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Original article

Evaluation of caffeine as a test drug for CYP1A2, NAT2 and CYP2E1 phenotyping in man by *in vivo* versus *in vitro* correlations‡

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Caffeine is used to phenotype subjects *in vivo* for the cytochrome P450 isoforms CYP1A2 and CYP2E1, and for *N*-acetyltransferase type 2 (NAT2). However, how much of the variation in phenotyping parameters may be attributed to variations in CYP1A2 and CYP2E1 activities has not been determined. Therefore, this study intraindividually compared enzyme activities and/or content in liver samples with pharmacokinetic parameters of caffeine *in vivo* after administration of a test dose in 25 patients undergoing hepatectomy. Parameters measured *in vitro* were the high affinity components of caffeine 3-demethylation and phenacetin *O*-deethylation, microsomal CYP1A2 and CYP2E1 immunoreactivity, and cytosolic sulfamethazine *N*-acetylation. Caffeine parameters *in vivo* included caffeine clearance from plasma and/or saliva, paraxanthine/caffeine ratios in plasma and saliva, plasma theophylline/caffeine ratio, and several metabolite ratios from spot urine sampled 6 h postdose. Correlations between parameters were determined using weighted linear regression analyses. Caffeine clearance and paraxanthine/caffeine ratios correlated most highly to intrinsic clearance of caffeine 3-demethylation and to CYP1A2 immunoreactivity (r = 0.58–0.82), whereas urinary CYP1A2 ratios correlated less strongly with CYP1A2 parameters *in vitro*. Assignment of acetylator phenotype by urinary NAT2 ratios was concordant with sulfamethazine *N*-acetylation *in vitro*. In contrast to CYP1A2 paramters *in vitro*, CYP2E1 immunoreactivity was not related to the theophylline/caffeine plasma ratio. CYP1A2 activity, thus, is the major determinant of caffeine clearance and the paraxanthine/caffeine ratios *in vivo*, of which the saliva ratio 6 h postdose appears as the most advantageous parameter. The results confirm that phenotyping using caffeine provides valid estimates of CYP1A2 and NAT2 activity.

Keywords: phenotyping, cytochrome P450, *N*-acetyltransferase, caffeine

Introduction

Limited validity of phenotyping for xenobiotic metabolizing enzymes

Xenobiotic metabolizing enzymes mediate both the elimination of foreign compounds and the formation of cytotoxic or mutagenic metabolites (Guengerich, 1993). Pronounced variations in the catalytic activities exist for many of these enzymes in human populations, due to both genetic and environmental influences (Gonzalez & Idle, 1994). Therefore, specific methods for measuring the activity of single enzymes *in vivo* (phenotyping) may provide important clues regarding the effect of enzyme inducers or inhibitors, and for assessing the relationship between exposure to chemicals and

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cancer incidence (Ioannides & Parke, 1993; Kadlubar, 1994).

Enzyme phenotyping is performed by administration of a 'probe' drug possessing a particular biotransformation pathway that has been shown (usually by *in vitro* experiments) to be selectively mediated by the enzyme in question. Depending on the quantitative importance of this metabolic step, either overall drug pharmacokinetic parameters or specific metabolite ratios in different body fluids are used to assess enzyme function *in vivo*. However, many factors may adversely affect the ability of such a parameter to accurately predict the activity of a specific enzyme, including: (i) the potential for drug elimination or specific metabolite formation by multiple related enzyme isoforms with overlapping substrate selectivities; (ii) the occurrence of complex patterns of competing or sequential biotransformation pathways, which will vary with drug dosage according to enzyme kinetic constants that are unique to each pathway; and (iii) variations due to nonmetabolic factors (especially in urinary metabolite ratios) such as renal excretion processes.

Indeed, the extent to which a phenotyping parameter reflects enzyme activity has been poorly defined in many instances. Since this information cannot be derived from *in vitro* data only, empirical evaluation of phenotyping methods is essential (Kalow & Tang, 1993).

