Plasma 4beta-hydroxycholesterol - an endogenous CYP3A metric?

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Abstract

We assessed the suitability of 4beta-hydroxycholesterol (4bOH-C) as endogenous CYP3A phenotyping metric.

4bOH-C and its ratio over cholesterol (4bOH-C/C) were determined in five cocktail phenotyping studies, with and without co-medication with a potential CYP3A inhibitor. These parameters were compared to established midazolam-based CYP3A metrics: i.v. (M-Cl) and apparent oral clearance (M-Cl/F) reflecting hepatic and overall activity, respectively.

In a common evaluation of periods without co-medication, 4bOH-C and 4bOH-C/C correlated slightly positively to midazolam metrics: M-Cl (r=0.239 and 0.348), M-Cl/F (r=0.267 and 0.353); p(one-sided)<0.05. Co-medication with lopinavir/ritonavir, causing a strong decrease in midazolam metrics, showed a mild decrease of cholesterol metrics. However, intake of propiverine gave opposite results for midazolam and cholesterol based metrics.

The information currently available does not justify the use of 4bOH-C for estimation of basal CYP3A activity. Further studies addressing temporal variation of local CYP3A activity are needed to assess its role as biomarker during CYP3A inhibition.

Introduction

Phenotyping for drug metabolizing enzymes (DME) or transporters enables the identification of potential factors affecting drug metabolism and disposition including drug-drug interactions(1). Phenotyping for CYP3A seems to be of special importance because about half the currently prescribed drugs are metabolized with participation of enzymes belonging to this family, particularly CYP3A4. However, selection of a proper CYP3A phenotyping substrate and metric is still a matter of discussion, although to date numerous CYP3A test substrates have been proposed, including midazolam, erythromycin, alprazolam, alfentanil, nifedipine, dapsone, omeprazole, dextromethorphan, and others.(2) At the same time identification of an appropriate endogenous CYP3A substrate and corresponding metrics would be a promising alternative allowing for simplification and maybe a broader application of the phenotyping approach. The oxidation of the endogenous compound cortisol to form 6beta-hydroxycortisol has been shown to be a CYP3A dependent pathway in humans. Consequently urinary excretion of 6beta-hydroxycortisol and its ratio to 17-hydroxycorticosteroids in 24 h urine sampling have been proposed as suitable phenotyping markers for the assessment of CYP3A activity.(3, 4) However, while this may be used to monitor CYP3A induction, the sensitivity of the respective metrics to detect small changes in the enzyme activity is low because of pronounced background variation(3). Recently Bodin at al. provided evidence for the role of CYP3A4 in *in vitro* hydroxylation of cholesterol to 4beta-hydroxycholesterol (4bOH-C), which was identified as one of the major oxysterols in man.(5) Following the observation that patients treated with antiepileptics like carbamazepine, phenytoin, or phenobarbital, which are strong inducers of several cytochrome P450 enzymes, had up to 20-fold higher plasma concentrations of this metabolite as compared to patients treated with antiepileptics not causing induction, the authors incubated cholesterol with recombinant human CYP3A4, CYP1A2, CYP2C9 and CYP2B6. Accordingly, only for CYP3A4, measurable formation of

4bOH-C has been identified, thus proving the key role of the enzyme for conversion of cholesterol to 4bOH-C.(5) Moreover, treatment with other known inductors of CYP3A like ursodeoxycholic acid, rifampicin or efavirenz was also shown to be related to increased plasma concentrations of 4bOH-C in patients.(5-8) Diczfalusy et al. demonstrated that 4bOH-C plasma concentration was correlated to the number of active *CYP3A5*1* alleles, in three different ethnic populations of healthy Swedes, Tanzanians and Koreans.(9) CYP3A5 is closely related to CYP3A4 and shows almost the same substrate spectrum, but its expression, in contrast to the ubiquitous CYP3A4, is limited to the carriers of at last one *CYP3A5*1* allele (CYP3A5 expressers) and subject to a pronounced inter-individual and interracial variability.(10) Moreover, the authors observed a good correlation between 4bOH-C and the metabolic ratio of quinine/3-hydroxyquinine in plasma, which is a parameter proposed for CYP3A phenotyping.(9, 11) Accordingly, 4bOH-C has been suggested as potential endogenous biomarker of CYP3A activity.(9, 12).

However, the available data on CYP3A phenotyping by 4bOH-C is quite limited and the metric has not been fully validated. Thus, the aim of our assessment is to expand the existing evidence base and provide clarification with respect to suitability of plasma concentrations of 4bOH-C as a metric for CYP3A activity in humans.

Results

Data from 52 healthy, male subjects, and 27 HIV-positive male and female patients participating in one of five clinical cocktail phenotyping studies (termed A – E) were considered for this evaluation, however, two of the healthy volunteers participated in two studies each (for study designs and populations see also Method section and Table 1). Table 2 shows means and standard deviations of 4bOH-C (given in ng/mL, which is the unit used in previously published data) and the metabolic ratio (molar) of 4bOH-C over cholesterol (4bOH-C/C) as well as those of CYP3A phenotyping parameters based on midazolam, i.e.

midazolam i.v. clearance (M-Cl) and apparent oral clearance (M-Cl/F), sorted by study and, for interaction studies B – E, by Reference and Test period. Tables 3 and 4 display the results of a correlation analysis between midazolam phenotyping parameters and, respectively, 4bOH-C concentrations and 4bOH-C/C ratios. Taking into account the data of all healthy volunteers estimated across all studies for periods without co-administration of a potential CYP3A inhibitor, both cholesterol metrics were statistically significantly ($p<0.05$, one sided), however, weakly correlated to midazolam metrics reflecting hepatic (M-Cl) and total CYP3A activity (M-Cl/F). The correlation to M-Cl/F was somewhat stronger (M-Cl/F: $r=0.27$ and $r=0.35$ vs. M-Cl: $r=0.24$ and $r=0.35$, respectively, for 4bOH-C and 4bOH-C/C). At the same time, the 4bOH-C/C ratio performed slightly better than the 4bOH-C concentration alone (see Figure 1). For individual studies, as well as Test and Reference periods within one study, the coefficient of correlation r was occasionally in the opposite of the expected direction, and failed to reach statistical significance in most cases. (Tables 3 and 4).

