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Simultaneous assessment of CYP3A4 and CYP1A2 activity *in vivo* with alprazolam and caffeine

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Alprazolam (ALP) and caffeine (CAF) were suggested as probe drugs for the activities of CYP3A4 and CYP1A2, respectively. We investigated the disposition of oral ALP (1 mg) and CAF (100 mg) in 17 normal volunteers to establish and validate a procedure for the simultaneous assessment of CYP3A4 and CYP1A2 enzyme activity. Nine received ALP alone, ALP and CAF and CAF alone in an open three-way crossover study to test for pharmacokinetic interaction. Four received ALP after a 2-day pretreatment with ketoconazole, an inhibitor of CYP3A4, and four normal volunteers received ALP after 4 days of rifampin, an inducer of CYP3A4. AUC values of ALP and CAF administered alone were not different from AUC values when both drugs were administered combined, indicating that there is no metabolic interaction. The ratio formed of paraxanthine and CAF correlated significantly with systemic CAF clearance at 3, 4, 6, 8, 10 and 24 h. There was a strong correlation between AUC values of ALP and CAF and the plasma concentration obtained 6, 8, 10, or 24 h after ingestion of the drug. Ketoconazole and rifampin pretreatment significantly changed AUC values of ALP (mean AUC values in µg/l h: ALP = 242.2, ALP + ketoconazole = 426.2, ALP + rifampin = 28.4, ANOVA $F = 17.7$, $P < 0.001$). We conclude that ALP and CAF can be administered simultaneously for the assessment of CYP activity. Plasma concentrations 6, 8, 10, and 24 h after drug ingestion reflect AUC of ALP and CAF and therefore in-vivo CYP3A4 and CYP1A2 activity, respectively. Pharmacogenetics 9:725–734 © 1999 Lippincott Williams & Wilkins

Keywords: cytochrome P450, CYP3A4, CYP1A2, metabolic activity

Introduction

Many researchers use caffeine as a probe drug to determine the metabolic rate of CYP1A2. Demethylation of this trimethylxanthine accounts for the major part (> 90%) of caffeine (CAF) metabolism in humans (Cornish & Christman, 1957; Callahan *et al.*, 1982; Ullrich *et al.*, 1992). Of the three possible demethylation reactions, 84% account for the 3-position resulting in the formation of paraxanthine (Kalow & Tang, 1993). It has been demonstrated that CAF metabolism, and 3-demethylation in particular, is mainly mediated by CYP1A2 (Butler *et al.*, 1989; Gu *et al.*, 1992; Tassaneeyakul *et al.*, 1992, 1994). CYP1A2 also catalyses 1- and 7-demethylation of CAF (Tassaneeyakul *et al.*, 1992; Kalow & Tang, 1993; Tassaneeyakul *et al.*, 1994; Ha *et al.*, 1996). Some investigators use metabolic ratios calculated from CAF and metabolite concentrations in collected urine as a measure of CYP activity. However, the validity of this method is controversial (Fuhr & Rost, 1994; Miners & Birkett, 1993; Rost & Roots, 1994; Miners & Birkett, 1996). Factors affecting the metabolic ratio of urinary metabolites are differences in urinary flow, the rate of excretion by the kidney and the formation of secondary metabolites by other enzymes (Kalow & Tang, 1993). In addition, metabolic ratios are not linearly correlated with the clearance of an investigated drug. The caveats of metabolic ratios in assessing metabolic enzyme activity have comprehensively been reviewed recently (Tucker *et al.*, 1998).

With respect to CYP3A4, several index reactions for in-vivo use have been suggested such as erythromycin *N*-demethylation (Watkins *et al.*, 1989), dapsone *N*-hydroxylation (May *et al.*, 1994), dextromethorphan *N*-demethylation (Gorski *et al.*, 1994), alfentanil demethylation (Krivoruk *et al.*, 1994), cortisol 6β-hydroxylation (Joellenbeck *et al.*, 1992), lidocaine

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(Oellerich *et al.*, 1990), nifedipine (Schellens *et al.*, 1991) and midazolam (Thummel *et al.*, 1994). However, correlation of CYP3A4 activity, as predicted by the different probes, is poor or absent (Kinirons *et al.*, 1993; Krivoruk *et al.*, 1994; Stein *et al.*, 1996). With the exception of the erythromycin breath test, substrate and metabolite concentrations of these compounds are mostly measured in urine. Many factors other than metabolism in the liver influence urinary recovery. It is likely that these factors are of considerable difference between the listed compounds. Nifedipine has been administered simultaneously with other probes to assess the differential influence of induction and inhibition with measurement of plasma concentrations (Schellens *et al.*, 1989). Reflection of CYP3A4 activity by erythromycin is compromised by its inhibitory and inducing properties on CYP3A4. Recently, alprazolam (ALP) has been suggested as a CYP3A4 probe *in vitro* (von Moltke *et al.*, 1993; Schmider *et al.*, 1996) as well as *in vivo* (Greenblatt *et al.*, 1993; Greene *et al.*, 1995; Yasui *et al.*, 1996). Oral bioavailability of ALP is almost complete at 80–100% (Greenblatt & Wright, 1993) and it is a low extraction drug (Greenblatt *et al.*, 1998), indicating that presystemic extraction plays only a minimal role compared with other CYP3A4 substrates such as midazolam and that liver blood flow is a minor determinant of the metabolic rate. Low impact of presystemic extraction is desirable if the probe drug is aimed to primarily reflect liver enzyme acitivity. Clearance of ALP is entirely dependent on CYP3A4 mediated 1- and 4-hydroxylation (von Moltke *et al.*, 1993; Yasui *et al.*, 1996; Venkatakrishnan *et al.*, 1998). ALP clearance is largely induced by the CYP3A4 inducer carbamazepine (Furukori *et al.*, 1998) and inhibited by the CYP3A4 inhibitor itraconazole (Yasui *et al.*, 1998). Thus, determination of ALP disposition reflects individual CYP3A4 catalytic activity. We investigated the disposition of oral ALP and CAF in volunteers to establish and validate a simultaneous CYP3A4 and CYP1A2 phenotyping procedure.

