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Effect of propiverine on cytochrome P450 enzymes: a cocktail interaction study in healthy volunteers*

D. Tomalik-Scharte, A. Jetter, M. Kinzig-Schippers, A. Skott, F. Sörgel, T. Klaassen, D. Kasel, S. Harlfinger, O. Doroshenko, D. Frank, J. Kirchheiner, M. Bräter, K. Richter, T. Gramatté, U. Fuhr

Department of Pharmacology, Clinical Pharmacology, University of Cologne, Germany (D.T.S., A.J., T.K., D.K., S.H., O.D., D.F., J.K., U.F.); IBMP - Institute for Biomedical and Pharmaceutical Research, Nürnberg-Heroldsberg, Germany (M.K.S., A.S., F.S.); APOGEPHA Arzneimittel GmbH, Dresden, Germany (M.B., T.G.); Institute of Clinical Pharmacology, University of Technology Dresden, Department of Pharmacology, Germany (K.R.); University of Duisburg - Essen, Germany (F.S.)

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Corresponding author:

Dorota Tomalik-Scharte M.D.

Department of Pharmacology, Clinical Pharmacology, University of Cologne

Gleueler Str. 24

50931 Köln, Germany

Phone: +49-221-4783403, Fax: +49-221-4787011

e-mail: dorota.tomalik-scharte@uk-koeln.de

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A list of non-standard abbreviations used in the paper: propiverine, propiverine hydrochloride; F_{hepatic} , hepatic availability (of midazolam); F_{oral} , absolute bioavailability (of midazolam); $F_{\text{intestinal}}$, intestinal availability of midazolam; UR_{6-12h} (OH-tolbut.+ carboxytolbut.)/tolbut., urinary molar concentration ratio (hydroxytolbutamide + carboxytolbutamide)/tolbutamide 6 – 12 hours postdose; CL/F, oral clearance; A_{e0-8h} 4'-OH-mephenytoin, amount of mephenytoin dose excreted as 4'-hydroxymephenytoin in urine 0- 8 hours postdose.

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Abstract

The study was conducted to assess a possible *in vivo* effect of propiverine, an anticholinergic drug to treat urinary incontinence and related disorders, on the activity of intestinal 3A4 and of hepatic CYP3A4, CYP2C9, CYP2C19 and CYP1A2. The activity of the respective CYPs was measured using the following metrics of selective substrates given as a tailored low-dose phenotyping cocktail: intestinal availability of midazolam (2 mg orally), clearance of midazolam (1 mg i.v.), apparent clearance of tolbutamide (125 mg orally), urinary excretion of 4'-hydroxymephenytoin 0 - 8 hours postdose (50 mg mephenytoin orally), and the paraxanthine/caffeine plasma ratio 6 hours postdose (150 mg caffeine orally). These metrics were determined in 16 healthy young men at the end of 7 days treatment with 15 mg of propiverine (Test) or placebo (Reference) twice daily. All phenotyping drugs were quantified by LC-MS/MS. Chronic propiverine treatment reduced hepatic and intestinal CYP3A4 activity slightly to 0.89-fold and 0.80-fold, respectively (90% CI for Test/Reference ratios 0.85 – 0.93 and 0.72 – 0.89), with the combined effect resulting in a 1.46-fold increase in AUC of oral midazolam (90% CI 1.36 - 1.57). Propiverine had no relevant effect on CYP2C9, CYP2C19 and CYP1A2 (90% CI for Test/Reference ratios 0.93-1.00 , 0.84-0.96 and 0.97-1.07, respectively). All study drugs were well tolerated. In conclusion, propiverine has a minor potential to cause drug-drug interactions.

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Propiverine hydrochloride, described below as propiverine, is indicated for the treatment of urinary incontinence, as well as urinary urgency and frequency in patients who have either idiopathic detrusor overactivity (overactive bladder) or neurogenic detrusor overactivity (detrusor hyperreflexia) from spinal cord injuries, e.g. transverse lesion paraplegia. It exhibits antagonistic effects towards muscarinic acetylcholine receptors and calcium channel modulating properties (Siegmund et al., 1990; Yono et al., 1999; Madersbacher and Mürtz, 2001).

After oral administration, propiverine is rapidly and almost completely absorbed from the gastrointestinal tract. The maximal serum concentration is reached approximately 90 minutes after a single-dose of 15 mg. Propiverine undergoes extensive presystemic metabolism via N-oxidation to propiverine-N-oxide with involvement of cytochrome P450 enzymes. Mean elimination half-life after chronic administration of propiverine is about 15 hours. The major fraction of propiverine and its metabolites is eliminated in the urine (Haustein and Hüller, 1988; Siepman et al., 1998). Information on the enzymes mediating Phase I metabolism of propiverine has been obtained in several in vitro systems (APOGEPHA, data on file). The primary metabolic route involves the oxidation of the piperidyl-N and is mediated by CYP3A4 and flavin-containing monooxygenases 1 and 3 and leads to the formation of the much less active N-oxide. CYP2C9 and CYP2C19 may also mediate a fraction of overall propiverine elimination, whereas other enzymes including (e.g., CYP1A2 and CYP2D6) were involved to a minor extent.

Several studies concerning possible drug-drug interactions of propiverine have been published. Müller et al. demonstrated that there is no effect of CYP2D6 genotype on propiverine biotransformation in humans, so that no effect of CYP2D6 inhibitors on propiverine pharmacokinetics is expected (Müller et al., 1993). Results of a study performed in rats provided no evidence for propiverine to cause relevant drug-drug interactions (Borchert et al., 1986). Studies on the effect of propiverine on drug metabolizing enzymes in human and rat hepatocyte cultures demonstrated an increase in CYP3A4 mRNA and protein expression, although the corresponding enzyme activities were inhibited (APOGEPHA, data on file). In preliminary studies in human liver microsomes, propiverine showed competitive inhibition of CYP2D6 with K_i values more than 30-fold above propiverine plasma peak

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concentrations (APOGEPHA, data on file). Recently, Walter et al. reported that the drug administered in rats in doses about 100-times above the therapeutic doses in humans is a phenobarbital-type enzyme inducer (Walter et al., 2003). In summary, propiverine appears to interact with CYPs mainly as a substrate. The available, mainly preclinical data suggest that any clinically relevant interaction of propiverine with cytochrome P450 enzymes as a substrate, inhibitor and/or inducer would mainly concern CYP3A4 and possibly also CYP2C9 or CYP2C19, but data with respect to induction or inhibition of CYPs are incomplete.

Whereas in vitro studies using human liver microsomes or expressed enzymes provide only preliminary information about CYPs that are likely to be affected clinically, in vivo phenotyping provides a more conclusive information on how the test drug might interfere with the respective CYPs in patients (Blakey et al., 2004). Phenotyping, i.e. estimation of enzyme activity by administration of a selective substrate for this enzyme and subsequent determination of pharmacokinetic parameters reflecting its activity, is considered to be the most favorable method for assessment of actual metabolic capacities resulting from both intrinsic and extrinsic factors (Streetman et al., 2000a). A major shortcoming of this procedure is the necessity of performing several phenotyping studies, often in sequential hierarchy, to characterize the effect of a test drug on the activity of all important cytochrome P450 enzymes. Here, this problem was overcome by simultaneous administration of low dosages of validated probe drugs in a tailored “cocktail” approach, providing information on several metabolic pathways in a single experimental session and minimizing the confounding influence of intrasubject variability over time (Frye et al., 1997; Tanaka et al., 2003).

Thus, the present study was conducted to evaluate a possible in vivo effect of propiverine on the activity of human CYPs, using a two-arm cross-over cocktail phenotyping approach. The primary objective was to obtain information about the effects on intestinal CYP3A4 and on hepatic CYP3A4, CYP2C9 and CYP2C19. Secondly, effects on CYP1A2 and CYP2D6 were to be investigated.

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Methods

The study protocol was reviewed and approved the Ethics Committee of the Faculty of Medicine of the University of Cologne.

Seventeen male Caucasian volunteers participated in the trial after having provided written informed consent. One early drop-out, unrelated to the study medication, occurred. In the 16 subjects who completed the study, the respective means and ranges for the age and body mass index were 29 (23 - 42) years and 23.5 (21.4 – 26.6) kg/m². All subjects were non-smokers and healthy as confirmed on the basis of an extensive pre-study examination.