The effect of co-medication on CYP3A activity has been evaluated in interaction studies B – E. In studies C and D, no statistically significant changes with respect to both cholesterol metrics were observed upon administration of the tested drug in the Test period (see Table 3 and 4, ratios Test/Reference), which was in concordance with the assessment of CYP3A activity based on midazolam metrics, also not showing any relevant interaction potential of the co-medication in those studies (data not shown). In contrast, in study B, chronic co-administration of propiverine resulted in mild but statistically significant increase of both cholesterol parameters (see Table 3 and 4), but this was not supported by the midazolam metrics indicating a change in the opposite direction: the mean reductions (90%CI) of M-Cl and M-Cl/F, respectively, were 0.91-fold (0.88-fold - 0.94-fold) and 0.68-fold (0.63-fold - 0.73-fold). In study E, chronic co-administration of a drug regimen containing ritonavir boosted lopinavir resulted in a rather small reduction of 4bOH-C and 4bOH-C/C (see Table 3 and 4) whereas at the same time, pronounced reductions of M-Cl to the 0.24-fold (90%CI,

0.20-fold – 0.29-fold) and M-Cl/F to the 0.18-fold (90%CI, 0.15-fold – 0.23-fold) were observed. Figure 2 shows the mean changes in midazolam and cholesterol phenotyping metrics upon co-administration of both regimens, propiverine and ritonavir boosted lopinavir.

Taking into account genotyping results, 49 subjects (69%) were homozygote carriers of *CYP3A5*3*, 16 subjects (23%) were heterozygote for this allele, and six subjects (8%), four of whom were of African origin, had the wild type genotype (*CYP3A5*1/*1*). The mean concentration of 4bOH-C and the mean 4bOH-C/C ratio increased with the number of the active *CYP3A5*1* alleles, however this did not reach statistical significance (data not shown).

Discussion

In the present evaluation, we studied the suitability of cholesterol based metrics, i.e. 4bOH-C and the 4bOH-C/C ratio in plasma as endogenous biomarkers of CYP3A activity and observed that only a small part of the variation in midazolam and cholesterol based metrics, respectively, shares the same source, and that cholesterol based metrics may reflect decreased CYP3A activity by co-administration of CYP3A inhibitor only if these have a strong effect.

Despite the crucial role of CYP3A in drug metabolism, a reliable estimation of its actual activity is still challenging and a perfect phenotyping metric has not been identified yet. Using an endogenous substrate would obviously be the simplest and safest phenotyping method. For that reason, we focused on the usefulness of 4bOH-C, and the related parameter, the 4bOH-C/C ratio, which may eliminate potential bias due to variations in plasma cholesterol, and compared these to established midazolam based CYP3A metrics (13). In fact, in the common evaluation of all healthy volunteers, both cholesterol metrics were statistically significantly correlated to i.v. clearance and, even more, apparent oral clearance of midazolam. However, this correlation was weak, and not consistently reflected in individual studies. Although the 4bOH-C/C ratio seems to perform slightly better than 4bOH-C alone, the difference was minor, thus confirming earlier assumptions that 4bOH-C is not considerably influenced by cholesterol concentrations.(7, 9)

One of the aims of our evaluation was to study the appropriateness of cholesterol based metrics to reflect possible changes in CYP3A activity by drug-drug interactions. In two interaction studies (C and D), lack of an effect on CYP3A was concordantly reflected by both midazolam and cholesterol based metrics. However, in the propiverine interaction study (study B), a small significant increase of 4bOH-C and the 4bOH-C/C ratio, suggesting induction of CYP3A, was in contrast to changes in midazolam metrics indicating inhibition of CYP3A.(14) Finally, in the ritonavir boosted lopinavir interaction study, cholesterol based

metrics reflect a moderated CYP3A inhibition, whereas midazolam based metrics indicate a strong inhibition of CYP3A activity. Noteworthy, these results are well in agreement with those of Josephson et al., who found a minor mean decrease of 4bOH-C of 11% following four weeks of therapy with ritonavir boosted lopinavir, both dosed like in our study, whereas a combination of atazanavir and ritonavir resulted in a higher and statistically significant reduction of 18% in this parameter.(6)

Both the weak correlation between cholesterol and midazolam based CYP3A metrics and the in part contradictory results in the interaction studies clearly show that cholesterol and midazolam metrics do not measure exactly the same thing. Indeed, the problem of discordant results of independent CYP3A metrics has been addressed previously. An early example of a discordance is a lacking correlation between erythromycin breath test and midazolam clearance, both established and widely used CYP3A metrics.(15) Masica et al., who compared alprazolam, triazolam, and midazolam clearance as CYP3A metrics, concluded that no accurate prediction of pharmacokinetics of any other CYP3A substrate by CYP3A phenotyping would be possible.(16) Moreover, as different CYP3A substrates may vary not only in their biopharmaceutic properties (solubility and permeability), but also with respect to their properties as substrates to various transporters, the general usefulness of CYP3A phenotyping has been challenged.(17) However, Kharasch et al. demonstrated an almost identical magnitude of induction and inhibition of CYP3A activity, as a result of, respectively, rifampicin and troleandomycin administration by means of midazolam and alfentanil based metrics.(18) In our data, we cannot confirm that midazolam and 4bOH-C reflect CYP3A inhibition in the same way. However, we suggest that identification and quantification of covariates beyond CYP3A activity which determine the results of CYP3A phenotyping may overcome these current limitations of individual CYP3A metrics including those based on 4bOH-C.

It may well be that processes beyond CYP3A activity involved in 4bOH-C formation, such as participation of other enzymes not yet tested, or in 4bOH-C elimination and/or transport are the reason for these discrepancies. Bodin at al., who showed that CYP3A4, but not CYP1A2, CYP2C9 and CYP2B6, is involved in formation of 4bOH-C *in vitro*, indeed did not test further enzymes.(5).