Methods

DESIGN

The protocol was reviewed and approved by the local ethics committee. The study comprised 16 men and 1 woman who were all healthy normal volunteers (age range 22–55 years), after each gave their written informed consent. All were ambulatory nonsmoking adults, with no evidence of medical disease or drug abuse and taking no other medication. Pharmacokinetic interaction between ALP and CAF was investigated in an open three-way crossover study with nine normal volunteers. Individuals received each of the following three treatment conditions: (i) 1 mg ALP (Tafil®, Upjohn, Germany), (ii) 1 mg ALP and 100 mg CAF (Coffeinum purum®, Berlin-Chemie, Germany) simultaneously, (iii) 100 mg CAF. Three normal volunteers started with concurrent treatment conditions (i), (ii), and (iii), respectively. At least 7 days elapsed between trials. The effect of ketoconazole (KET), an inhibitor of CYP3A4, and rifampin (RIF), an inducer of CYP3A4, on ALP disposition was investigated in eight normal volunteers. Four normal volunteers received ALP after a 2-day pretreatment with 200 mg b.i.d. KET and four normal volunteers after 4 days of 450 mg once daily RIF.

POWER ANALYSIS

A power analysis was performed before the study which was reviewed by a biostatistician. Power of the interaction study should be sufficient to identify a difference of at least 25%. With a sample size of seven individuals, a 25% difference in ALP clearance is detected with a power of 80%, assuming a standard deviation of ALP clearance of 0.17 ml min^{-1} kg^{-1} (Blyden *et al.*, 1988). With a sample size of eight individuals, a difference in mean CAF clearance of 0.375 (25%) is detected with a power of 80% based on the population parameters given by Benet *et al.* (1996).

PROCEDURES

Individuals receiving KET and RIF pretreatment reported 2 and 4 days, respectively, before ALP administration to the outpatient Clinical Pharmacology Research Unit. They received the pretreatment medication and the first dose was administered under observation of the investigator. All individuals were instructed to refrain from CAF containing beverages and food as well as from grapefruits and grapefruit juice for at least 2 days before taking the study medication and to fast on the morning of the study day until 12.00 h. On the study day individuals entered the Research Unit at 08.00 h, where they received ALP, ALP and CAF or CAF. They remained in the Research Unit for the next 10 h. Plasma samples were collected at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, and 24 h. Samples were centrifuged, and the plasma was separated and frozen until the time of assay.

ANALYSIS OF PLASMA CONCENTRATIONS

ALP, CAF, and paraxanthine concentrations were determined with a Shimadzu high-performance liquid chromatography (HPLC) system consisting of a LC–9A HPLC pump, an SLC-6B autosampler, CTO–6A column oven, and an SPD-6AV ultraviolet detector. Chemical reagents and drug entities were analytical grade and purchased from commercial sources.

Analysis of CAF plasma concentration

Paraxanthine and CAF were quantified in plasma as described by Grant *et al.* (1984) but using 10%

methanol in the mobile phase. The lower limit of quantification was 0.02 mg/l for paraxanthine and 0.03 mg/l for CAF. Interassay coefficients of variation were 8.8% for paraxanthine and 8.0% for CAF at a concentration of 5 mg/l for both compounds (n = 20).

Analysis of alprazolam plasma concentrations

1 ml of plasma was extracted after addition of 50 µl saturated sodium bicarbonate and 10 µl of a 100 mg/l oxazepam solution (internal standard) in 7 ml dichloromethane. The organic layer was evaporated to dryness under a gentle stream of nitrogen at 40 °C and redissolved in 100 µl mobile phase. 50 µl were injected onto a 150 × 3 mm Inertsil ODS-3 5 µm column (VDS Optilab, Montabaur, Germany) at a flow rate of 1 ml/min with a mobile phase consisting of 30% acetonitrile, 70% 50 mM KH_2PO_4 adjusted to a pH of 6. Absorbance was monitored at 222 nm. The lower limit of quantification was 0.5 µg/l and the recovery was 90%. The intraassay and interassay coefficients of variation were 3.73% (n = 10) and 5.50% (n = 6), respectively, at a concentration of 7.5 µg/l.

ANALYSIS OF DATA

The slope (λ_Z) of the terminal log-linear phase of each plasma concentration–time curve was determined by linear regression analysis. This slope was used to calculate the apparent elimination half-life ($t_{1/2-\lambda z}$). Area under the plasma concentration–time curve from time zero until the last detectable concentration was determined by the linear trapezoidal method. The residual area was added to this area, extrapolated to infinity, calculated as the final concentration divided by λ_Z, yielding the total area under the plasma concentration–time curve ($AUC_{()-\infty}$). The peak plasma concentration and the time of peak concentration were taken as measured. Oral clearance was calculated as the administered dose divided by $AUC_{()-\infty}$. The molar metabolic concentration ratio paraxanthine/CAF as an index of CYP1A2 activity was calculated according to Rostami-Hodjegan *et al.* (1996).

