	7638 ± 1971	8504 ± 2011	9626 ± 1758	9905 ± 2876
EC_{50} ± SD (μmoles/L)	0.09 ± 0.06[a]	0.15 ± 0.10[a]	0.20 ± 0.08	0.22 ± 0.04
AUEC ± SD (beats•mmHg•h/min)	57952 ± 16193[bc]	71838 ± 17949	85435 ± 15382	84359 ± 23239

Emax: maximum effect; EC50: Concentration producing half of maximum effect; Results presented as mean ± standard deviation; [a]$P < 0.05$ EM vs PM; [b]$P < 0.05$ EM - PCB vs EM – DPH; [c]$P < 0.05$ EM - PCB vs PM – PCB.

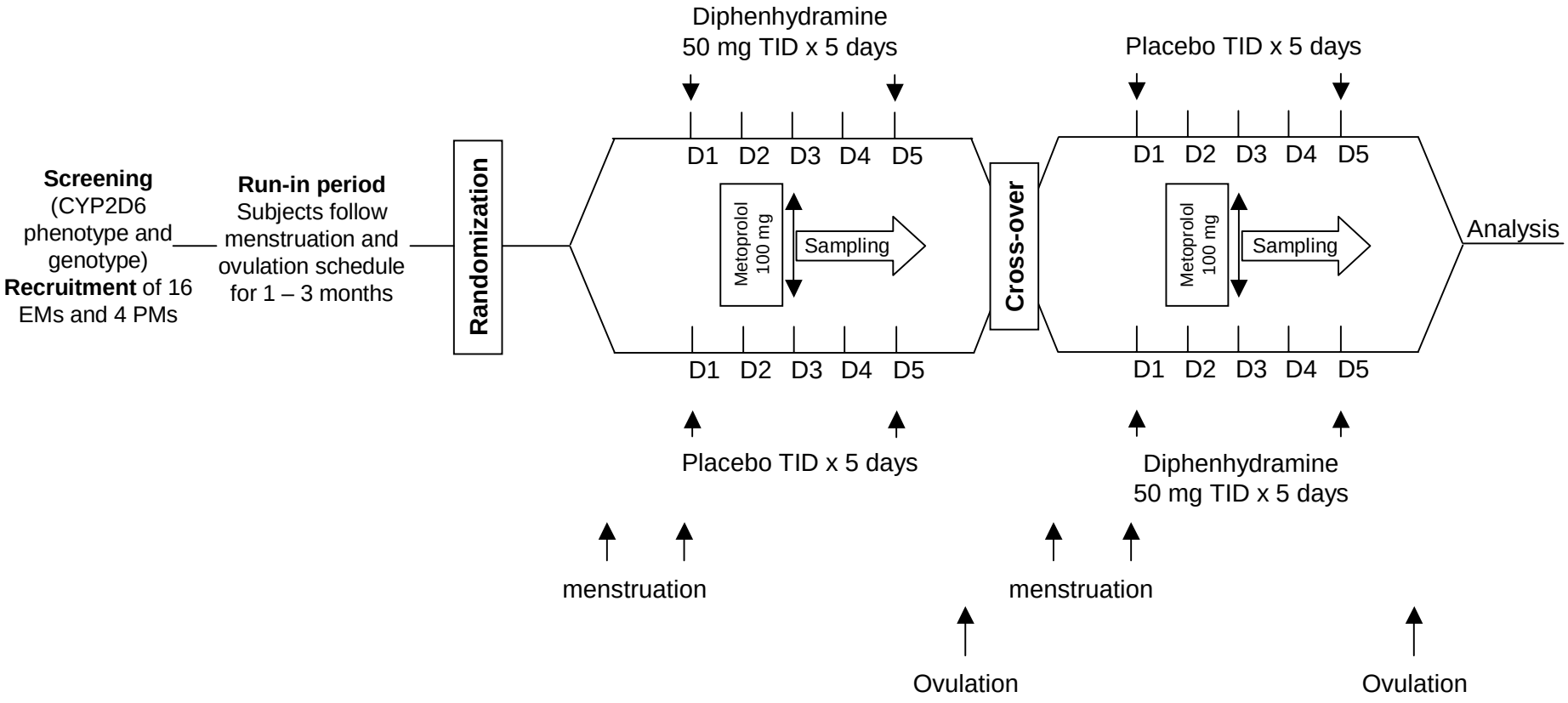

Figure 1

JPET Fast Forward. Published on February 17, 2005 as DOI: 10.1124/jpet.104.081109
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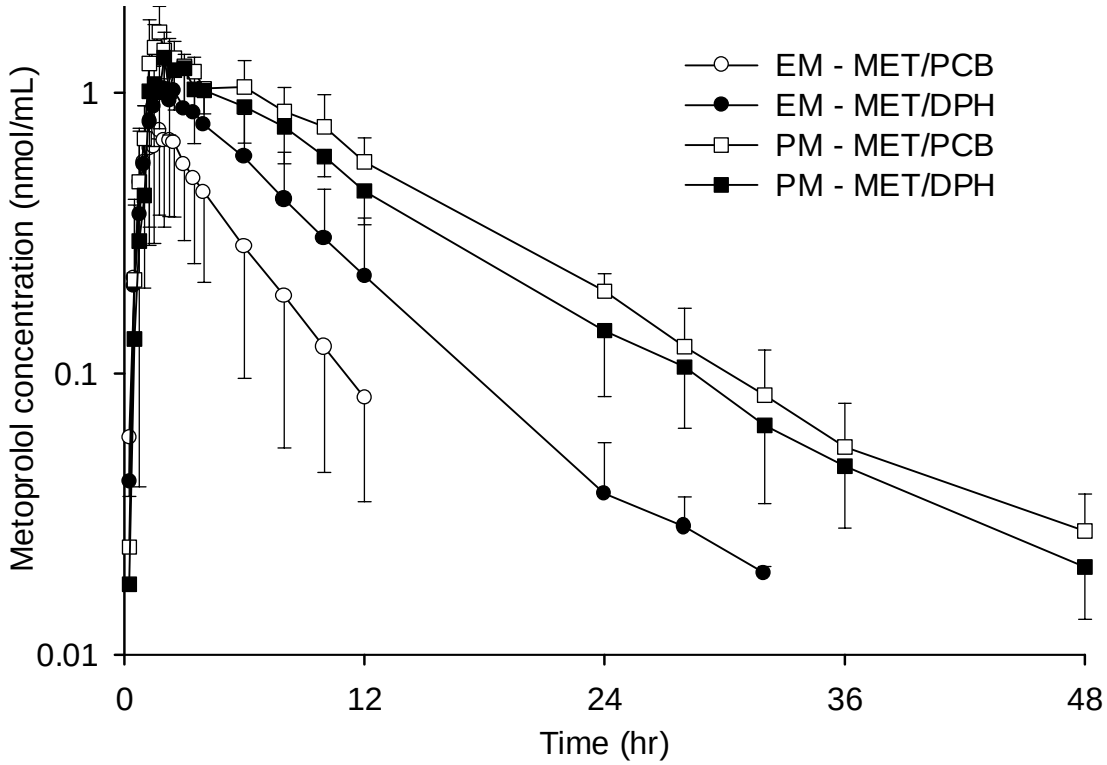