However, a closer look suggests that the data do not necessarily exclude that both procedures reflect primarily CYP3A activity despite the discrepancies. Midazolam has a short elimination half-life of approximately 1.8 hours (19), while that reported for 4bOH-C is between around 60 hours and 17 days. (12, 20) Thus, midazolam-based metrics reflect actual hepatic (Cl) or hepatic and intestinal (Cl/F) CYP3A activity, while 4bOH-C would represent a weighted average of CYP3A activity over a period of several days, and it is unclear to which extent gut wall CYP3A may contribute to cholesterol hydroxylation during intestinal absorption. Short-term fluctuations in CYP3A activity could therefore be another reason for the observed discrepancies. There is some evidence that CYP3A activity has circadian variation.(21, 22) Furthermore, the degree of a competitive inhibition of CYP3A depends on actual local concentration, and there are very pronounced and rapid changes in gut wall and hepatic concentrations of a drug administered to assess its interaction with CYP3A in vivo. Oral midazolam in the studies reported here has been administered together with the respective test drugs, thus maximal concentrations of the interacting drugs were present at the enzyme at this time. Therefore, midazolam based metrics would be expected to capture maximal inhibition while cholesterol based metrics would be expected to reflect average inhibition. Ritonavir is reported as being the most potent CYP3A inhibitor and the extent of CYP3A inhibition quantified by means of midazolam phenotyping is clearly in concordance with the recently published data by Kharasch et al., who determined profound CYP3A inhibition upon

treatment with ritonavir using alfentanil, another CYP3A substrate with a short elimination half-life(23, 24). In addition, ritonavir is also an inducer of CYP3A.(6) Thus, assuming that both cholesterol and midazolam based metrics would depend virtually exclusively on CYP3A activity, an increase of overall CYP3A expression during ritonavir administration with a pronounced inhibition of CYP3A primarily shortly after administration which fades between administrations of individual ritonavir doses would be plausible and also compatible with all results. A similar explanation may hold true for the results obtained with propiverine, if this drug also causes some CYP3A induction. However, whereas induction of hepatic cytochrome P450 enzymes was observed following chronic administration of high doses of propiverine in rats(25), no respective data in man is available.

The complexity of relationships between CYP3A activity and 4bOH-C concentrations is also reflected by data from Wide et al. (26), who found some inconsistency with respect to the time course of the increase in the cholesterol parameter and induction of the enzyme. Whereas CYP3A induction due to carbamazepine intake was definitely complete at the end of a two weeks period (as based on plasma concentrations of carbamazepine and its metabolite), 4bOH-C continued to increase till eight weeks after therapy onset suggesting that CYP3A activity is not the only factor regulating the concentration of this endogenous biomarker.(26) Similarly in studies B and E, following the administration of propiverine and ritonavir/lopinavir for, respectively, one or two weeks a new steady state of 4bOH-C might not have been reached considering its very long elimination half-life.

Beyond considering temporal variations in CYP3A activity, it is tempting to speculate on the site of cholesterol hydroxylation and the effective concentrations of cholesterol at the binding site of the enzyme. As cholesterol is present in huge concentrations in plasma, only a tiny fraction may reach the enzyme; otherwise, cholesterol would compete for any other substrate

and probably block CYP3A metabolism because substrate saturation was obtained above 100 µM (5).

A final aspect which may contribute to the discordant results for midazolam and cholesterol phenotyping is the differing substrate specificity between CYP3A4 and CYP3A5.(27) Although not confirmed in our evaluation, CYP3A5 polymorphism has been shown to significantly affect the cholesterol biomarker, whereas it has no influence on midazolam phenotyping.(9, 28)

In summary, the current understanding of CYP3A and cholesterol 4-hydroxylation is not sufficient to establish 4bOH-C concentrations as endogenous CYP3A biomarker. Empirical data available suggest that cholesterol-based phenotyping metrics may be useful to assess CYP3A activity changes by strong CYP3A inducers and inhibitors. The current information does, however, not justify the use of 4bOH-C for estimation of basal CYP3A activity or to assess small changes in CYP3A activity. Further studies are needed to assess the appropriateness of individual CYP3A markers when taking short-term fluctuations of CYP3A activity and other covariates such as transporter activities into account.

Methods

Cholesterol based metrics 4bOH-C and its ratio to cholesterol were evaluated in five clinical cocktail phenotyping studies (termed A – E), in which assessment of hepatic and overall (hepatic and intestinal) CYP3A activity has been previously studied using midazolam based metrics, respectively, i.v. clearance (M-Cl) and apparent oral clearance (M-Cl/F). Four of these studies (A - D) were performed in healthy, male, Caucasian volunteers and one study (E) in HIV positive male and female patients (five of them of African origin). Midazolam metrics were obtained upon low-dosed, semi-simultaneous oral (2 mg in studies A – D and 1.5 mg in study E) and i.v. (1 mg) administration of the drug (Dormicum®, Hoffmann-La Roche AG, Germany) given as part of the phenotyping cocktail. In study A, oral midazolam was given 1.5 hours after administration of the other cocktail components including i.v. midazolam. In studies B - E, i.v. midazolam was given four hours following the cocktail administration including oral midazolam.(14, 28-30). For the cocktail administrations, the subjects were under standardized fasting conditions. Whereas in study A the phenotyping cocktail was given only once (pilot cocktail study), studies B – E consisted of two periods (Test and Reference) with a co-administration of a potential CYP3A inhibitor (studies B - D) and a strong one (E) in Test period, respectively (see Table 1). In studies C and D, the identity of the investigational products, both not licensed, tested in one period with respect to the effect on CYP activity, is confidential. Data obtained in studies B and E have been published previously.(14, 29) All studies were approved by the ethics committee of the Medical Faculty of the University of Cologne, Germany, and were performed in accordance with the Declaration of Helsinki, and corresponding European and International guidelines. All participants gave their written informed consent. Study designs, demographics and baseline characteristics of the participants are displayed in Table 1.

Midazolam was quantified by LC-MS/MS as described.(14, 29) Midazolam i.v. clearance and apparent oral clearance were calculated by compartmental population pharmacokinetic

analysis using NONMEM software (V version 1.1, NONMEM Project Group, University of California at San Francisco, 1998).(28)

Concentrations of 4bOH-C and cholesterol to calculate the metabolic ratio of 4bOH-C over cholesterol (C/C) were quantified in plasma samples derived from blood collections performed just before i.v. administration of midazolam in study A and before oral administration of midazolam (together with other oral cocktail compounds) in studies B – E. The respective plasma samples were stored frozen at below -65 °C until analysis. Cholesterol in plasma was analyzed by gas chromatography-flame ionization detection using 5α-cholestane as internal standard.(31) Cholesterol concentration were within the clinical reference range in all studies and respective periods, Test and Reference.