Statistical significance of differences of $AUC_{()-\infty}$ were determined by Student's t-test for paired samples or by analysis of variance and Student–Newman–Keuls test as a post hoc procedure where appropriate. Correlation of single point plasma concentrations with $AUC_{()-\infty}$ were determined with Pearson product–moment linear regression analysis. Correlation of paraxanthine/CAF ratios with oral clearance corrected for body weight ($Cl_{oral/kg}$) was evaluated by nonparametric Spearman rank correlation. For all statistical procedures, $P < 0.05$ was considered significant.

Results

Pharmacokinetic parameters of ALP alone and administered in combination with CAF as well as of CAF alone and in combination with ALP were not significantly different (Table 1, Figs 1 and 2). Thus, there was no mutual interference in the disposition of these compounds. Pharmacokinetic parameters of both compounds are in the same range as reported previously (Greenblatt & Wright, 1993; Greenblatt *et al.*, 1993; Fuhr & Rost, 1994; Kaplan *et al.*, 1997). There was no correlation between ALP and CAF $AUC_{()-\infty}$ values within the individuals (r = 0.16, P = 0.53).

None of the individuals had detectable plasma ALP concentrations before ALP application. ALP C_{max} ranged from 6–25 µg/l and t_{max} ranged from 0.5–4 h. No significant differences in these parameters were detected if ALP was administered alone or in combination with CAF or after pretreatment with either RIF or KET. Pretreatment with RIF, a known potent inducer of CYP3A4 significantly reduced ALP $AUC_{()-\infty}$ and $t_{1/2-\lambda z}$ and significantly increased $Cl_{oral/kg}$ (Table 2, Fig. 1). Pretreatment with KET, a known potent inhibitor of

Table 1. Pharmacokinetic interaction of alprazolam (ALP) and caffeine (CAF). Differences were statistically not significant (paired Student's t-test) in either group

	Alprazolam pharmacokinetics (n = 9)		*Caffeine pharmacokinetics* (n = 8)	
	ALP	*ALP + CAF*	*CAF*	*CAF + ALP*
C_{max} (µg/l)	15.2 ± 1.5	15.6 ± 1.2	1589 ± 202	1756 ± 153
t_{max} (h after dose)	1.2 ± 0.4	1.3 ± 0.4	1.2 ± 0.4	0.7 ± 0.1
$t_{1/2-\lambda z}$(h)	14.1 ± 1.4	17.2 ± 1.1	5.3 ± 0.6	6.1 ± 1.3
$AUC_{()-\infty}$(µg/lh)	242.2 ± 37.9	260.3 ± 31.3	11105 ± 1589	14090 ± 2297
$Cl_{oral/kg}$(ml/min/kg)	1.1 ± 0.1	0.9 ± 0.1	2.1 ± 0.4	2.3 ± 0.6

All values arithmetic means ± standard error; C_{max}, peak plasma concentration; t_{max}, time to reach C_{max}; $t_{1/2-\lambda z}$, half-life of the elimination phase; $AUC_{()-\infty}$, area under the concentration versus time curve extrapolated to infinity; $Cl_{oral/kg}$, oral clearance corrected for body weight.