Study design

The study had a single-centre, controlled, open, cross-over design with period-balanced randomly allocated sequences Reference - Test, Test - Reference. Between the two periods, there was a 7-day wash-out interval.

According to the primary objective to obtain information about the effects of propiverine on intestinal CYP3A4 and on hepatic CYP3A4, CYP2C9 and CYP2C19, the evaluation of the respective metrics (Table 1) was mandatory. If the study showed pronounced inhibition of these enzymes indicating high propiverine concentrations at hepatic CYP binding sites which might result in relevant in vivo effects on enzymes for which propiverine had only a minor effect in vitro, effects on CYP1A2 and CYP2D6 activity were to be evaluated additionally. Although there was no such pronounced inhibition, propiverine effects on CYP1A2 were also evaluated, prompted by the recent additional data (Walter et al. 2003). We also decided to evaluate the effects on CP2D6 but these will be reported separately because extensive additional evaluations are required to assess the validity of dextromethorphan metabolic ratios in urine and plasma for the quantification of drug-drug interactions (Streetman et al 2000a, Özdemir et al. 2004, Borges et al. 2005).

In the Test period, 15 mg propiverine hydrochloride (1 tablet Mictonorm®, APOGEPHA Arzneimittel GmbH, Dresden, Germany) were administered orally in the morning and in the evening for 7 days at approximately 7:00 and 19:00 o'clock, respectively. In the Reference period, placebo tablets were administered instead. For each drug intake, the volunteers

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reported to the ward. They were instructed to abstain from fluid and from food intake from 1 hour prior to each dosing until 1 hour thereafter.

For phenotyping with a six-probe metabolic cocktail, the volunteers were hospitalized from 13 hours prior to cocktail administration on study day 7 in each study period till 24 hours thereafter. Phenotyping drugs were administered in the morning on day 7 (profiling day) of each period as follows: 125 mg of tolbutamide (a quarter of a tablet Tolbutamid RAN®), 50 mg of mephenytoin (half a tablet Epilan Gerot Tabletten®), 150 mg of caffeine (3 tablets Percoffedrinol®) and 30 mg of dextromethorphan-HBr (1 capsule Hustenstillerratiopharm®) were administered together with the propiverine or placebo dose for evaluation of CYP2C9, CYP2C19, CYP1A2 and CYP2D6, respectively. One minute thereafter, 2 mg of midazolam (Dormicum®) were given orally for intestinal CYP3A4.

Finally, 1 mg of midazolam was administered intravenously 4 hours after oral propiverine / placebo administration for hepatic CYP3A4. Table 1 shows an overview of the cocktail drugs and the respective phenotyping metrics used to assess the *in vivo* activity of CYPs. Study participants abstained from alcohol, methylxanthines and grapefruit from 72 h before first dosing until 48 h after the last dosing. Food and fluid were standardised during hospitalisation. The subjects were fasted from 9 hours before until 6 hours after cocktail dosing and adhered to a relaxed recumbent position during 6 hours following the cocktail administration.

In the Test period, blood for the subsequent determination of plasma concentrations of propiverine and its main metabolite was sampled 3 minutes prior to the administration of the morning dose on day 7 and 2:00, 3:57, 8:00 and 11:57 hours thereafter to demonstrate exposure. In each study period, blood samples for phenotyping were collected 3 min prior to dosing in the morning of day 7 and 0:10, 0:20, 0:30, 0:45, 1:00, 1:20, 1:40, 2:00, 2:30, 3:15, 3:57, 4:08, 4:15, 4:30, 4:50, 5:15, 6:00, 7:00, 8:00, 10:00, 11:57, 14:00, 16:00 and 24:00 hours postdose. Furthermore, urine was collected predose and during -00:10-02:00, 02:00-03:57, 03:57-06:00, 06:00-08:00, 08:00-12:00, 12:00-24:00 h post-dose intervals. For the respective sampling intervals, pH values were measured, and urine volumes were determined by means of weighing of urine containers.

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Bioanalytical and genotyping assays

Propiverine and propiverine-N-oxide

Plasma samples of 0.5 mL were diluted with 0.5 mL formic acid (9 %) and extracted by an ASPEC XL Sample Processor (Gilson, Middleton, USA) employing 1 mL, 30 mg NEXUS solid phase extraction cartridges (Varian, Darmstadt, Germany). The eluates were evaporated to dryness in a stream of air, redissolved in 100 μ L mobile phase, and 20 μ L were injected for HPLC. Chromatographic separation was achieved using a 125 x 2 mm, 5 μ LiChrospher 60-5 select B column (E. Merck, Darmstadt, Germany) and isocratic elution with 70% acetonitrile and 30 % phosphate buffer (2 mmol/L; pH 7.3). Two wavelengths, 202 nm and 220 nm, were used simultaneously for detection. Moreover, the full UV spectrum in the range of 200 to 400 nm was saved for confirming the identity of the analytes.

A linear concentration range from 15.6 ng/mL to 2000 ng/mL for both analytes (propiverine and propiverine-N-oxide) was used for validation. The lowest points of the standard curves were set as the lower limit of quantification (LLOQ). Standard curves were weighted with the factor 1/concentration and showed correlation coefficients >0.998. All concentration data was calibrated externally. The accuracy [% bias] for propiverine and propiverine-N-oxide ranged from -3.5% and +7.4%. Precision [% CV] was found to be between 0.8% and 5.5% for propiverine and between 1.1% and 11.7% for propiverine-N-oxide.

As for all assays precision and accuracy were calculated as the mean coefficient of variation (CV) of all samples used for validation and the mean relative deviation of validation samples from their nominal values, respectively.

Tolbutamide, 4-hydroxytolbutamide and 4-carboxytolbutamide

The method for the quantification of tolbutamide and its metabolites 4-hydroxytolbutamide and 4-carboxytolbutamide in urine and plasma by LC-MS/MS has been published (Jetter et al., 2004). In urine linear calibration curves could be fitted over a concentration range from 12.5 to 50000 ng/mL for tolbutamide, and from 50 to 50000 ng/mL for 4-hydroxytolbutamide and 4-carboxytolbutamide. Intra- and interday accuracy were within -8.8% and +14.0% for all analytes. Both intra- and interday precision were found to be between 1.0% and 12.6% for tolbutamide, 4-hydroxytolbutamide and 4-carboxytolbutamide. Additionally, up to twenty-fold dilution of samples with blank human urine was tested during method validation.

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In plasma calibration curves were linear between 15 and 15000 ng/mL for tolbutamide. Intra- and interday accuracy ranged from +1.6% to +3.3% and intra- and interday precision was between 0.7% and 8.4%.

In both assays, chlorpropamide was used as internal standard. The calibration curves were calculated from the peak area ratios of analyte / internal standard and the nominal analyte concentrations using linear regression with $1/\text{concentration}^2$ weighting. In all cases, coefficient of correlation of the weighted standard curves was at least 0.997. The lowest concentration of the calibration curves was set as the LLOQ.

4'-hydroxymephenytoin

The method for the quantification of 4'-hydroxymephenytoin with 4'-methoxymephenytoin as internal standard in urine by LC-MS/MS has been published in detail (Klaassen et al., 2004). Linearity of the weighted ($1/\text{concentration}$) calibration curves over a concentration range from 15 to 10000 ng/mL was expressed in correlation coefficients >0.999 . The LLOQ for 4'-hydroxymephenytoin, corresponding to the lowest concentration of quality control samples, was 20 ng/mL. Accuracy [% bias] ranged from -1.6% to +2.3% and precision [% CV] was between 4.4% and 8.6%.

Midazolam

The method for the quantification of midazolam in plasma by LC-MS/MS has been reported (Kasel et al., 2002). A standard curve using weighted quadratic polynomial regression with weights of $1/\text{concentration}^2$ was fitted over a concentration range from 0.09 to 186 ng/mL with coefficients of correlation higher than 0.998. All concentration data were calibrated externally. The LLOQ for midazolam, corresponding to the lowest concentration of quality control samples, was 0.44 ng/mL. Intra- and inter-day accuracy [% bias] ranged from +3.0% to +11.4%. Intra- and inter-day precision [% CV] were within 3.2% and 14.1%, respectively.