The concentration of the oxysterol 4bOH-C (as trimethylsilyl-derivative) was determined by an highly sensitive and specific isotope dilution methodology (gas chromatography-mass spectrometry) using [26.26.26.27.27.27-$^{2}H_{6}$] 4beta -hydroxycholesterol as internal standard.(5) Authentic 4beta-hydroxycholesterol was delivered by Steraloids, Inc. (Newport RI, USA) and deuterium labelled 4beta-hydroxycholesterol was synthesized as published previously.(32) The respective inter and intra-day precision were 2.1% and 2.7% and inter and intra-day accuracy were 2.9% and 3.3%. The low limit of quantification was 3.0 ng/mL and the linear range was 0.3 – 300 ng/mL. Long-term and freeze-thaw stability was proven.

4bOH-C and 4bOH-C/C were compared to the midazolam metrics by correlation analysis (separately for all studies and periods and jointly for the periods without co-medication in the studies with healthy volunteers A - D). To this end Spearman's rank correlation coefficient was used, and one-sided p-values were calculated since the anticipated direction of relationship, if any, was obvious. Because two healthy volunteers taking part in study B were later recruited for study C or D, respectively, their data derived from study B only were used for the common evaluation of periods without co-medication.

Furthermore, for studies B – E, the interactions between co-medications and cholesterol

phenotyping metrics were evaluated as a bioequivalence problem.(33) For each interaction study, point estimates for the true ratios of respective parameters in the periods without (Reference) and with co-medications (Test), the corresponding 90% confidence intervals (CI) and intra-subject coefficients of variations (CV) were calculated. An interaction was assumed to be absent when both the point estimate and the 90% CI were entirely within the bioequivalence boundaries of 80–125% of the Reference values. Genotyping for determination of the most frequent single nucleotide polymorphism *CYP3A5*3* has been described previously.(28)

All calculations were done by SPSS (version 14.0, SPSS Inc., Chicago, IL, USA.) and WinNonlin™, Version 2.1 (Pharsight Corp., Palo Alto, CA, USA.)

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References

(1) Fuhr, U., Jetter, A. & Kirchheiner, J. Appropriate phenotyping procedures for drug metabolizing enzymes and transporters in humans and their simultaneous use in the "cocktail" approach. *Clinical pharmacology and therapeutics* **81**, 270-83 (2007).

(2) Streetman, D.S., Bertino, J.S., Jr. & Nafziger, A.N. Phenotyping of drug-metabolizing enzymes in adults: a review of in-vivo cytochrome P450 phenotyping probes. *Pharmacogenetics* **10**, 187-216 (2000).

(3) Galteau, M.M. & Shamsa, F. Urinary 6beta-hydroxycortisol: a validated test for evaluating drug induction or drug inhibition mediated through CYP3A in humans and in animals. *European journal of clinical pharmacology* **59**, 713-33 (2003).

(4) Kovacs, S.J., Martin, D.E., Everitt, D.E., Patterson, S.D. & Jorkasky, D.K. Urinary excretion of 6 beta-hydroxycortisol as an in vivo marker for CYP3A induction: applications and recommendations. *Clinical pharmacology and therapeutics* **63**, 617-22 (1998).

(5) Bodin, K. *et al.* Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4. *The Journal of biological chemistry* **276**, 38685-9 (2001).

(6) Josephson, F. *et al.* CYP3A induction and inhibition by different antiretroviral regimens reflected by changes in plasma 4beta-hydroxycholesterol levels. *European journal of clinical pharmacology* **64**, 775-81 (2008).

(7) Marschall, H.U. *et al.* Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans. *Gastroenterology* **129**, 476-85 (2005).

(8) Niemi, M. *et al.* Effect of SLCO1B1 polymorphism on induction of CYP3A4 by rifampicin. *Pharmacogenetics and genomics* **16**, 565-8 (2006).

(9) Diczfalusy, U. *et al.* 4Beta-hydroxycholesterol is a new endogenous CYP3A marker: relationship to CYP3A5 genotype, quinine 3-hydroxylation and sex in Koreans, Swedes and Tanzanians. *Pharmacogenetics and genomics* **18**, 201-8 (2008).

(10) Wang, Y.H., Jones, D.R. & Hall, S.D. Differential mechanism-based inhibition of CYP3A4 and CYP3A5 by verapamil. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 664-71 (2005).

(11) Mirghani, R.A., Ericsson, O., Tybring, G., Gustafsson, L.L. & Bertilsson, L. Quinine 3-hydroxylation as a biomarker reaction for the activity of CYP3A4 in man. *European journal of clinical pharmacology* **59**, 23-8 (2003).

(12) Bodin, K. *et al.* Metabolism of 4 beta -hydroxycholesterol in humans. *The Journal of biological chemistry* **277**, 31534-40 (2002).

(13) Gorski, J.C., Jones, D.R., Haehner-Daniels, B.D., Hamman, M.A., O'Mara, E.M., Jr. & Hall, S.D. The contribution of intestinal and hepatic CYP3A to the interaction between midazolam and clarithromycin. *Clinical pharmacology and therapeutics* **64**, 133-43 (1998).

(14) Tomalik-Scharte, D. *et al.* Effect of propiverine on cytochrome P450 enzymes: a cocktail interaction study in healthy volunteers. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 1859-66 (2005).

(15) Kinirons, M.T. *et al.* Failure of erythromycin breath test to correlate with midazolam clearance as a probe of cytochrome P4503A. *Clinical pharmacology and therapeutics* **66**, 224-31 (1999).

(16) Masica, A.L., Mayo, G. & Wilkinson, G.R. In vivo comparisons of constitutive cytochrome P450 3A activity assessed by alprazolam, triazolam, and midazolam. *Clinical pharmacology and therapeutics* **76**, 341-9 (2004).