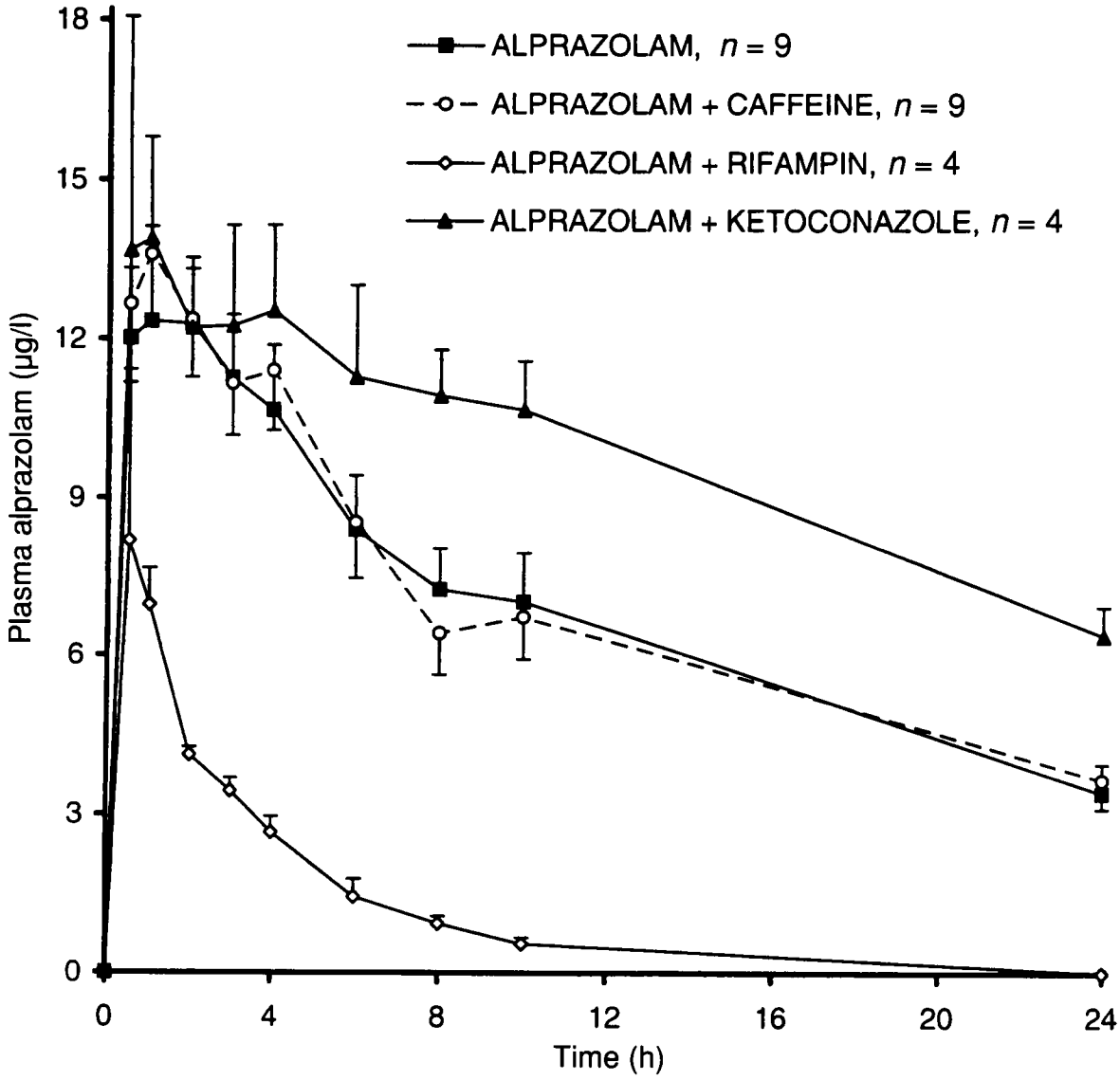

Fig. 1. Mean plasma alprazolam concentrations after 1 mg alprazolam was administered alone, with 100 mg caffeine, after 4 days of 450 mg once daily rifampin pretreatment, and after 2 days of 200 mg b.i.d. ketoconazole pretreatment. Error bars indicate the standard error of the mean.

CYP3A4, significantly increased ALP $AUC_{0-\infty}$ and $t_{1/2-\lambda z}$ and significantly decreased $Cl_{oral/kg}$ (Table 2, Fig. 1).

One individual did not refrain from CAF on both CAF study days and displayed markedly elevated CAF concentrations before CAF application. This individual was excluded from further analysis. Three additional individuals had detectable but very low plasma CAF concentrations before CAF application. Exclusion of these individuals from further analysis did not change results. Therefore, these individuals were included in the statistical evaluation. CAF C_{max} ranged from 1.1–2.8 mg/l and t_{max} ranged from 0.5–3 h. No significant differences in these parameters were detected if CAF was administered alone or in combination with ALP. Paraxanthine concentrations ranged from 0.24–0.95 mg/l between 4 and 10 h after CAF administration. Significant Spearman rank correlations between the paraxanthine/CAF ratio with $Cl_{oral/kg}$ of CAF was observed for plasma concentrations obtained 3 h ($r_s = 0.64$, $P = 0.02$), 4 h ($r_s = 0.69$, $P = 0.008$), 6 h ($r_s = 0.82$, $P = 0.002$), 8 h ($r_s = 0.67$, $P = 0.01$), 10 h ($r_s = 0.68$, $P = 0.01$), and 24 h ($r_s = 0.66$, $P = 0.04$) after CAF administration.

There was a highly significant and strong correlation of ALP and CAF $AUC_{0-\infty}$ with plasma ALP and CAF concentrations of the elimination phase, respectively (Figs 3 and 4). 6, 8, 10 and 24 h after administration. Pearson product–moment correlation coefficients for