Caffeine and paraxanthine

After addition of internal standard (7-(beta-hydroxyethyl)theophylline) to 150 μL plasma, plasma proteins were precipitated and the clear supernatant was evaporated to dryness. Thereafter, the pellets were redissolved and analyzed by LC-MS/MS (TSQ Quantum, Thermo Electron, San Jose, USA) in a validated assay. Chromatography was performed in 4.5 min using a 50 x 2.1 mm, 5 μm Hypersil Gold column (Thermo Electron, Runcorn, U.K.) and a

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linear gradient of methanol and formic acid (0.1%). Caffeine and paraxanthine were detected by positive electrospray ionization in the selected reaction monitoring mode with following ion transitions [m/z]: 195.23→138.16 for caffeine and 181.19→124.15 for paraxanthine. Peak areas were used for quantification of caffeine and peak height for that of paraxanthine, respectively.

Linear calibration curves with correlation coefficients >0.999 were observed for both substances in the concentration range 30-5000 ng/mL. The LLOQ was 30 ng/mL. The accuracy [% bias] for caffeine ranged between +0.9% and +3.8% and between +5.4% and +10.4% for paraxanthine. Precision [% CV] was better than 6.4% for caffeine and 5.9% for paraxanthine, respectively.

Genotyping

DNA was extracted from 5 ml EDTA blood samples using the MagNA Pure LC™ Total Nucleic Acid Isolation kit (Roche Diagnostics, Mannheim, Germany) and purified DNA was eluted with a low salt buffer and stored at 4°C. For *CYP2C19* genotyping, the *2 allele was determined using PCR-RFLP as described earlier (De Morais et al., 1994).

Data analysis

Pharmacokinetic analysis

Pharmacokinetic calculations were performed using Excel 97 (Microsoft corp., Seattle, WA, USA) and WinNonlin™, Version 2.1 (Pharsight Corp., Palo Alto, CA, USA) in the non-compartmental mode.

All metabolic ratios were calculated using molar concentrations and/or doses.

The respective phenotypic metrics used for determination of the activity of the cytochrome P450 enzymes and the additional pharmacokinetic parameters of the phenotyping substrates are shown in Table 1.

CYP3A4 activities were calculated by noncompartmental analysis of midazolam plasma concentrations (Figure 1) and checked by an independent compartmental analysis. For the noncompartmental evaluation, the AUC attributable to the oral midazolam dose was defined as the AUC following oral administration until the point of time just prior to intravenous administration, plus the AUC from the point of time when the concentration just prior to

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intravenous administration was reached again after intravenous administration to infinity. The point of time when the concentration just prior to intravenous administration was reached again after intravenous administration was obtained by log-linear extrapolation from the two adjacent points of the concentration vs. time profile. The AUC attributable to the intravenous dose was calculated as overall AUC minus the AUC attributable to the oral dose.

Clearance of i.v. midazolam was calculated using the formula: $Cl_{i.v.} = dose_{i.v.} / AUC_{0-\infty i.v.}$.

Hepatic availability of i.v. administered midazolam was calculated as $F_{hepatic} = 1 - Cl_{i.v.}/liver\ blood\ flow$ and the liver blood flow was estimated as 25.3 mL/kg body weight /min (Gorski et al., 1998; Lee et al., 2002). Intestinal availability of oral midazolam was calculated as :

$F_{intestinal} = F_{oral} / F_{hepatic}$, whereas absolute bioavailability following oral administration of midazolam was calculated as $F_{oral} = AUC_{oral} / AUC_{i.v.}$ corrected for dose differences.

Compartmental pharmacokinetics of midazolam were assessed by population pharmacokinetic analysis using the NONMEM software (V version 1.1, NONMEM Project Group, University of California at San Francisco, 1998). The most simple model, i.e. a one-compartment model with no intraindividual variation in any parameter, was stepwise expanded to more complex models if these were superior as evaluated by the plausibility of the parameter estimates and their 95% confidence intervals, by goodness-of-fit plots, and by a significant changes ($p < 0.05$) of the objective function value provided by NONMEM. Fitting was performed with the “first order conditional estimates” algorithm, taking interactions between the parameters into account.

The apparent clearance of tolbutamide was calculated as $Cl/F = dose_{oral} / AUC_{0-\infty oral}$.

Statistical analysis

Testing for a pharmacokinetic interaction was handled as an bioequivalence problem (Steinijans et al., 1991). Test and Reference treatments were compared with respect to the phenotyping metrics.

Lack of a clinically relevant interaction was assumed if 90% confidence intervals (CIs) for Test/Reference ratios of the phenotyping metrics were within the range 0.7-1.43. This range was used instead of the more narrow standard bioequivalence range of 0.8-1.25 because the elimination of most drugs is not limited exclusively to a single pathway, thus inhibition of a single enzyme usually does not fully show for therapeutic drugs. A statistically significant difference, however, was considered if unity was not included in the confidence intervals.

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Propiverine trough values prior to the last morning dose were compared to propiverine trough values prior to the previous dose by the same procedure in order to check for having reached steady state concentrations on profiling days.

For bioequivalence testing of the pharmacokinetic parameters and for the calculation of ANOVAs, the BioQ software, version 1.2.2 for Windows (E. Diletti, Byk Gulden Pharmaceuticals, Konstanz, Germany) was used.

For all primary phenotyping metrics a multiplicative model was assumed. Thus the Test versus Reference comparison is a ratio for all parameters.

On the basis of the anticipated intrasubject multiplicative coefficients of variation of up to 30% for the phenotyping metrics, a sample size of N=16 was considered to be sufficient to allow rejection of the respective null hypotheses "clinically relevant interaction present" with $\alpha = 0.05$ and a power of at least 80 % for all parameters if the true $\mu_{\text{test}}/\mu_{\text{reference}}$ ratios are within the 0.95 to 1.05 range (Diletti et al., 1991; Diletti et al., 1992).

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Results

Table 2 and 3 show the mean values of the phenotypic metrics (see table 1) and additional pharmacokinetic parameters for model substrates determined in Test and Reference periods. Table 4 displays the selected comparisons, i.e. point estimates and 90% confidence intervals for Test/Reference ratios. Figures 2 – 5 show the values of the important phenotyping metrics in all subjects in Test and Reference period.

In the noncompartmental analysis of midazolam pharmacokinetics, propiverine reduced clearance and increased intestinal availability. The extent of the reduction of hepatic and intestinal CYP3A4 activity was to 0.89-fold and to 0.80-fold, respectively, of the Reference period (90% CI for Test/Reference ratios 0.85 – 0.93 and 0.72 – 0.89), with the combined effect resulting in a 1.46-fold increase in AUC of oral midazolam (90% CI 1.36 - 1.57) (Table 4, Fig. 1, Fig. 2). For the compartmental analysis of midazolam pharmacokinetics used to check the results of the noncompartmental evaluation, the best fit was obtained by a two-compartment model with first-order absorption and intraindividual variation in each parameter. The presence of co-medication with propiverine had a major effect on the values of midazolam clearance and bioavailability (changes in NONMEM objective function by -32, $p < 0.005$, and -91, $p < 0.005$, respectively). In this evaluation, clearance decreased to 0.90-fold (95% CI 0.86-0.95) in the presence of co-medication with propiverine; the point estimates for clearance were 24.4 and 27.0 L/h with and without co-medication, respectively. Intestinal midazolam availability and oral bioavailability increased by a factor of 1.29 (90% CI 1.15-1.39) and 1.32 (95% CI 1.22-1.42), respectively, upon co-medication with propiverine. The point estimates for oral bioavailability were 0.34 and 0.45, mean values for intestinal availability were 0.57 and 0.71 without and with co-medication, respectively. Furthermore body weight was a significant covariate for clearance (increase of 0.85 % per kg of body weight) and for both central and peripheral volume of distribution (increase of 0.73 % per kg of body weight). In summary, these results fully confirmed those obtained by the noncompartmental analysis.