(17) Benet, L.Z. There are no useful CYP3A probes that quantitatively predict the in vivo kinetics of other CYP3A substrates and no expectation that one will be found. *Molecular interventions* **5**, 79-83 (2005).
(18) Kharasch, E.D., Thummel, K.E. & Watkins, P.B. CYP3A probes can quantitatively predict the in vivo kinetics of other CYP3A substrates and can accurately assess CYP3A induction and inhibition. *Molecular interventions* **5**, 151-3 (2005).
(19) Jochemsen, R., van Rijn, P.A., Hazelzet, T.G., van Boxtel, C.J. & Breimer, D.D. Comparative pharmacokinetics of midazolam and loprazolam in healthy subjects after oral administration. *Biopharmaceutics & drug disposition* **7**, 53-61 (1986).
(20) Diczfalusy, U., Kanebratt, K.P., Bredberg, E., Andersson, T.B., Bottiger, Y. & Bertilsson, L. 4beta-hydroxycholesterol as an endogenous marker for CYP3A4/5 activity. Stability and half-life of elimination after induction with rifampicin. *British journal of clinical pharmacology* **67**, 38-43 (2009).
(21) Klotz, U. & Ziegler, G. Physiologic and temporal variation in hepatic elimination of midazolam. *Clinical pharmacology and therapeutics* **32**, 107-12 (1982).
(22) Klotz, U. & Reimann, I.W. Chronopharmacokinetic study with prolonged infusion of midazolam. *Clinical pharmacokinetics* **9**, 469-74 (1984).
(23) Kharasch, E., Bedynek, P., Park, S., Whittington, D., Walker, A. & Hoffer, C. Mechanism of Ritonavir Changes in Methadone Pharmacokinetics and Pharmacodynamics: I. Evidence Against CYP3A Mediation of Methadone Clearance. *Clinical pharmacology and therapeutics*, (2008).
(24) Kharasch, E., Bedynek, P., Walker, A., Whittington, D. & Hoffer, C. Mechanism of Ritonavir Changes in Methadone Pharmacokinetics and Pharmacodynamics: II. Ritonavir Effects on CYP3A and P-Glycoprotein Activities. *Clinical pharmacology and therapeutics*, (2008).
(25) Walter, R., Ullmann, C., Thummler, D. & Siegmund, W. Influence of propiverine on hepatic microsomal cytochrome p450 enzymes in male rats. *Drug metabolism and disposition: the biological fate of chemicals* **31**, 714-7 (2003).
(26) Wide, K., Larsson, H., Bertilsson, L. & Diczfalusy, U. Time course of the increase in 4beta-hydroxycholesterol concentration during carbamazepine treatment of paediatric patients with epilepsy. *British journal of clinical pharmacology* **65**, 708-15 (2008).
(27) Emoto, C. & Iwasaki, K. Enzymatic characteristics of CYP3A5 and CYP3A4: a comparison of in vitro kinetic and drug-drug interaction patterns. *Xenobiotica; the fate of foreign compounds in biological systems* **36**, 219-33 (2006).
(28) Tomalik-Scharte, D. *et al.* No role for the CYP3A5*3 polymorphism in intestinal and hepatic metabolism of midazolam. *European journal of clinical pharmacology* **64**, 1033-5 (2008).
(29) Wyen, C. *et al.* Effect of an antiretroviral regimen containing ritonavir boosted lopinavir on intestinal and hepatic CYP3A, CYP2D6 and P-glycoprotein in HIV-infected patients. *Clinical pharmacology and therapeutics* **84**, 75-82 (2008).
(30) Klaassen, T. *et al.* Assessment of urinary mephenytoin metrics to phenotype for CYP2C19 and CYP2B6 activity. *European journal of clinical pharmacology* **64**, 387-98 (2008).
(31) Lutjohann, D. *et al.* High doses of simvastatin, pravastatin, and cholesterol reduce brain cholesterol synthesis in guinea pigs. *Steroids* **69**, 431-8 (2004).
(32) Breuer, O. Identification and quantitation of cholest-5-ene-3 beta,4 beta-diol in rat liver and human plasma. *Journal of lipid research* **36**, 2275-81 (1995).
(33) Steinijans, V.W., Hartmann, M., Huber, R. & Radtke, H.W. Lack of pharmacokinetic interaction as an equivalence problem. *International journal of clinical pharmacology, therapy, and toxicology* **29**, 323-8 (1991).

Figure legends

Figure 1
Correlation between 4bOH-C (left) and 4bOH-C/C (right) in plasma and i.v. clearance of midazolam; pooled data from 50 healthy volunteers (studies A - D) in the period without co-medication (Reference); Spearman's rank correlation coefficient = 0.239 (p = 0.048) and 0.348 (p = 0.007), respectively, for 4bOH-C and 4bOH-C/C; 4bOH-C, 4beta-hydroxycholesterol in plasma; 4bOH-C/C, molar ratio of 4beta-hydroxycholesterol over cholesterol in plasma;

Figure 2
Effect of ritonavir boosted lopinavir (left) and propiverine (right) on cholesterol (open symbols) and midazolam (closed symbols) CYP3A metrics; whiskers represent 90% confidence intervals; 4bOH-C, 4beta-hydroxycholesterol in plasma; 4bOH-C/C, molar ratio of 4beta-hydroxycholesterol over cholesterol in plasma; M-Cl, i.v. clearance of midazolam; M-Cl/F, apparent oral clearance of midazolam

Table 1 Design of studies A – E and the respective demographics of the study populations

Study	Design	Number of subjects available for the evaluation	Age [years]	BMI [kg/m^2]
A	single period pilot cocktail study;	n=8[a] healthy male Caucasian volunteers	29 (25 - 36)	24.0 (21.2 – 26.4)
B	open, randomized, cross-over study (Test and Reference period) with chronic (one week) co-administration of propiverine 15 mg b.i.d. (Test) or placebo (Reference); cocktail administration: together with the morning dose of propiverine (Mictonorm®, APOGEPHA Arzneimittel GmbH, Germany) or placebo on day 7	n=16 healthy male Caucasian volunteers	29 (23 - 42)	23.5 (21.5 – 26.6)
C	open, randomized, cross-over study (Test and Reference period) with co-administration of an investigational drug (Test) or placebo (Reference) given as single dose on two consecutive days; cocktail administration: two hours after administration of the investigational drug or placebo on day 2	n=12 (11)[b] healthy male Caucasian volunteers	38 (24 - 49)	24.5 (20.0 – 29.3)
D	double-blind, randomized, cross-over study (Test and Reference period) with chronic (one week) co-administration of an investigational drug given once a day (Test) or placebo (Reference); cocktail administration: together with administration of the investigational drug or placebo on day 7	n=16 (15)[b] healthy male Caucasian volunteers	29 (21 - 43)	24.1 (20.4 – 27.8)
E	prospective, two periods, open, fixed sequence study prior to (Reference period) and with chronic, i.e. at least 14 days co-administration of ritonavir (100mg) boosted lopinavir (400mg) (Kaletra, Abbott, Wiesbaden, Germany) b.i.d. with zidovudine and lamivudine, respectively, 300mg and 150mg, given b.i.d. (Test period); cocktail administration: together with antiretroviral treatment	n=27[c] HIV positive male and female Caucasian and African origin (n=6) patients	39 (25 - 60)	23.6 (16.4 – 36.7)

BMI, body mass index; age and BMI are given as mean and (range);
[a]Ten subjects participated in this study, however data from eight subjects only were available for the present evaluation. [b]One subject from this study already participated in study B. [c]28 subjects participated in this study, however, data from 27 subjects only were available for the present evaluation.