A variety of methods have been proposed for using caffeine (1,3,7-trimethylxanthine) as an *in vivo* probe to predict the activities of several human drug-metabolizing enzymes (for review, see Kalow & Tang (1993)). These include the cytochrome P450 enzymes CYP1A2 and CYP2E1 (nomenclature: see Nelson *et al.* (1993)) and the arylamine *N*-acetyltransferase type 2 (NAT2). A detailed scheme of the metabolic steps of caffeine and the enzymes involved has recently been published (Rost & Roots, 1994).

CYP1A2 phenotyping using caffeine

The human cytochrome P450 isoform CYP1A2 is known to play an important role in the metabolism of environmental procarcinogens, including those of the aromatic amine class (Shimada *et al.*, 1989; Guengerich, 1993). The enzyme also degrades several clinically important drugs such as theophylline (Fuhr *et al.*, 1992a), paracetamol (Patten *et al.*, 1993), verapamil (Kroemer *et al.*, 1993), imipramine (Lemoine *et al.*, 1993) and clozapine (Pirmohamed *et al.*, 1995). Whilst the existence of a genetic polymorphism for CYP1A2 is equivocal (Kalow & Tang, 1991; Butler *et al.*, 1992), the enzyme is clearly inducible. Its activity may be increased considerably by xenobiotics, such as cigarette smoke and polycyclic aromatic hydrocarbons from other sources (Campbell *et al.*, 1987), and by omeprazole (Rost & Roots, 1994; Rost *et al.*, 1994). Although the role of CYP1A2 in carcinogenesis has not yet been proven in earlier epidemiological studies (Kadlubar *et al.*, 1992, 1994), recent results of a case-control study (Lang *et al.*, 1994) suggest that a high CYP1A2 activity is a risk factor for colorectal neoplasm. Finally, inhibition of CYP1A2, for instance by quinolone antibiotic agents (Fuhr *et al.*, 1992b) or serotonine reuptake inhibitors (Brøsen *et al.*, 1993), may result in a clinically important increase in theophylline toxicity. For these reasons, the utility of a reliable CYP1A2 phenotyping test is obvious.

In vivo, relevant primary metabolism of caffeine in man is by demethylation at positions 1, 3, and 7 (Arnaud & Welsch, 1982). The 3-demethylation pathway, which is responsible for some 80% of primary caffeine metabolism *in vivo*, is mediated specifically by CYP1A2 *in vitro* (Butler *et al.*, 1989). The other two demethylation pathways of caffeine and the subsequent breakdown of dimethylxanthines is catalysed in part by CYP1A2 (Fuhr *et al.*, 1992a; Gu *et al.*, 1992; Tassaneeyakul *et al.*, 1994a). Although other cytochromes P450, especially CYP1A1, may also contribute to primary caffeine metabolism (Berthou *et al.*, 1991; Eugster *et al.*, 1993, Fuhr *et al.*, 1994; Tassaneeyakul *et al.*, 1994a), it is generally accepted that the high affinity site of hepatic caffeine 3-demethylation is CYP1A2, and that this reaction may be used in human liver microsomes to determine CYP1A2 activity (Tassaneyakul *et al.*, 1992).

Based on this rationale, a number of different parameters of caffeine biotransformation have been proposed as CYP1A2 markers (for review, see Kalow & Tang (1993)). These include systemic caffeine clearance using plasma or saliva samples, paraxanthine to caffeine ratios in plasma or saliva (Fuhr & Rost, 1994), pulmonary excretion of ^{13}C following a test dose of caffeine labelled at the 3-methyl group, and a variety of urinary caffeine metabolite ratios. Although all these parameters certainly reflect CYP1A2 activity, at least to some extent, and are highly intercorrelated, it remains to be established whether the contribution of CYP1A2 to the expression of any of them is sufficient to make it a truly reliable estimator of enzyme activity *in vivo* (Kalow & Tang, 1993). The possible contribution of enzymes other than CYP1A2 (mainly CYP1A1) to primary caffeine metabolism *in vivo* and the effect of renal clearance on some urinary metabolite ratios (Tang *et al.*, 1994b; Tucker *et al.*, 1995) may be the most important confounding factors.