Propiverine had no relevant effect on CYP2C9 (Fig. 3), CYP2C19 (Fig. 4) and CYP1A2 (Fig. 5). The respective 90% CIs for Test/Reference ratios were 0.93-1.00, 0.84-0.96 and 0.97-1.07 (Table 4).

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The volume of urine excreted was similar between treatments: mean \pm SD in Test and Reference were 3466 ± 1268 mL and 2744 ± 966 , respectively, within 24 hours. The mean urinary pH ranged between 6.7 and 7.3 during daytime and between 6.2 and 6.5 at night, respectively, in both treatments.

Figure 6 depicts mean concentrations of propiverine and its metabolite propiverine-N-oxide in steady state, measured after the last morning administration in the Test period on study day 7. As the 90% confidence interval of the ratio of trough evening propiverine concentration and its trough morning concentration on study day 7 in Test period was 0.85 – 1.07 (point estimate was 0.95), it was assumed that steady state was reached.

Safety results:

Forty-one adverse events occurred during the study. Most of them like dry mouth or accommodation problems were clearly explained with antagonistic effects of propiverine towards muscarinic acetylcholine receptors. On the profiling days, the most frequent adverse events were dizziness, tiredness and sedation which were closely temporally related to the intravenous administration of midazolam. Forty adverse events were classified as mild while one adverse event only was moderate in severity (dizziness in one subject). All adverse events were transient and resolved shortly after the administration of the cocktail, when related to the phenotyping drugs, or after the cessation of propiverine therapy, when dependent on its anticholinergic properties. Notably, an extensive monitoring of sedation and hypoglycemic symptoms on profiling days did not reveal any relevant abnormalities. Clinical examinations, vital signs, subjects' well-being, and clinical laboratory data did not provide evidence for health impairment caused by the study drugs. Together, all study drugs were well tolerated.

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Discussion

The aim of this study was to evaluate a possible effect of propiverine hydrochloride intake at therapeutic doses on the activity of important human cytochrome P450 enzymes. Our results indicate that propiverine has a modest inhibitory activity on CYP3A4 and negligible effects on other CYPs.

The exposure of propiverine and its main metabolite, propiverine-N-oxide, at steady-state during the Test period is in agreement with published data (Siepmann et al., 1998). The selected dosing scheme has been shown to be therapeutically effective and is used clinically (Alloussi et al., 2005). This allowed the characterization of eventual changes in the activity of the respective metabolic enzymes under similar conditions as in clinical practice.

The cocktail approach, which is proposed as a screening method for assessment of drug-drug interactions *in vivo*, has been used for almost two decades (Schellens et al., 1988; Streetman et al., 2000a.; Tanaka et al., 2003). Unfortunately, no general agreement has been reached on the applicability of the cocktail approach in clinical investigations and during drug development (Zhou et al., 2004). Here, the low-dose cocktail strategy could be shown to be an effective tool to assess the drug-drug interactions profile of propiverine *in vivo*. All probe substrates used in this cocktail approach have been previously shown to be enzyme selective substrates without relevant mutual interaction *in vivo* (Bruce et al., 2001; Blakey et al., 2004; Endres et al., 1996; Frye et al., 1997; Streetman et al., 2000b; Palmer et al., 2001; Wang et al., 2001; Zhu et al., 2001). The composition of the cocktail used here was tailored primarily with respect to the previous information obtained in *in vitro* studies, suggesting that any clinically relevant interaction of propiverine with cytochrome P450 enzymes as a substrate, inhibitor and/or inducer would mainly concern CYP3A4 and possibly also CYP2C9 and/or CYP2C19. Nevertheless, the inclusion of optional cocktail components allowed a further evaluation of propiverine effects.

Phenotyping for CYP3A4 activity with midazolam is a well established method although it is elaborate due to requirement of multiple blood collections (e.g. Streetman et al., 2000a). A

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problem is the independent regulation of intestinal and hepatic CYP3A4 (Gorski et al., 1998; Lee et al., 2002; Tsunoda et al., 1999). For this reason, two different metrics have to be used for estimation of both activities. In the present study, for the evaluation of hepatic and intestinal CYP3A4 activity, a sequential administration of midazolam, first orally and then intravenously, was conducted. Application of the oral midazolam dose concomitantly with the morning dose of propiverine was considered to be adequate for assessment of potential interaction between both drugs during absorption when the concentration at enzyme site is maximal. The time interval of 4 hours between the oral and intravenous midazolam administration allowed absorption and distribution of the oral midazolam to be completed. Therefore, it was possible to attribute the two components of the obtained midazolam AUC to the respective midazolam doses (see figure 1). Lee et al. (2002) have shown that such phenotyping results obtained with semi-simultaneously administered midazolam were not different from those for administration of midazolam on two separate occasions. The validity of the procedure is supported by the similar results obtained in this study by both noncompartmental and compartmental methods. Both midazolam doses, 2 mg orally and 1 mg intravenously, were chosen to obtain similar concentrations after the two administrations, taking into account the mean oral bioavailability of midazolam of approximately 40%. For phenotyping with midazolam, dosage considerations are very important because of safety reasons. Due to low dosages of the drug in our study, the adverse events observed in the subjects were very mild. Based on the clearance of intravenously administered midazolam and intestinal availability of orally administered midazolam, which were the phenotypic metrics for hepatic and intestinal CYP3A4 activity, respectively, chronic treatment with propiverine had some inhibitory effect on CYP3A4. For hepatic and intestinal CYP3A4 activity in the Test period, we observed a mean reduction to 0.89-fold and to 0.80-fold of the activity observed in the Reference period, respectively. The extent of these effects taken separately was considered not to be clinically relevant as the 90 % confidence interval for Test/Reference ratios of the metrics were within the predefined limits of 0.7-1.43. However, the combined effect on intestinal and hepatic CYP3A4 resulted in 1.46-fold increase in AUC of oral midazolam. This effect of chronic therapy with propiverine would be considered as relevant even taking inherent variation in midazolam pharmacokinetic parameters into account. The intrasubject coefficients of variation observed in our study were 6.8%, 17.6% and 11.6%, respectively, for clearance of i.v. administered midazolam, intestinal availability and AUC of oral midazolam. These results are similar to those published by Kharasch et al.,

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who demonstrated the interday coefficient of variation in midazolam clearance of $19\% \pm 12\%$ (Kharasch et al., 1999).

For the assessment of the CYP2C9 activity, both urinary ratio (hydroxytolbutamide + carboxytolbutamide)/ tolbutamide 6-12 hours postdose, which was proposed as a convenient phenotyping metric (Veronese et al., 1990), and tolbutamide plasma data were determined. Based on the urinary metabolic ratio of tolbutamide, there was a statistically significant and clinically relevant inhibition of the CYP2C9 activity due to the chronic treatment with propiverine as the confidence interval of the ratio Test/Reference exceeded the tolerance zone of 0.70 - 1.43. In contrast, using plasma data (i.e., oral tolbutamide clearance) as well as tolbutamide plasma concentration 24 hours postdose, a slight statistically significant inhibition of CYP2C9 which was observed which was without clinical significance according to the definition used. In a recent paper by Jetter et al., no correlation could be found between the proposed urinary metabolic ratio and the tolbutamide plasma clearance in 26 healthy volunteers following administration of 125 mg of the drug (Jetter et al., 2004). On the other hand, the authors observed a close correlation between tolbutamide plasma clearance and 24-h plasma tolbutamide concentration and thus they proposed the latter parameter as a simple metric for determining CYP2C9 activity. Moreover, a close relationship between tolbutamide plasma pharmacokinetics and genetic CYP2C9 polymorphisms was confirmed following administration of 125 mg tolbutamide (Jetter et al., 2004) as well as 500 mg tolbutamide (Lee et al., 2003; Kirchheiner et al., 2002). For that reason, we assumed the plasma-based CYP2C9 metric to be more reliable and do not expect any pronounced interaction between propiverine and drugs metabolized by CYP2C9. CYP2C9 phenotyping with a standard tolbutamide dose i.e. 500 mg was reported to be possibly accompanied by hypoglycemia (Streetman et al., 2000a). This problem, however, could be minimized by co-administration of oral glucose (Lee et al., 2003) or, as in our study, was avoided by using a very low tolbutamide dose of 125 mg.