Table 2 Mean values and (standard deviations) of both cholesterol based metrics 4bOH-C and 4bOH-C/C and midazolam metrics in all studies. For interaction studies B – D, the results are given separately for Reference and Test (=with co-medication) period

Study		Cholesterol phenotyping parameters		Midazolam phenotyping parameters	
		4bOH-C [ng/mL]	4bOH-C/C x10^4 [-]	M Cl [L/h]	M Cl/F [L/h]
A	-	20.45 (5.86)	0.12 (0.04)	22.43 (3.56)	37.23 (7.51)
B	Reference	23.87 (4.99)	0.14 (0.03)	26.97 (5.32)	81.99 (18.44)
	Test	28.70 (7.29)	0.18 (0.03)	24.45 (5.36)	55.86 (15.05)
C	Reference	22.81 (9.13)	0.13 (0.05)	17.02 (4.11)	55.94 (23.58)
	Test	23.74 (8.08)	0.13 (0.04)	17.44 (3.85)	56.85 (26.70)
D	Reference	18.75 (5.28)	0.11 (0.03)	20.97 (5.74)	71.27 (27.11)
	Test	18.10 (5.47)	0.10 (0.02)	20.58 (5.28)	65.60 (28.24)
E	Reference	42.72 (33.70)	0.28 (0.20)	25.17 (6.42)	44.42 (21.24)
	Test	31.16 (23.11)	0.19 (0.12)	6.51 (2.91)	8.49 (4.04)

4bOH-C, 4beta-hydroxycholesterol in plasma given in ng/mL, 4bOH-C/C,molar ratio of 4beta-hydroxycholesterol over cholesterol in plasma; Ref, Reference period; Test, Test period with co-medication of potential inhibitor; M-Cl, i.v. clearance of midazolam; M-Cl/F, apparent oral clearance of midazolam

Table 3 Correlation of 4beta-hydroxycholesterol concentrations in plasma to midazolam based CYP3A metrics

Comparison	all healthy vol. (Ref)	Study A	Study B (Ref)	Study B (Test)	Study C (Ref)	Study C (Test)	Study D (Ref)	Study D (Test)	Study E (Ref)	Study E (Test)
n	50[a]	8	16	16	12	12	16	16	27	27
r_s to M-Cl	**0.239**	-0.071	0.235	-0.168	-0.098	0.070	**0.559**	0.216	0.079	-0.131
p single-sided	**0.048**	0.433	0.190	0.267	0.381	0.415	**0.012**	0.210	0.347	0.258
r_s to M Cl/F	**0.267**	0.048	0.082	-0.338	0.231	0.273	**0.638**	0.132	0.098	-0.143
p single-sided	**0.031**	0.455	0.381	0.100	0.235	0.196	**0.004**	0.313	0.313	0.239
Ratio Test/Reference for 4bOH-C (90% Cl)	n. a.	n. a.	1.190 (1.083–1.308)		1.068 (0.988–1.154)		0.970 (0.863-1.089)		0.730 (0.529-1.008)	
Intrasubject CV (%)			15.2		10.5		18.9		27.0	

Ref, Reference period; Test, Test period with co-medication of potential inhibitor; M-Cl, i.v. clearance of midazolam; M-Cl/F, apparent oral clearance of midazolam; 4bOH-C, 4beta-hydroxycholesterol; CI, confidence interval; intrasubject CV, intrasubject coefficient of variation; r_s, Spearman's rank correlation coefficient; statistically significant ($p<0.05$, one-sided test) results are printed in bold.

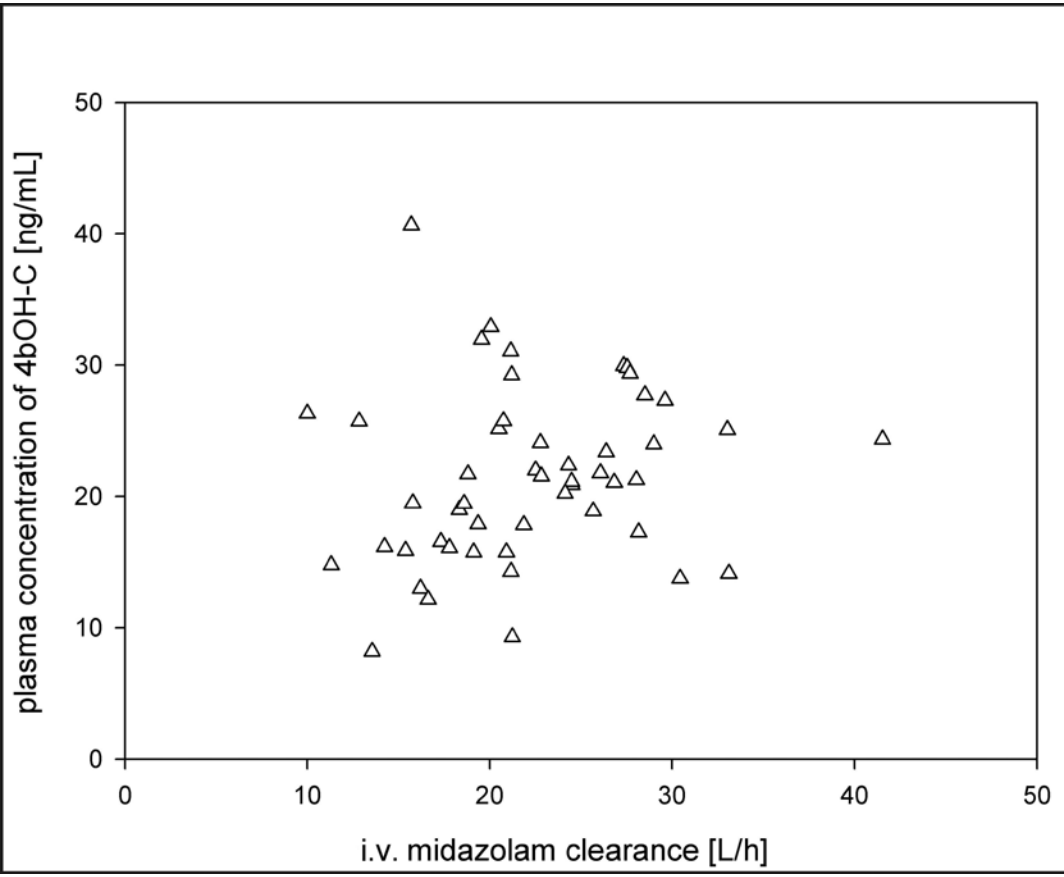
[a]One subject from each study C and D previously participated in study B, so that, for these subjects, the data derived from study B only were used for the common evaluation of periods without co-medication in healthy volunteers