NAT2 phenotyping using caffeine

Caffeine has also found utility as a probe for the activity of the genetically polymorphic isoform 2 of arylamine *N*-acetyltransferase (NAT2). This enzyme metabolizes a variety of arylamine, hydrazine and heterocyclic amine chemicals, including drugs such as isoniazid, hydrazine, dapsone, amrinone, procainamide and sulphonamides (Weber & Hein, 1985). Deficiency in NAT2 is found in up to 60% of Caucasian subjects and is of considerable clinical importance in drug therapy with its substrates (Grant *et al.*, 1992). Like CYP1A2, NAT2 has been implicated in pathways of arylamine carcinogen bioactivation (Hein *et al.*, 1992). Indeed, several epidemiological studies have led to the suggestion that genetically 'slow acetylators' have an increased risk for the occurrence of bladder cancer and a decreased risk for colorectal cancer, but results contrary to these hypotheses have also been reported (Hein *et al.*, 1993; Rodriguez *et al.*, 1993; Kadlubar, 1994; Lang *et al.*, 1994).

In vivo, urinary ratios of caffeine are used for NAT2 phenotyping in man. The formation of 5-acetylamino-6-formylamino-3-methyluracil (AFMU) from an unknown intermediate product of paraxanthine, the major primary metabolite of caffeine, is catalysed by NAT2 (Grant *et al.*, 1984). After administration of a caffeine test dose, NAT2 activity is estimated by the molar concentration of AFMU and/or its decomposition product 5-acetylamino-6-amino-3-methyluracil (AAMU) in urine relative to that of 1-methylxanthine, the alternative paraxanthine metabolite (Grant *et al.*, 1984; Tang *et al.*, 1987; Kalow & Tang, 1993; Rost & Roots, 1994). *In vitro* activity of NAT2 can be determined specifically by acetylation of sulfamethazine in hepatocyte cytosol (Grant *et al.*, 1992).

The urinary caffeine metabolite ratios provided concordant results with *in vivo* phenotype determined using the classical 'polymorphic' (NAT2) substrate sulfamethazine (Grant *et al.*, 1983), with sulfamethazine acetylation rates measured in human liver cytosols (Grant *et al.*, 1990), and with NAT2 acetylator genotypes determined using molecular diagnostic techniques (Hickman & Sim, 1991).

CYP2E1 phenotyping using caffeine

Recent data has also indicated that the 7-demethylation of caffeine to theophylline (1,3-dimethylxanthine), is mediated by CYP2E1 additional to CYP1A2 (Gu *et al.*, 1992; Tassaneeyakul *et al.*, 1994a). CYP2E1 is known to metabolize a variety of low molecular weight suspect carcinogens like acrylonitrile, benzene, nitrosamines and vinyl halides (Guengerich, 1993). The enzyme is inducible by ethanol (Perrot *et al.*, 1989). Both substantial interindividual variation in CYP2E1 level and activity (Lucas *et al.*, 1993) and a DNA sequence polymorphism in the gene that encodes it have been observed (Ingelman-Sundberg *et al.*, 1993). Current estimates of CYP2E1 activity *in vivo* using chlorzoxazone (Kharasch *et al.*, 1993) may be contaminated by CYP1A2 activity (Hotta *et al.*, 1994). Based upon a change of caffeine urinary metabolite pattern in relation to consumption of ethanol, Tang and colleagues (1994a) have recently proposed the use of caffeine metabolite ratios for CYP2E1 phenotyping.

In the present study, we have compared parameters of caffeine metabolism capacity *in vivo* with known catalytic and/or immunological markers of CYP1A2, NAT2, and CYP2E1 content and activity in hepatic enzyme preparations of patients undergoing partial hepatectomy. The objective of this work was to evaluate to which extent the caffeine-derived phenotyping parameters indeed reflect these enzyme activities.