Mephenytoin as a probe drug for CYP2C19 is well established and used in epidemiological studies as well as in clinical pharmacological research (Tamminga et al., 2001). Urinary recovery of 4'-hydroxymephenytoin in an 8-hour urine sample has been shown to discriminate precisely between poor and extensive metabolizers of mephenytoin (Wedlund et al., 1984; Wedlund et al., 1985). In our study, a statistically significant small difference between excreted amount of 4'-hydroxymephenytoin in Test and Reference period did not reach the magnitude of expected clinical relevance so we conclude that there is no clinically

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relevant effect of chronic treatment with propiverine on the activity of CYP2C19. The high interindividual coefficient of variation of the CYP2C19 metric was the result of the participation of a poor metabolizer (*CYP2C19**2/*2) for mephenytoin in the study who showed minimal urine concentrations of 4'-hydroxymephenytoin as compared to the other participants. For safety reasons, the study subjects were administered a low dose of mephenytoin i.e. 50 mg so that sedation, which is a frequently reported adverse reaction of mephenytoin, was not observed.

Based on the paraxanthine/caffeine plasma ratio 6 hours postdose, which is extensively validated for assessment of CYP1A2 activity (Fuhr et al. 1996, Streetman et al. 2000a), we found no inducing (or inhibitory) effect of chronic treatment with propiverine on the CYP1A2 function, indicating that the characterization of propiverine as a phenobarbital-type inducer at high doses in animals (Walter et al 2003) is not predictive for an effect of therapeutic propiverine doses on CYP1A2 in humans.

In contrast to other existing cocktails (e.g. Blakey et al., 2004; Frye et al., 1997; Palmer et al., 2001), the cocktail used here has several useful peculiarities, including: semisequential midazolam administration, allowing the assessment of hepatic and intestinal CYP3A4 activity within one day; low doses for all substances minimizing the risk for adverse reactions; the inclusion of optional cocktail components; and the use of fully validated metrics for all CYPs included.

Finally, as the intraindividual coefficients of variation for all phenotyping metrics did not exceed 30 % and were clearly below this margin for most substances, the sample size proved to be sufficient (Diletti et al., 1991; Diletti et al., 1992) and may be even decreased further in such studies, depending on the enzymes to be assessed.

Our results suggest that propiverine may cause relevantly increased concentrations of drugs only if they undergo extensive first pass metabolism by both intestinal and hepatic CYP3A4. Examples for such drugs include verapamil, saquinavir, and felodipine. However, a very pronounced concentration increase for such drugs is not expected as the influence of chronic treatment with propiverine seems to be small compared to classical enzyme inhibitors such as ketoconazole or grapefruit juice. There was no relevant effect of chronic treatment with

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propiverine on CYP2C9 , CYP2C19 and CYP1A2 so that no interaction between propiverine and drugs metabolized by these cytochrome P450 enzymes is expected.

In summary, propiverine has a minor potential to cause drug-drug interactions. The application of this tailored cocktail is an effective and well tolerated method for assessment of drug-drug interaction in healthy volunteers.

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Footnotes

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Reprint request to: Dr. Dorota Tomalik-Scharte, Department of Pharmacology, Clinical
Pharmacology, University of Cologne, Gleueler Str. 24, 50931 Köln, Germany
e-mail: dorota.tomalik-scharte@uk-koeln.de

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Legends to figures

Figure 1: Mean midazolam plasma concentration vs. time curves of 16 volunteers in Test and Reference period

Figure 2: Comparison of clearance of intravenously administered midazolam, intestinal availability of oral midazolam and $AUC_{0-\infty}$ of orally administered midazolam as phenotyping metrics of CYP3A4 activity in Reference and Test period in all study participants (noncompartmental analysis)

Figure 3: Comparison of molar urinary ratio (hydroxytolbutamide + carboxytolbutamide)/tolbutamide in urine 6-12 hours after tolbutamide administration, concentration of tolbutamide 24 h postdose and oral tolbutamide clearance as phenotyping metrics of CYP2C9 activity in Reference and Test period in all study participants

Figure 4: Amount of mephenytoin excreted as 4'-hydroxymephenytoin (%) in urine during 8 hours postdose as a phenotyping parameter of CYP2C19 activity in Reference and Test period in all study participants

The individual with very low values in both periods is considered to have low-activity CYP2C19 alleles.

Figure 5: paraxanthine/caffeine plasma ratio 6 hours postdose as a phenotyping parameter of CYP1A2 activity in Reference and Test period in all study participants

Figure 6: Mean plasma concentrations of propiverine and propiverine-N-oxide on day 7 in Test period (ng/mL)

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Tables

Table 1: Probe substrates and the respective phenotyping metrics used in the “cocktail” approach in the study (for calculation of metrics, see methods section).

Cytochrome P450 enzymes	Probe substrate	Dose and administration route	Matrix sampled	Pharmacokinetic parameters determined (main phenotyping metrics are printed in bold)
hepatic CYP3A4	midazolam	1mg/ intravenously	plasma	$AUC_{0-\infty}^a$ i.v., clearance of i.v. midazolam , F_{hepatic}^b
intestinal CYP3A4	midazolam	2 mg/orally	plasma	F_{oral}^c , $F_{\text{intestinal}}^d$, $AUC_{0-\infty}^a$ oral, C_{max}^e oral, t_{max}^f oral, $t_{1/2, \lambda z}^g$
CYP2C9	tolbutamide	125 mg/orally	urine and plasma	UR_{6-12h} (OH-tolbut.+ carboxytolbut.)/tolbut. ^h , $AUC_{0-\infty}^a$, C_{max}^e , t_{max}^f , $t_{1/2, \lambda z}^g$, CL/Fⁱ, tolbutamide plasma concentration 24 postdose
CYP2C19	mephenytoin	50 mg/orally	urine	Ae_{0-8h} 4'-OH-mephenytoin^j
CYP1A2	caffeine	150mg/orally	plasma	paraxanthine/caffeine plasma ratio 6 hours postdose

^a $AUC_{0-\infty}$ - area under the plasma concentration vs. time curve extrapolated to infinity

^b F_{hepatic} - hepatic availability of midazolam

^c F_{oral} - absolute bioavailability of midazolam

^d $F_{\text{intestinal}}$ - intestinal availability of midazolam; to enable a better comparison, in the text changes in intestinal CYP3A4 activity were calculated as the inverse of changes in $F_{\text{intestinal}}$

^e C_{max} - maximum plasma concentration

^f t_{max} - time to reach maximum plasma concentration

^g $t_{1/2, \lambda z}$ - terminal half life

^h UR_{6-12h} (OH-tolbut.+ carboxytolbut.)/tolbut. - urinary molar concentration ratio (hydroxytolbutamide + carboxytolbutamide)/tolbutamide 6 – 12 hours postdose

ⁱCL/F - oral clearance of tolbutamide

^jAe_{0-8h} 4'-OH-mephenytoin - amount of mephenytoin dose excreted as 4'-hydroxymephenytoin in urine 0-8 hours postdose

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Table 2: Mean pharmacokinetic parameters^a of midazolam in plasma for Test and Reference period (non-compartmental evaluation)

Pharmacokinetic parameter (unit)	Phenotyping metric for	mean (%CV) in Test period	mean (%CV) in Reference period
clearance of intravenous midazolam (L/h)	hepatic CYP3A4	26.0 (20.3)	29.2 (20.1)
F _{intestinal} (-)	intestinal CYP3A4	0.646 (23.2)	0.518 (22.7)
F _{hepatic} (-)	-	0.775 (5.4)	0.749 (5.6)
F _{oral} (-)	-	0.501 (22.7)	0.388 (21.7)
AUC _{0-∞ oral} midazolam (ng/mL • h)	-	39.9 (30.0)	27.3 (23.8)
AUC _{0-∞ i.v.} midazolam (ng/mL • h)	-	38.2 (20.1)	34.1 (20.2)
c _{max oral} midazolam (ng/mL)	-	14.2 (23.1)	10.5 (26.9)
t _{½,λz} midazolam (h)	-	3.02 (23.3)	2.98 (29.6)
t _{max oral} midazolam (h)	-	0.630 (27.2)	0.661 (34.2)

^aGeometric means (geometric CV) are given for all parameters except for t_{max} where arithmetic mean (arithmetic CV) is given, CV – coefficient of variation. For pharmacokinetic parameters, see legend to Table 1.