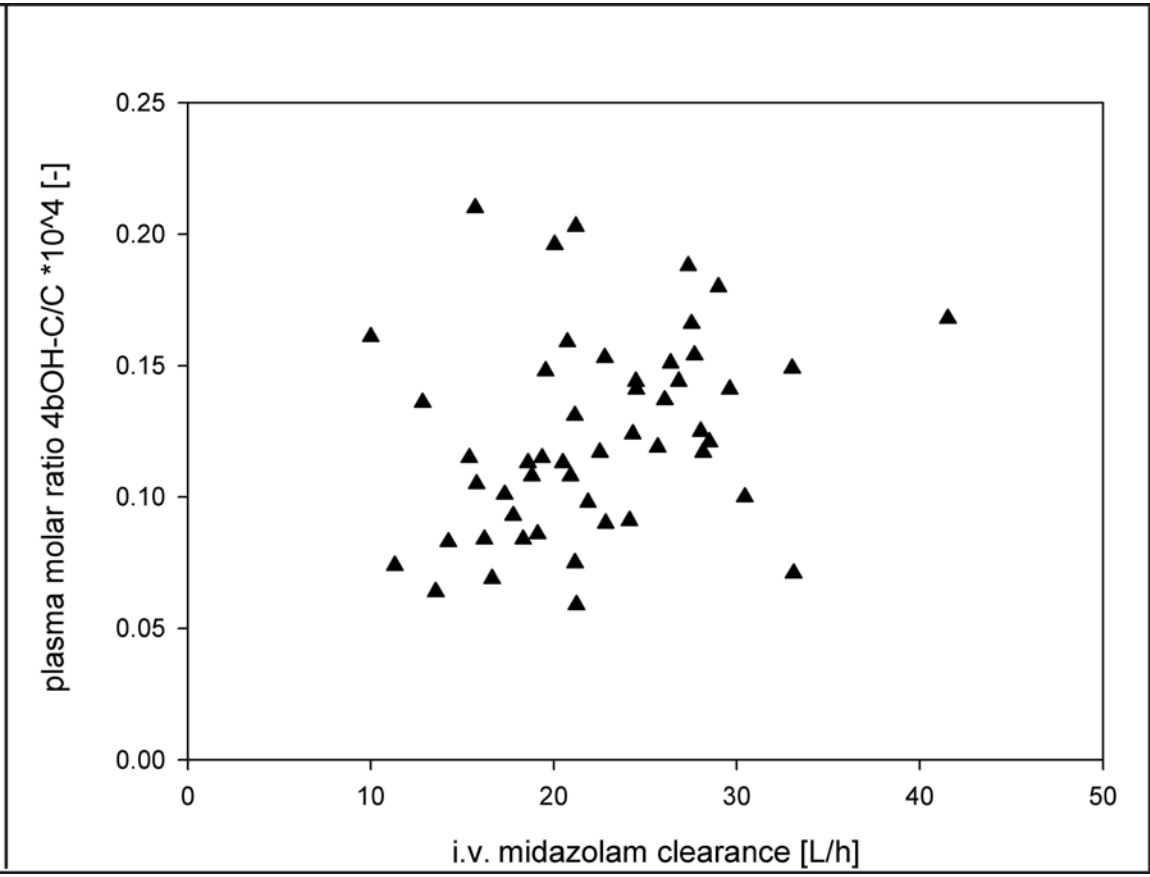
Table 4 Correlation of 4beta-hydroxycholesterol/cholesterol concentration ratios in plasma to midazolam based CYP3A metrics

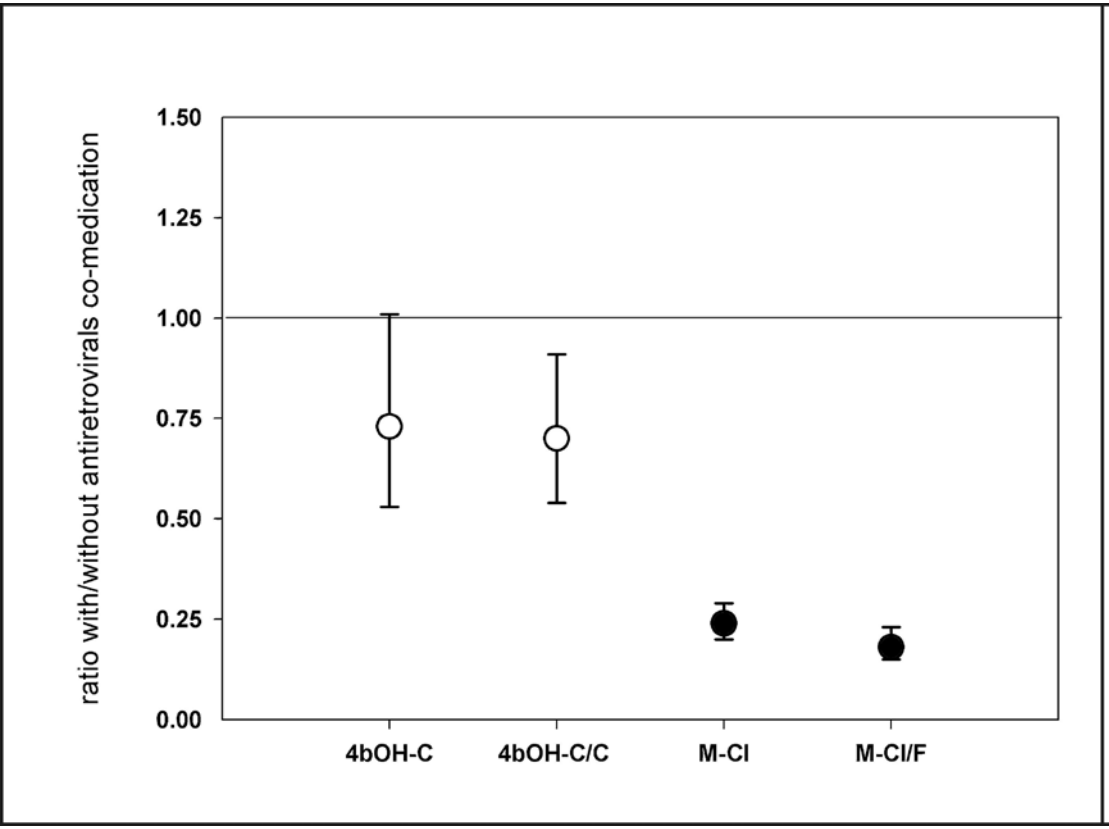
Comparison	all healthy vol. (Ref)	Study A	Study B (Ref)	Study B (Test)	Study C (Ref)	Study C (Test)	Study D (Ref)	Study D (Test)	Study E (Ref)	Study E (Test)
n	50[a]	8	16	16	12	12	16	16	27	27
r_s to M-Cl	**0.348**	0.286	**0.476**	0.353	-0.189	0.147	**0.506**	0.297	0.219	-0.254
p single-sided	**0.007**	0.246	**0.031**	0.090	0.278	0.324	**0.023**	0.132	0.137	0.101
r_s to M Cl/F	**0.353**	0.310	0.332	0.288	0.133	0.224	**0.776**	0.394	0.168	-0.255
p single-sided	**0.006**	0.228	0.104	0.139	0.340	0.242	**0.000**	0.065	0.200	0.100
Ratio Test/Reference for 4bOH-C/C (90% Cl) intrasubject CV (%)	n. a.	n. a.	1.279 (1.162-1.409) 15.5		1.058 (0.985-1.136) 9.7		0.994 (0.912-1.084) 13.9		0.702 (0.540-0.912) 26.5	