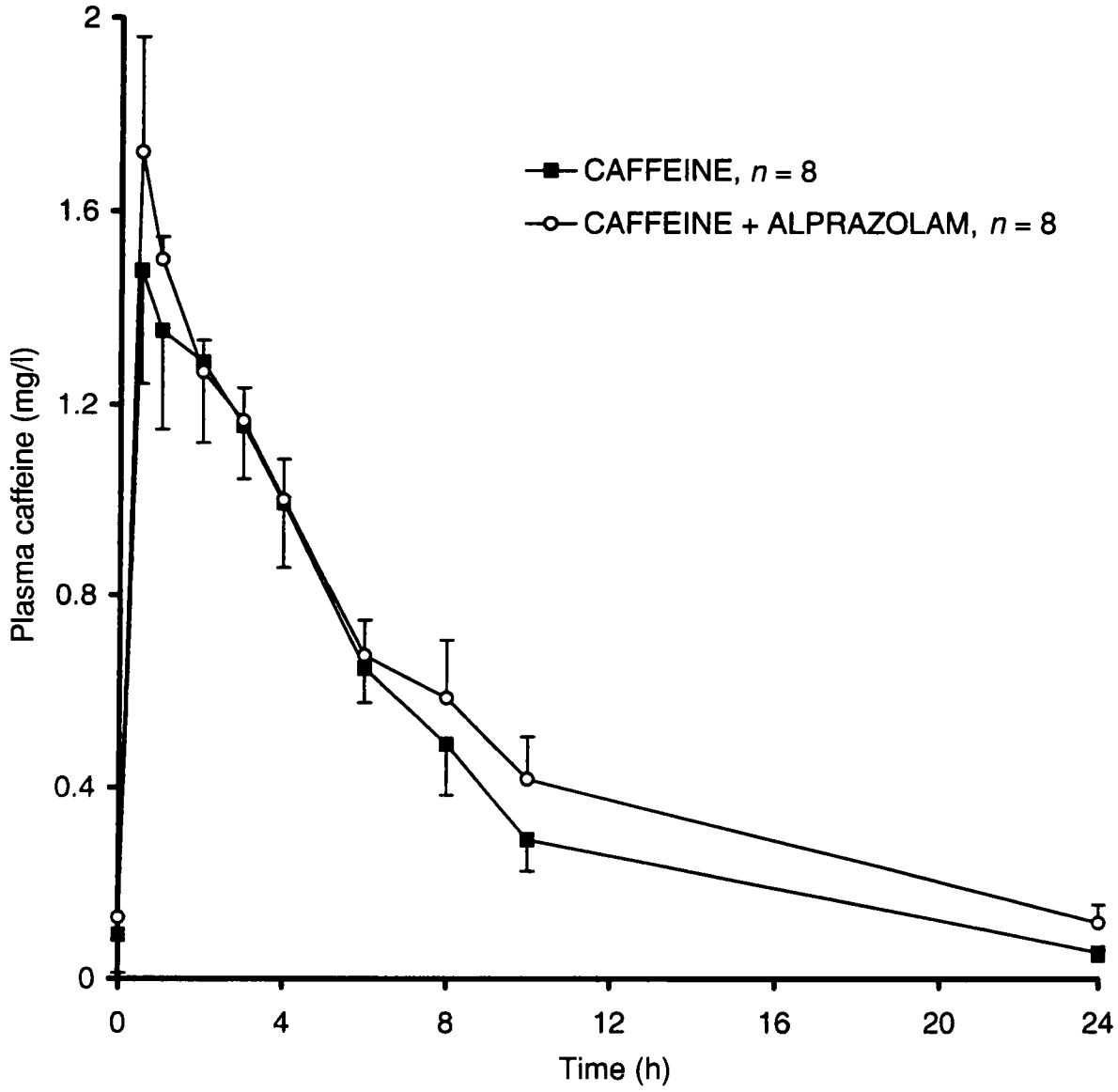

Fig. 2. Mean plasma caffeine concentrations after 100 mg caffeine was administered alone and with 1 mg alprazolam. Error bars indicate the standard error of the mean.

ALP were 0.88, 0.93, 0.96, and 0.99, respectively (P in all cases < 0.001; Table 3). For CAF, the correlation coefficients at the same time points were 0.81, 0.83, 0.84 and 0.81, respectively (P in all cases < 0.001; Table 3). At 24 h, four individuals had no detectable CAF concentration. Oral clearance of ALP (not adjusted for body weight) is estimated by the equation:

$$Cl_{oral}\,[l/h] = \text{dose}\,[mg]/(A \times \text{plasma ALP concentration}_{TIME}\,[\mu g/l] + B) \quad \text{(Eqn 1)}$$

and Cl_{oral} of CAF by the equation:

$$Cl_{oral}\,[l/h] = \text{dose}\,[mg]/(C \times \text{plasma CAF concentration}_{TIME}\,[mg/l] + D) \quad \text{(Eqn 2)}$$

Parameters A, B, C, and D for the respective plasma concentrations at time points 6–24 h (plasma ALP concentration$_{TIME}$, plasma CAF concentration$_{TIME}$) are displayed in Table 3. Equation 2 is applicable only for a test dose of 100 mg because CAF kinetics are known to be nonlinear in the range between 70 and 300 mg (Cheng *et al.*, 1990). ALP kinetics is linear for doses up to 10 mg (Wright *et al.*, 1997).

Discussion

A clinical test procedure for the combined use of alprazolam and caffeine as probes for the activity of CYP3A4

Table 2. Effect of coadministration of pretreatment with ketoconazole (^{pre}-KET) or rifampin (^{pre}-RIF) on pharmacokinetic parameters of alprazolam (ALP)

	ALP (n = 9)	*ALP* + ^{pre} *KET* (n = 4)	*ALP* + ^{pre} *RIF* (n = 4)	*F*-value
C_{max} (μg/l)	15.2 ± 1.5	16.4 ± 2.9	9.7 ± 2.6	2.2 (NS)
t_{max} (h after dose)	1.2 ± 0.4	1.0 ± 0.4	0.9 ± 0.1	0.1 (NS)
$t_{1/2-\lambda z}$(h)	14.1 ± 1.4	20.5 ± 2.2	2.6 ± 0.3	24.2 ($P < 0.0001$)
$AUC_{0-\infty}$(μg/lh)	242.2 ± 37.9	426.2 ± 43.1	28.4 ± 3.4	17.7 ($P < 0.0001$)
$Cl_{oral/kg}$(ml/min/kg)	1.1 ± 0.1	0.6 ± 0.1	8.3 ± 1.6	35.3 ($P < 0.0001$)

All values arithmetic means ± standard error; C_{max}, peak plasma concentration; t_{max}, time to reach C_{max}; $t_{1/2-\lambda z}$, half-life of the elimination phase; $AUC_{0-\infty}$, area under the concentration versus time curve extrapolated to infinity; $Cl_{oral/kg}$, oral clearance; *F*-value, *F*-value of factorial analysis of variance; NS, not significant.