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Table 3: Mean pharmacokinetic parameters^a of tolbutamide in urine and plasma, mephenytoin in urine and caffeine in plasma for Test and Reference period

Pharmacokinetic parameter (unit)	Phenotyping metric for	mean (%CV) in Test period	mean (%CV) in Reference period
UR _{6-12h} (OH-tolbut.+ carboxytolbut.)/tolbut. (-)	CYP2C9	525 (42.6)	664 (33.9)
AUC _{0-∞} tolbutamide (µg/mL•h)	-	160.9 (16.3)	154.6 (15.1)
CL/F tolbutamide (mL/min)	CYP2C9	12.93 (16.3)	13.44 (15.1)
c _{max} tolbutamide (µg/mL)	-	11.7 (11.1)	12.3 (12.3)
t _{max} tolbutamide (h)	-	3.99 (4.5)	3.80 (5.0)
t _{½, λz} tolbutamide (h)	-	8.27 (12.9)	7.70 (14.0)
tolbutamide plasma concentration 24 hours postdose (µg/mL)	CYP2C9	2.00 (27.4)	1.82 (30.9)
Ae _{0-8h} 4'-OH-mephenytoin (% of dose)	CYP2C19	18.2 (195)	19.3 (228)
paraxanthine/caffeine plasma ratio 6 hours postdose (-)	CYP1A2	0.49 (37)	0.51 (39)

^aGeometric means (geometric CV) are given for all parameters except for t_{max} where arithmetic mean (arithmetic CV) is given, CV – coefficient of variation. For pharmacokinetic parameters, see legend to Table 1.

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Table 4: Bioequivalence assessment for Test versus Reference period (main phenotyping metrics are bold)

Pharmacokinetic parameter	Point estimate of ratios Test/Reference	90 % confidence interval of ratios Test/Reference	Intrasubject CV (%)
clearance of intravenous midazolam	0.892	0.855 – 0.930	6.8
hepatic availability of oral midazolam	1.035	1.020 – 1.050	2.3
intestinal availability of oral midazolam	1.248	1.119 - 1.392	17.6
absolute availability of oral midazolam	1.292	1.171- 1.426	15.9
AUC _{0-∞} for oral midazolam	1.461	1.359 – 1.570	11.6
UR _{6-12h} (OH-tolbutamide + carboxytolbutamide) / tolbutamide.	0.791	0.679 - 0.922	24.9
CL/F of tolbutamide	0.962	0.929-0.997	5.7
tolbutamide plasma concentration 24 hours postdose	1.107	1.055 – 1.161	7.7
Ae_{0-8h} 4'-OH-mephenytoin	0.90	0.84 – 0.96	10.7
paraxanthine/caffeine plasma ratio 6 hours postdose	0.97	0.88 – 1.07	16.1

CV – coefficient of variation. For pharmacokinetic parameters, see legend to Table 1.