Figure 2

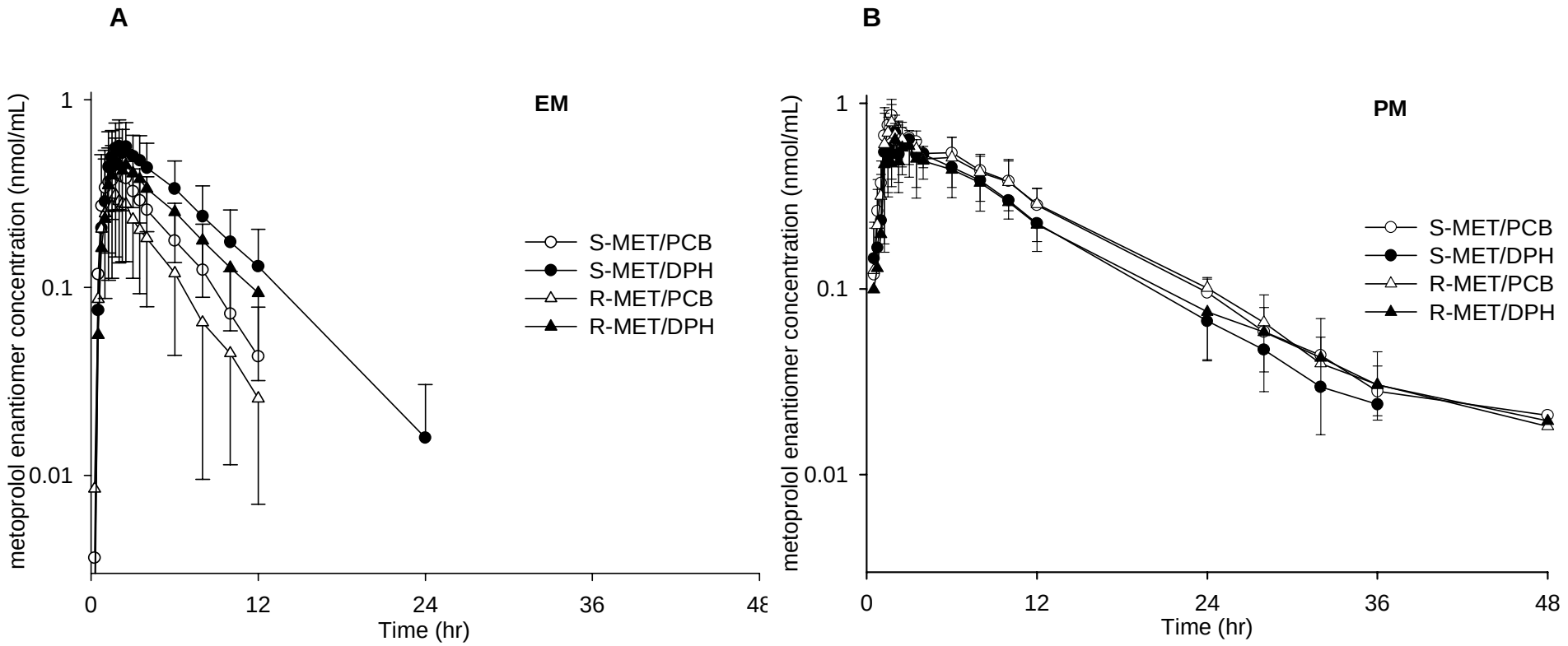
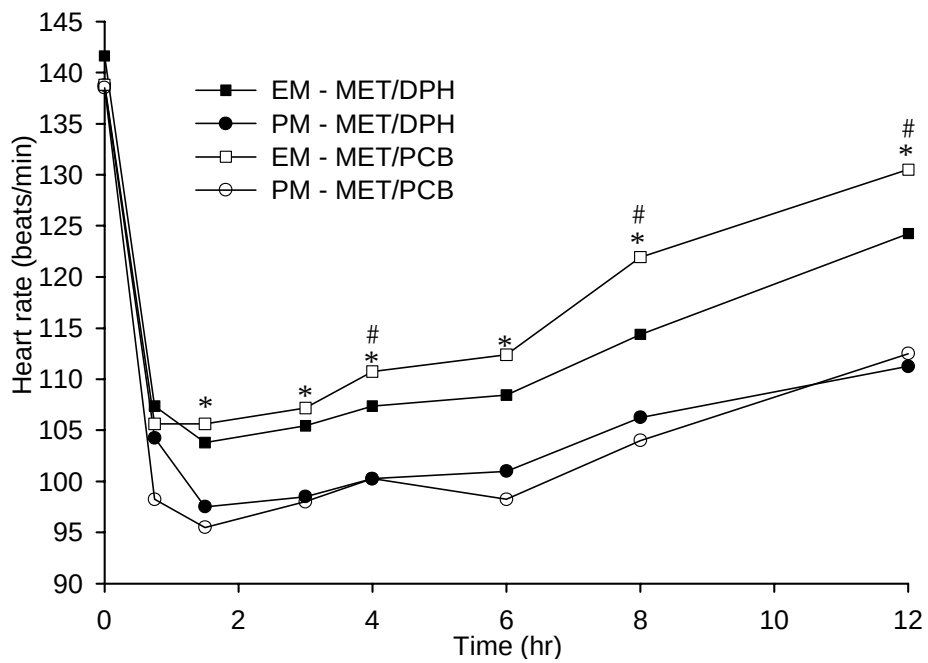