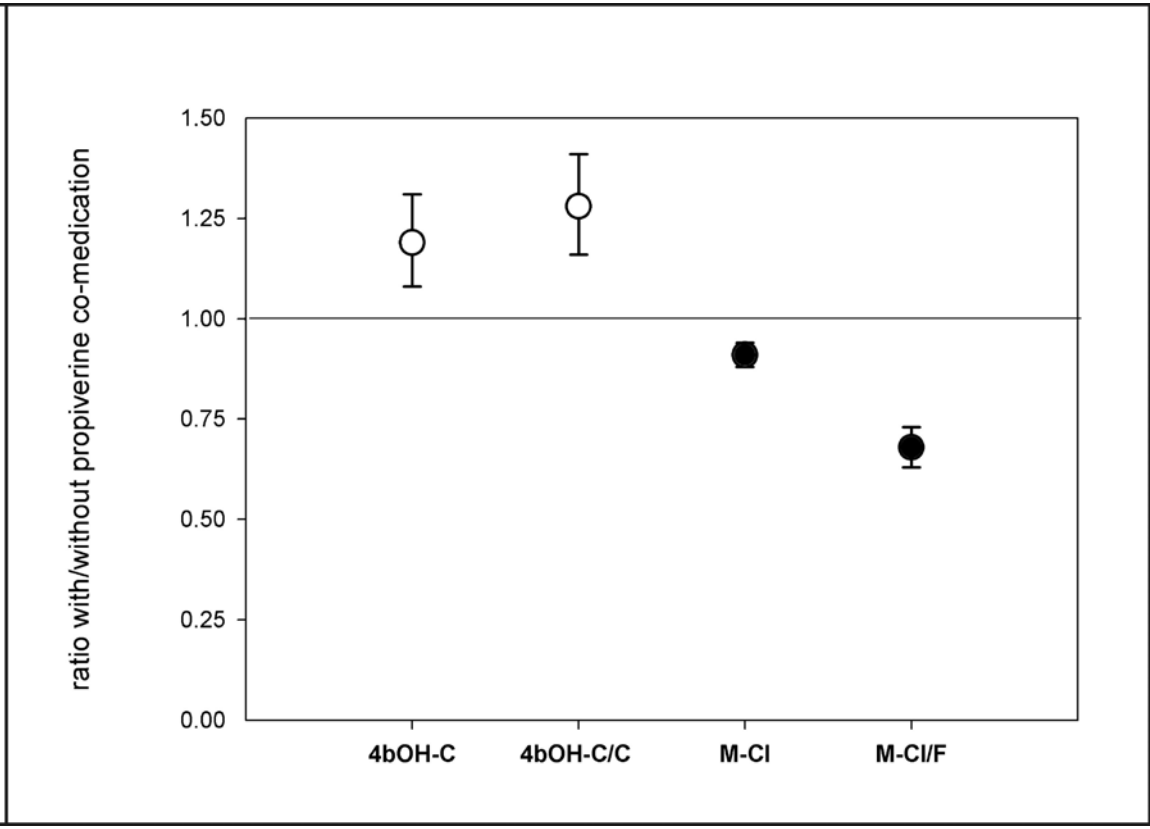
Ref, Reference period; Test, Test period with co-medication of potential inhibitor; M-Cl, i.v. clearance of midazolam; M-Cl/F, apparent oral clearance of midazolam; 4bOH-C/C,molar ratio of 4beta-hydroxycholesterol over cholesterol; CI, confidence interval; intrasubject CV, intrasubject coefficient of variation; r_s, Spearman's rank correlation coefficient; statistically significant ($p<0.05$, one-sided test) results are printed in bold.

[a]One subject from each study C and D previously participated in study B, so that, for these subjects, the data derived from study B only were used for the common evaluation of periods without co-medication in healthy volunteers


plasma concentration of 4bOH-C [ng/mL]
0
10
20
30
40
50
i.v. midazolam clearance [L/h]


plasma molar ratio 4bOH-C/C *10^4 [-]
0.00
0.05
0.10
0.15
0.20
0.25
0
10
20
30
40
50
i.v. midazolam clearance [L/h]


ratio with/without antiretrovirals co-medication
1.50
1.25
1.00
0.75
0.50
0.25
0.00
4bOH-C
4bOH-C/C
M-Cl
M-Cl/F


ratio with/without propiverine co-medication
1.50
1.25
1.00
0.75
0.50
0.25
0.00
4bOH-C
4bOH-C/C
M-Cl
M-Cl/F