Figure 1

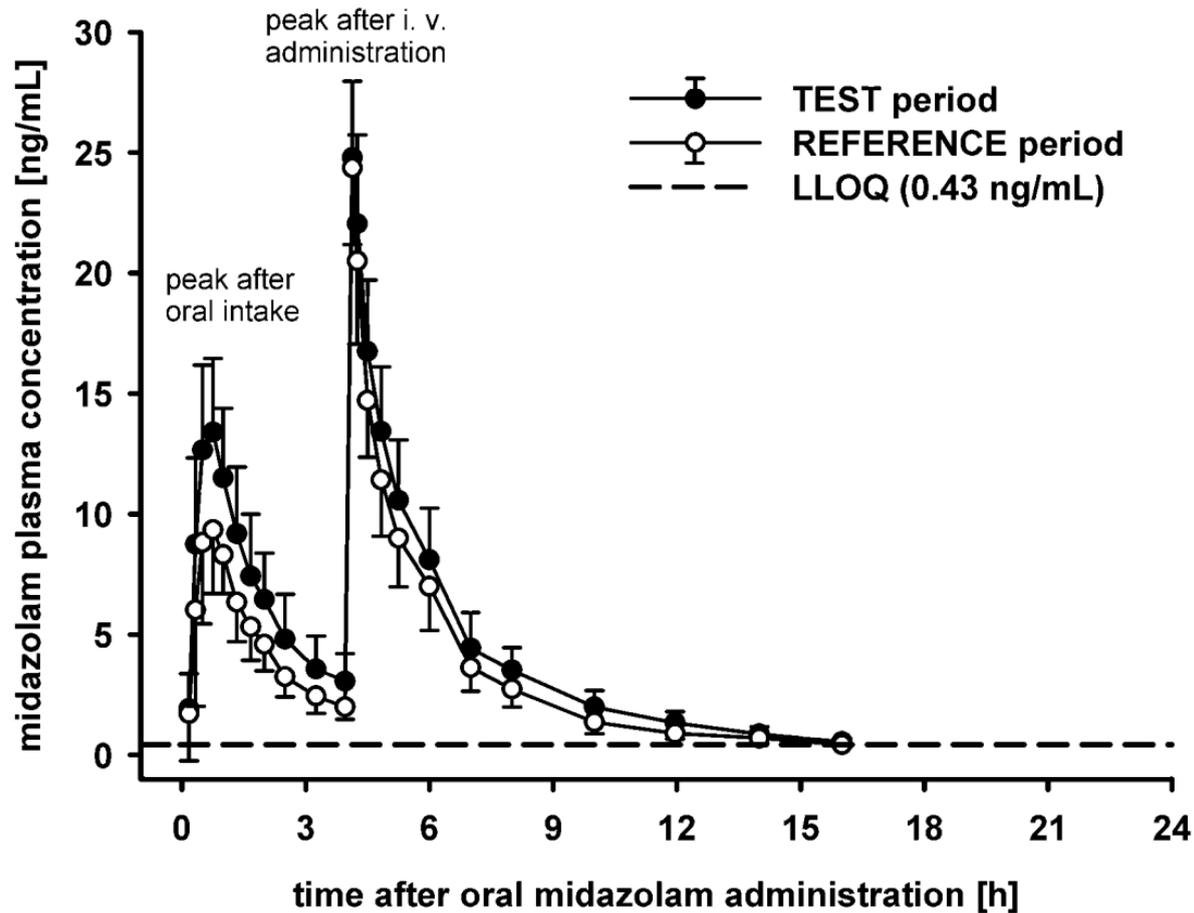


Figure 2

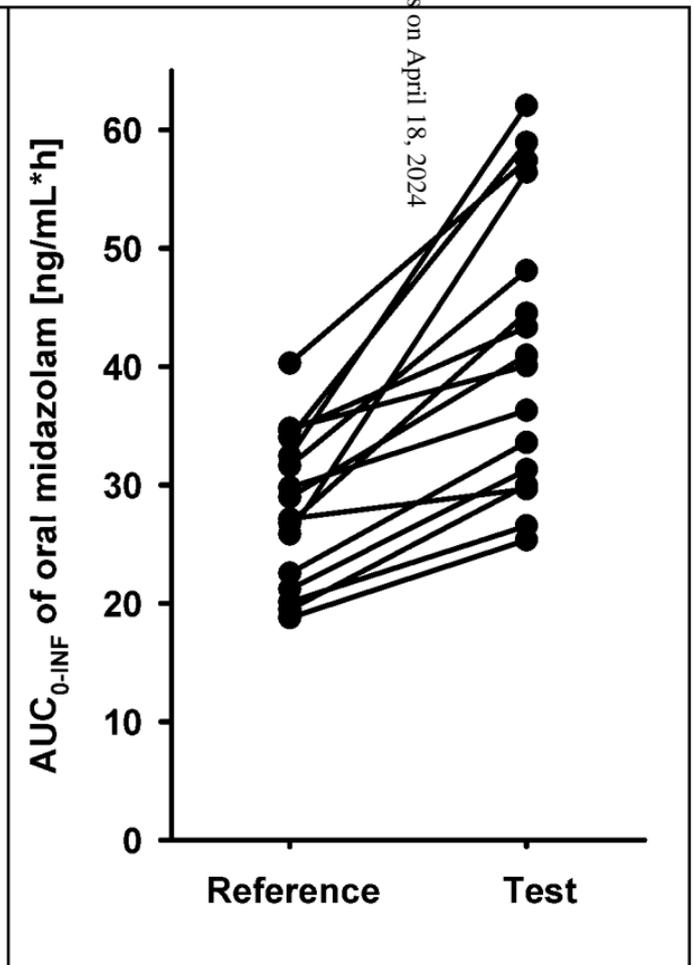
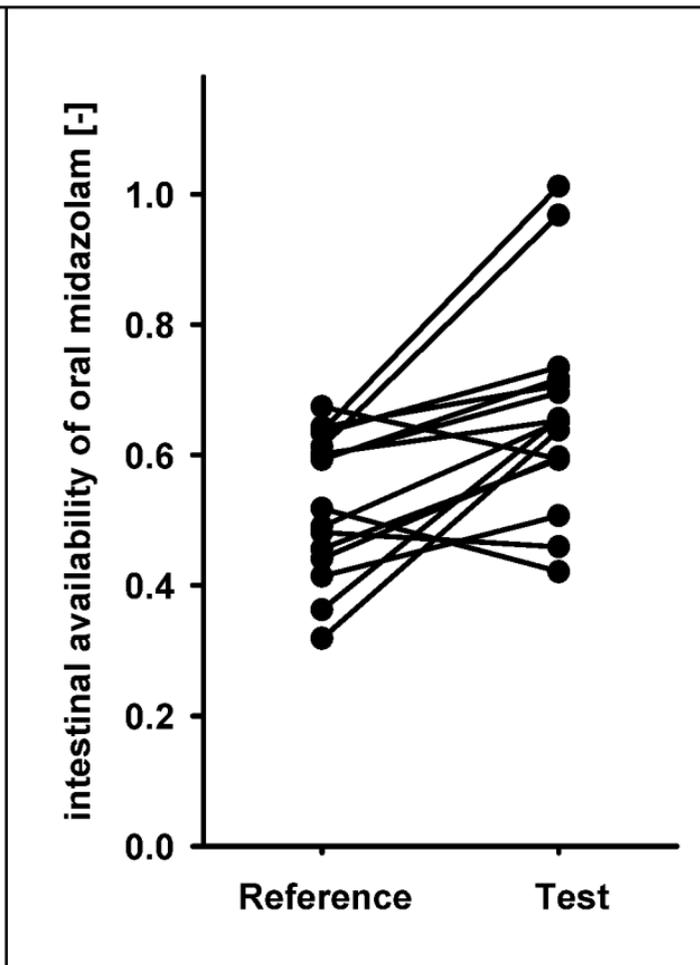
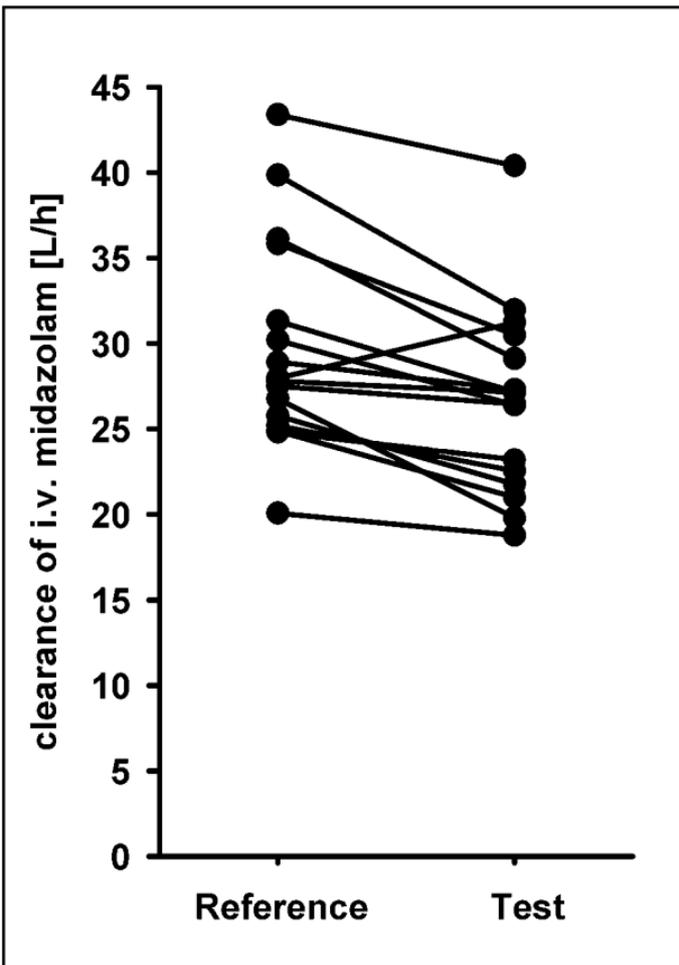