Table 3. Pearson product moment correlation coefficients of alprazolam (ALP) and caffeine (CAF) of plasma concentrations versus total area under the plasma concentration–time curve ($AUC_{0-\infty}$) and derived coefficients for the scaling equations 1 and 2 to extrapolite oral clearance from single concentrations drawn 6 to 24 h after ALP and CAF administration

	Time (h)			
	6	*8*	*10*	*24*
Alprazolam				
Pearson product moment correlation coefficient (r) of ALP_{TIME} concentr. versus ALP $AUC_{0-\infty}$	0.88	0.93	0.96	0.99
Coefficient of determination (r^2)	0.77	0.87	0.92	0.98
Parameter A of equation 1	0.031	0.038	0.038	0.063
Parameter B of equation 1	0.001	–0.007	–0.001	0.028
Caffeine				
Pearson product moment correlation coefficient (r) of CAF_{TIME} concentr. versus CAF $AUC_{0-\infty}$	0.81	0.83	0.84	0.81
Coefficient of determination (r^2)	0.66	0.69	0.71	0.66
Parameter C of equation 2	22.8	16.4	23.2	45.1
Parameter D of equation 2	–1.95	4.32	4.91	9.27

For all correlation coefficients, $P < 0.001$.

and CYP1A2 has been evaluated. ALP is a low extraction compound with a bioavailability ranging from 80–100%. Therefore it is reasonable to assume independence of ALP clearance from first pass metabolism and liver blood flow. Unlike erythromycin, ALP is neither an autoinhibitor of CYP3A4 nor an inducer of its own metabolism. A potential problem would arise if the substrate or its metabolites generated *in vivo* are also substrates, inhibitors, or inducers of the enzyme of interest (Watkins, 1994). Induction by RIF, a CYP3A4 inducer, and inhibition by KET, a CYP3A4 inhibitor of ALP metabolism confirms previous investigators who demonstrated CYP3A4 mediated induction of ALP clearance with carbamazepine (Furukori *et al.*, 1998) and reduction of ALP clearance with the CYP3A4 inhibitors ketoconazole (Greenblatt *et al.*, 1998) and itraconazole (Yasui *et al.*, 1998). Taken together with in-vitro evidence (von Moltke *et al.*, 1993; Schmider *et al.*, 1996), it is obvious that clearance of ALP is primarily if not entirely dependent on CYP3A4 activity. In addition, alprazolam at a dose of 1 mg displays extremely few side-effects, besides sedation, is well tolerated and, if administered overnight, sedation is not of primary concern. Thus, ALP can be considered an excellent probe for CYP3A4 activity.

ALP concentrations of 6–24 h after administration display strong correlations with total AUC values (Table 3). The 10 and 24 h coefficients of determination (r^2) indicate a predictive accuracy of 92% and 98%, respectively (Table 3). Thus the 24 h ALP plasma