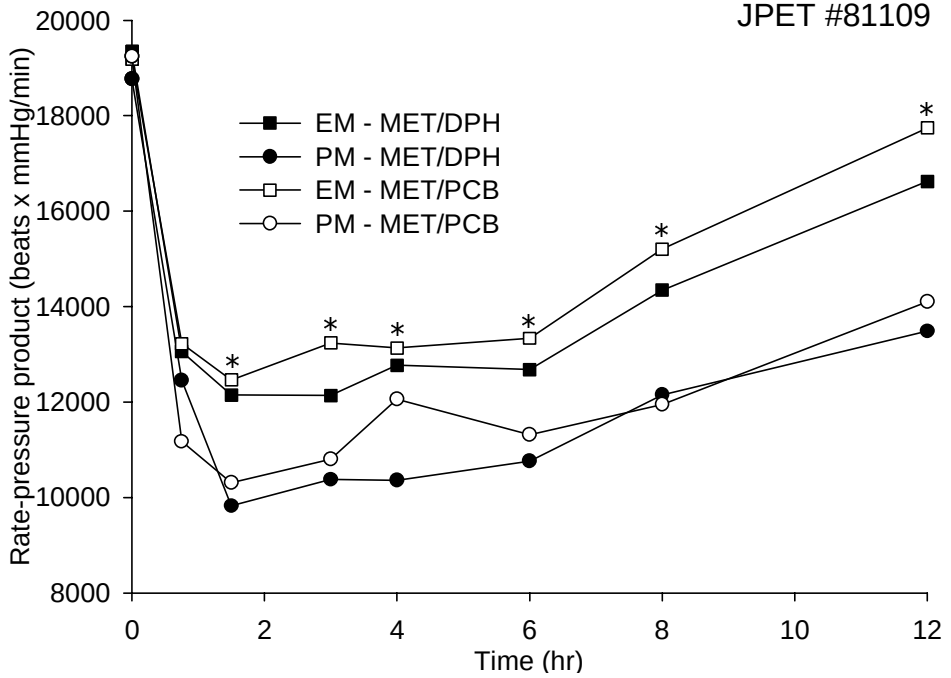
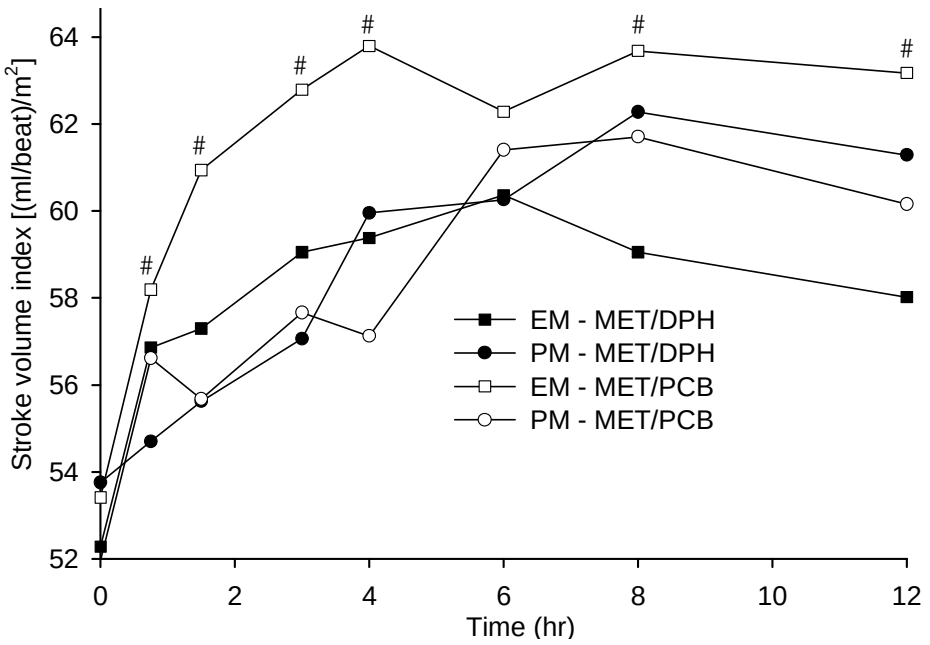