Figure 3

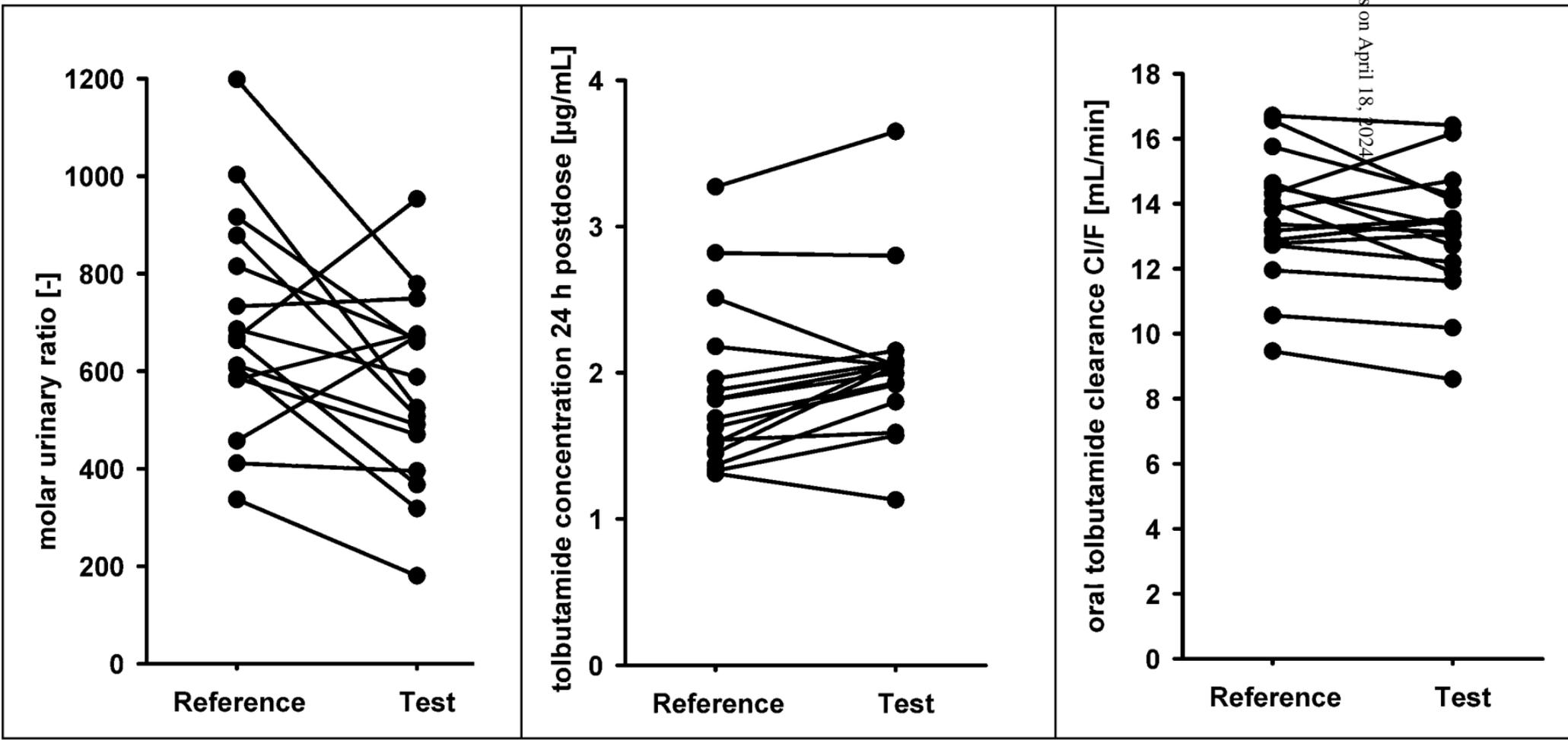


Figure 4

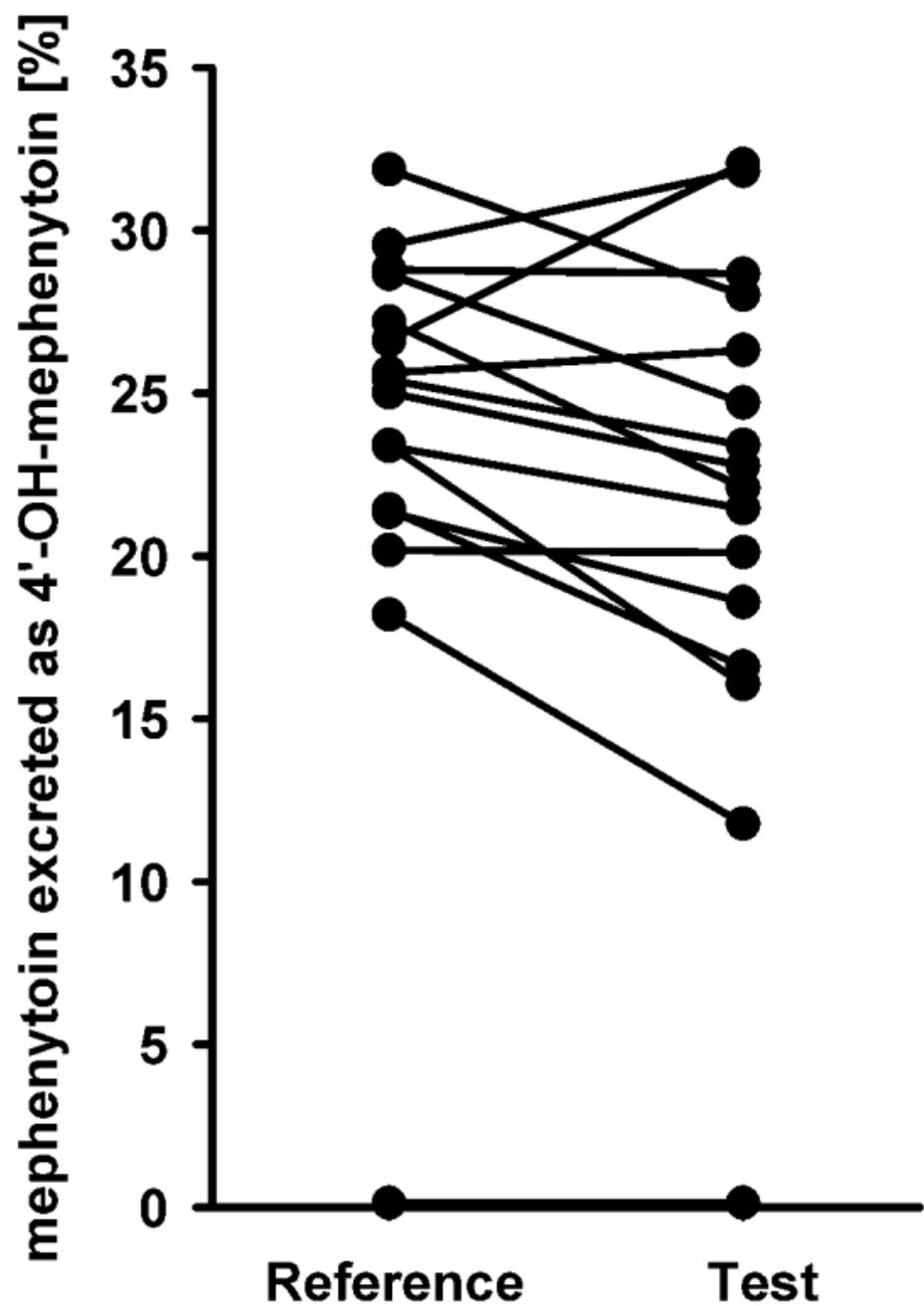


Figure 5

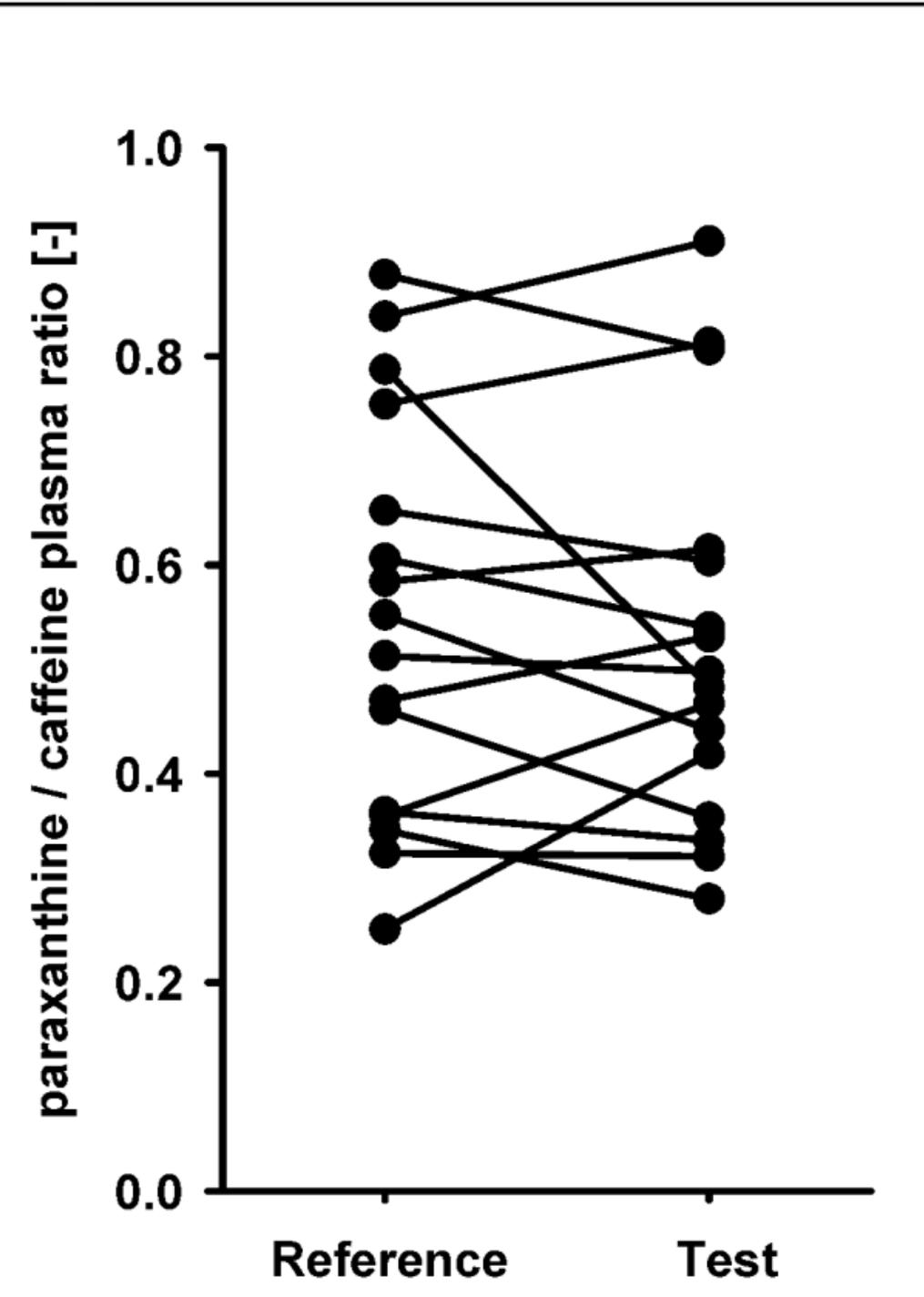


Figure 6