Patients and methods

PATIENTS

Patients undergoing partial hepatectomy in the Department of General Surgery of the University Hospital, Frankfurt a. M. from December 1991 to November 1992 and without severe diseases other than that making surgery necessary were asked to participate in the study. 25 patients (14 women, 11 men, age 56 ± 11 years, five smokers/20 nonsmokers, weight 72 ± 14 kg [mean ± SD]) gave their informed consent to take part in the investigation which was approved by the Ethics Committee of the University Hospital Frankfurt a. M., Germany. Surgery was in most cases required due to primary liver neoplasm or liver metastasis of other carcinoma. Individual data of the highly heterogeneous study population are shown in Table 1.

ESTIMATION OF FUNCTIONAL LIVER SIZE

Computed tomograms (CT) which were made for diagnostic reasons within one month prior to hepatectomy were used to estimate functional liver size. To this end, the liver and – if possible – the tumour were marked on the tomogram. The weight of these areas and the height of tomogram cuts (8–10 mm in most cases) were used to estimate gross liver volume including the tumour. The precision of such a procedure was better than 5% in a published investigation (Heymsfield *et al.*, 1979). To estimate functional liver size, the volume of the tumour had to be substracted from the gross liver size, since primary liver tumours do not express high levels of CYP1A2 (El Mouelhi *et al.*, 1987; McKinnon *et al.*, 1991). Expression of CYP1A2 in

Table 1. Characteristics of patients

No.	Sex	Age (years)	Weight (kg)	Smoking habits[a]	Diagnosis[d] M[b] Ca[c]	Funct. liver volume (ml)	Caffeine test available	Comedication during caffeine test	Liver sample available
1	M	81	75	NS	M[b] Ca[c] colon	NA[e]	Yes	Radix urticae	Yes
2	M	66	81	NS	M Ca colon	1370	Yes	Theophylline, acetylcysteine	Yes
3	F	39	56	S	FNH[d]	1243	Yes	–	Yes
4	F	60	57	S	Cyst	1659	Yes	Acetylcysteine, ranitidine	No
5	M	57	94	NS	M hypernephroma	2330	Yes	–	Yes
6	M	61	110	NS	M Ca pancreas	NA	Yes	Glibenclamide, molsidomine, gemfibrozil	No
7	F	43	60	S	M Ca mamma	1007	No	Fe^{2+}, thyroxine, methotrexate, endoxan, fluorouracil	Yes
8	F	72	90	NS	Cirrhosis	1360	Yes	Isosorbide dinitrate, thyroxine, glibenclamide, pentoxifylline	No
9	F	60	64	S	Ca bile ducts	1432	Yes	Bromazepam	Yes
10	F	54	70	NS	Ca bile ducts	1701	Yes	–	Yes
11	F	51	70	NS	M Ca colon	1323	Yes	–	Yes
12	M	49	95	NS	Ca bile ducts	2074	Yes	Captopril, sodium perchlorate	Yes
13	F	64	72	NS	M Ca colon	1588	Yes	K^+	Yes
14	F	48	70	NS	M carcinoid small intestine	1477	Yes	Carbimazole	Yes
15	M	64	72	NS	Liver cell Ca	1871	Yes	–	Yes
16	M	65	65	Unknown	M Ca colon	NA	Yes	Unknown	No
17	F	54	58	S	M Ca colon	2059	Yes	–	Yes
18	F	44	63	NS	Liver cell adenoma	1445	Yes	Thyroxine	Yes
19	M	60	77	NS	M Ca colon	NA	Yes	Lactulose	No
20	M	48	69	NS	M Ca colon	2010	Yes	–	Yes
21	F	53	53	NS	Liver cell Ca	1071	Yes	Etilefrine, estrogens	Yes
22	F	41	65	NS	FNH	1008	Yes	Metoprolol	Yes
23	F	36	52	NS	M Ca colon	1384	Yes	–	Yes
24	M	68	72	NS	Liver cell Ca	1166	Yes	Verapamil, chinidin sulfate, carduus marianus	Yes
25	M	53	90	NS	M Ca colon	1574	Yes	Mezlocillin	Yes