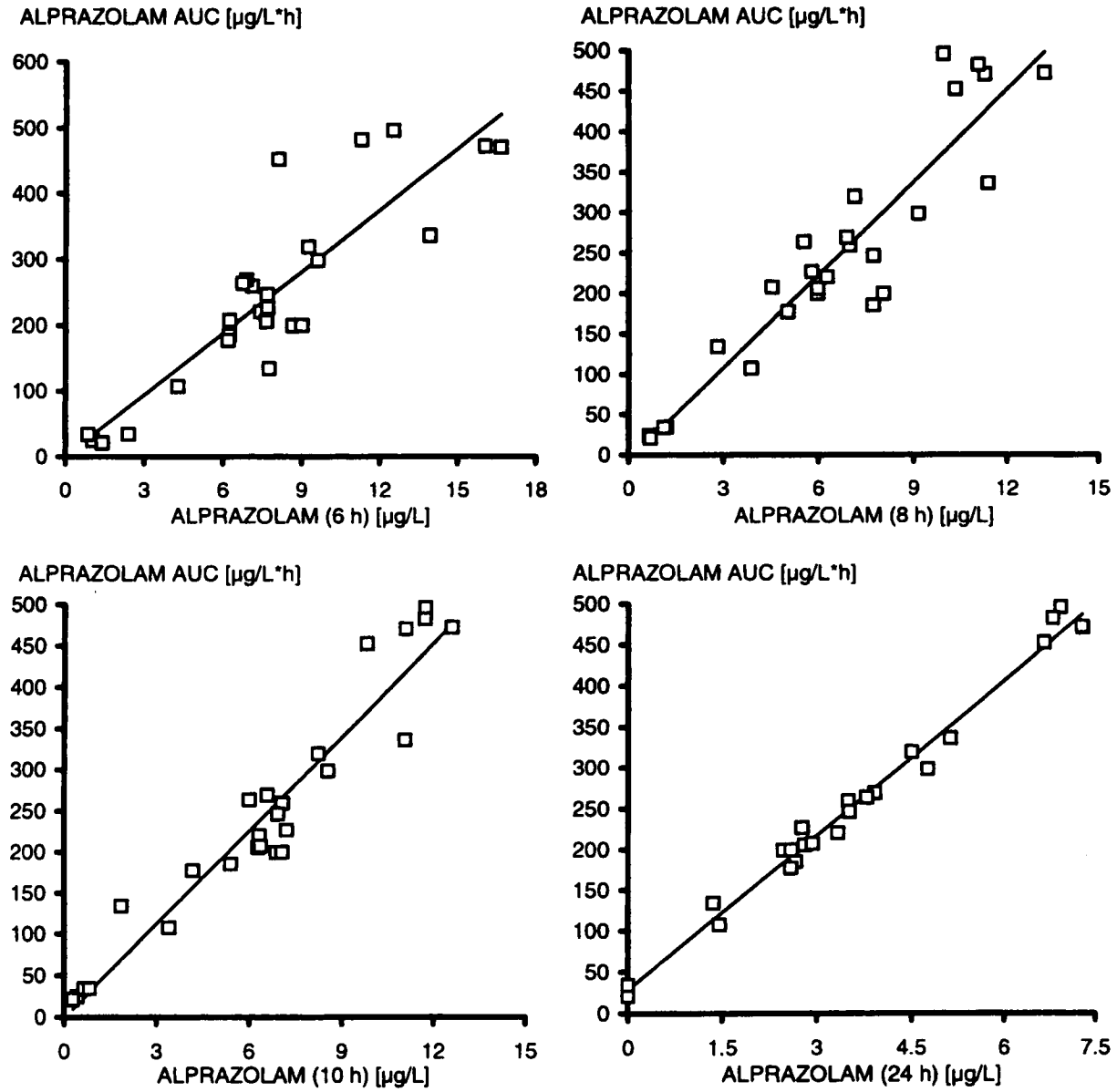

Fig. 3. Relation of plasma alprazolam concentrations 6, 8, 10, and 24 h after drug intake to the total area under the plasma concentration–time curve. Solid lines represent predicted values by linear regression. Correlation coefficients are listed in Table 3.

concentration allows extrapolation of the AUC with the greatest reliability utilizing Equation 1 and the respective coefficients as given in Table 3.

Figure 1 suggests non-linear pharmacokinetics of ALP when concomitantly administered with KET. However, KET was administered the last time with alprazolam and the inhibitory effect of this compound might have worn off over the 24 h time course of ALP concentration monitoring.

Hossain *et al.* (1997) observed increased clearance of ALP in smokers compared to nonsmokers, implying that ALP metabolism is induced in smokers. CYP1A2 is known to be induced by smoke. The authors concluded that CYP1A2 might be involved in ALP metabolism. If there is a significant involvement of CYP1A2 in ALP metabolism, ALP $AUC_{0-\infty}$ is expected to parallel CAF $AUC_{0-\infty}$ within the individuals since CAF is reflecting CYP1A2 activity. However, we did not observe such a correlation, ruling out a significant contribution of CYP1A2 to ALP metabolism. The finding by Hossain *et al.* is also interpretable as evidence that cigarette smoke induces CYP3A4.

As reported previously (Fuhr & Rost, 1994) the ratio of paraxanthine/CAF from 3–24 h after CAF administration reflected systemic CAF clearance. However, a convincing correlation with an r_s greater than 0.8, could