Figure 3







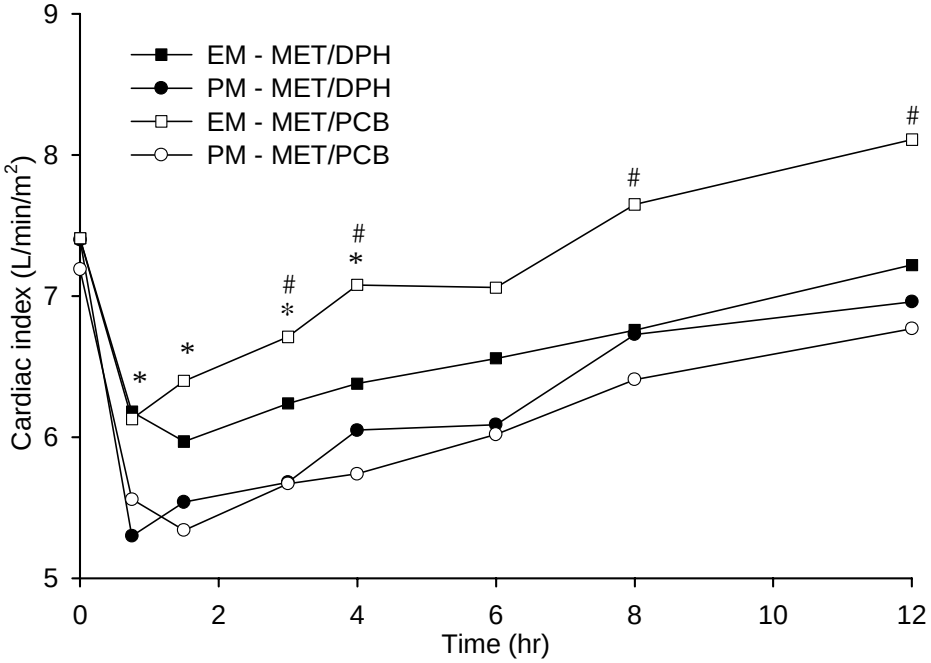


Figure 4