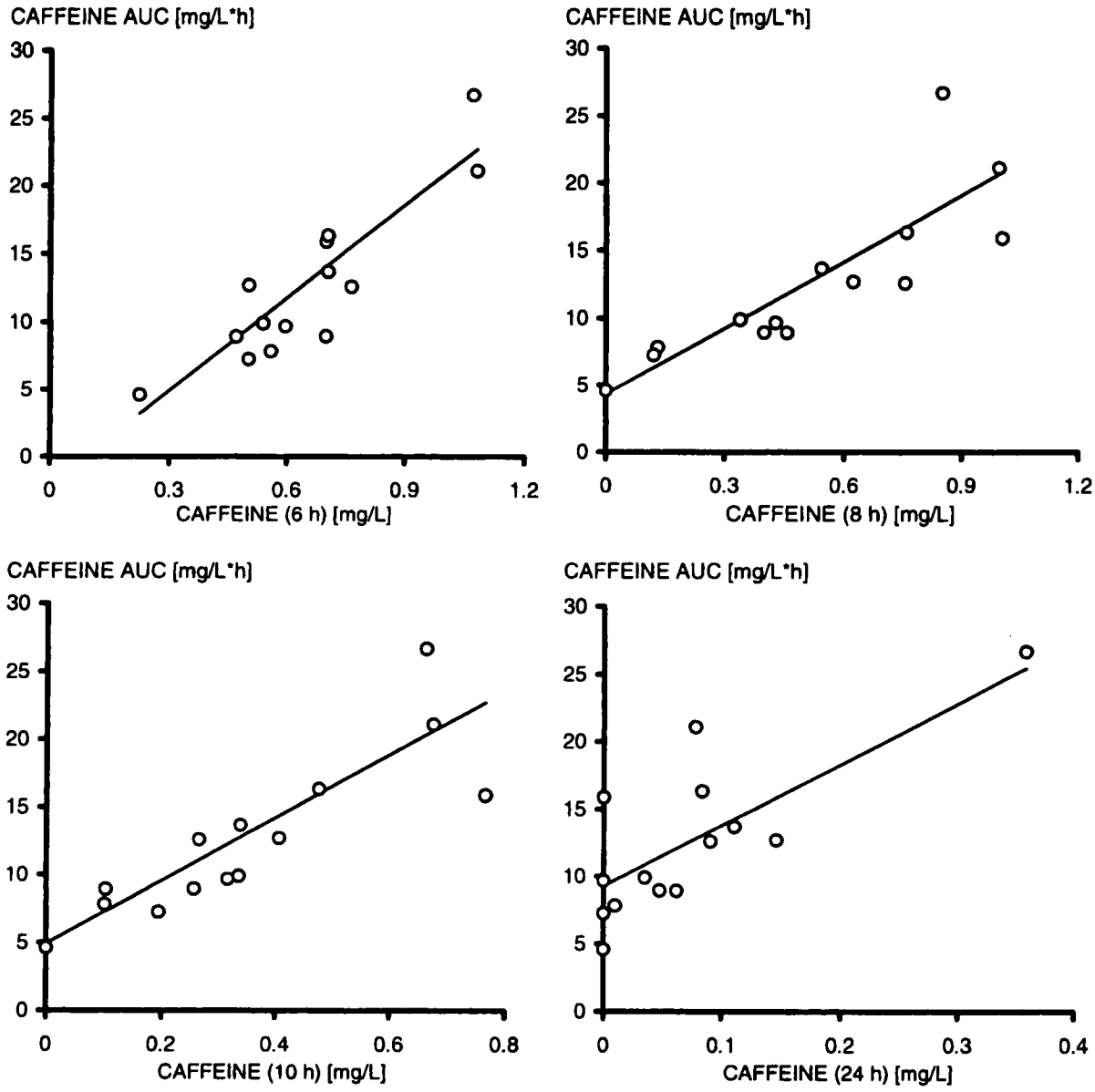

Fig. 4. Relation of plasma caffeine concentrations 6, 8, 10, and 24 h after drug intake to the total area under the plasma concentration–time curve. Solid lines represent predicted values by linear regression. Correlation coefficients are listed in Table 3.

only be observed at 6 h. Although metabolite ratios or metabolite-substrate ratios of index compounds are often utilized to evaluate the metabolic activity of cytochrome P450, it is obvious that these ratios are not linearly related to clearance of the respective compound. This is only of minimal relevance if metabolic acitivities are investigated in individuals, who display a metabolic activity within the normal range. At the extremes of the metabolic range, however, metabolite ratios reflect oral clearance with considerable distortion.

Plasma CAF concentrations at 6, 8, and 10 h displayed a stronger correlation with AUC compared with the paraxanthine/CAF ratio. There is a linear relationship between $AUC_{0-\infty}$ and plasma concentrations of the elimination phase (Fig. 4). Plasma concentrations of the elimination phase reflect systemic clearance of the index substrate with greater reliability because their relation to AUC and clearance is closer compared with the metabolite ratio and is linear. The coefficients of determination of all elimination phase concentrations assessed in this study ranged between 0.66 and 0.71 (Table 3) with the 10 h sample displaying the highest value. Although of limited predictive power, the 10 h plasma concentration appears to reflect the CAF AUC with greater accuracy than the often utilized paraxanthine/CAF ratio.

The validity of a reduced point analysis for the determination of the disposition of drugs has been demonstrated for antipyrine (Døssing *et al.*, 1982; Scavone *et al.*, 1988) and acetaminophen (Scavone *et al.*, 1990). In these studies, however, either two-point analysis were utilized to determine Cl_{oral} or one-point analysis that required to assume volume of distribution or calculate volume of distribution from published equations relating total body water to height, weight, age and sex. Accordingly, variance in these studies was comparably high and so were the sample sizes to demonstrate correlation between the different methods. In contrast, we determined $AUC_{0-\infty}$ in a kinetic study to derive scaling equations, allowing the estimation of $AUC_{0-\infty}$ from single point concentrations (equations 1 and 2). Estimation of clearance by this method is not influenced by volume of distribution because plasma concentrations of the elimination phase are dependent on this parameter to the same extend as $AUC_{0-\infty}$. Single-point determinations to reflect total AUC have been suggested previously for nifedipine (Soons *et al.*, 1992).

The strategy to assess the activity of multiple enzymes simultaneously previously has been called the 'cocktail approach' (Schellens *et al.*, 1989). As summarized in an earlier review (Brockmöller *et al.*, 1994), such cocktail approaches have great practical advantages and may even provide information that is not available by separate administration of the test compounds, but there are severe problems of mutual interactions of the test compounds which need to be ruled out in clinical studies.

There is no statistically significant mutual interference in disposition of ALP and CAF when both compounds are administered simultaneously. Based on the power analysis performed prior to study, a significant interaction between both test substances could be ruled out. Power of this study was even higher than anticipated prior to study since variability in this selected sample was lower compared with the published data. Also the inspection of the mean concentration–time curves (Figs 1 and 2) did not indicate any relevant interaction. Nevertheless, minor interactions may exist (Table 1).

An overnight administration of ALP is desirable to account for its sedative properties. Therefore a sampling time of 10 h after drug administration allows a convenient test procedure: administration of 1 mg ALP and 100 mg CAF at 22.00 h and blood sampling at 08.00 h the following morning. Individual clearances of ALP and CAF can then be calculated using equations 1 and 2, respectively, in combination with the coefficients provided in Table 3.

With this approach, clearances of ALP and CAF can be reliably estimated. Since clearances of these compounds are dependent on the activity of CYP3A4 and CYP1A2, this procedure provides a convenient tool to assess the catalytic activity of both enzymes simultaneously.

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