[a]S, smoker; NS, non-smoker.
[b]M, metastasis.
[c]Ca, carcinoma.
[d]FNH, follicular nodular hyperplasia.
[e]NA, not available.

metastases of other neoplasms is not probable because it is a hepatic enzyme. Depending on tumour type, it was possible to estimate tumour volume from CT data in 13 out of 25 patients. In only four cases could we measure tumour weight after separation from the operative specimen, since the specimen had to be examined microscopically to check for complete removal of the neoplasma. In these cases, tumour weight reached 77 ± 14% (mean ± SD) of the specimen weight. This value was used to estimate tumour size from operative specimen size in eight further patients where tumours could not clearly be delimited in the CT. A

validation of this procedure by comparison of functional liver size derived from tumour CT data relative to that derived from operative specimen size gave a value of 101 ± 17% (mean ± SD). This control was possible in nine patients. In summary, functional liver volume could be estimated in 21 out of 25 patients, with a presumed error of less than 20%. No CT was available in one patient, in three other patients partial hepatectomy was not carried out due to inoperability found during surgery.

PARAMETERS USED TO ESTIMATE ENZYME ACTIVITY *IN VIVO*

Within 2 days prior to partial hepatectomy, a caffeine test dose of approximately 165 mg, given as a cup of coffee prepared from 5 g of instant coffee (Nescafé®, Nestlé, Frankfurt a. M., Germany) was administered in the morning. For measurement of concentrations of caffeine and its metabolites, 5 ml blood samples (before administration of caffeine and scheduled 3, 6, and 9 h thereafter) and 2 ml saliva samples (before administration and scheduled 2, 3, 6, 9, 12, and 15 h thereafter) were taken. Since adherence to a strict urine collection protocol was not possible due to other investigations carried out on the preoperative days, urine sampling was restricted to a spot sample taken 6 h after administration.

Concentrations of caffeine and its metabolites in urine samples and in plasma samples drawn during surgery were measured according to Rost & Roots (1994). Sample preparation of this method includes an extraction step and is therefore very sensitive. All other plasma and saliva samples were prepared using protein precipitation and measured according to Fuhr *et al.* (1990), but 50 µl instead of the 22 µl described in the reference were injected onto the HPLC column. This method provides valuable results only for caffeine and praxanthine in the two matrices, but not for further caffeine metabolites.

The following pharmacokinetic parameters were calculated:

Systemic caffeine clearance

Using plasma concentrations: The terminal elimination constant k_{el}, elimination half life ($t_{1/2} = In(2)/k_{el}$), and virtual initial concentration C_0 was derived from the last (at least 3) data points of the concentration time profile using regression analysis after logarithmic transformation of concentrations. The calculated parameters were only used for further evaluation if the squared coefficient of correlation for estimation of the elimination constant reached at least $r^2 = 0.95$. If caffeine concentrations C_i exceeding the limit of quantification were detected in plasma prior to administration of the test dose, instead of measured concentrations C_t corrected concentrations $C_{t,korr} = C_t - (C_i \cdot e^{(-k_{el}\cdot(t-i))})$ were used. Since caffeine is absorbed rapidly and completely following oral administration (Benet & Williams, 1990), clearance was calculated as $k_{el} \cdot V_d$ and is presented as ml min^{-1} per kg body weight.

Using saliva concentrations: Saliva concentrations were transformed to plasma concentrations using division by the saliva to plasma relation of *0.790* (Fuhr *et al.*, 1993). The further procedure was as described above.

Using both plasma and saliva concentrations: The individual mean ratio of caffeine concentrations in saliva and in plasma was calculated using samples withdrawn at the same scheduled time, if applicable using a correction when true sampling times were different. This ratio was used to transform saliva to plasma concentrations. For calculation of kinetic parameters (see above), both measured plasma concentrations and plasma values derived from saliva concentrations were used in this case.

The ratios paraxanthine/caffeine in plasma and in saliva and the ratio theophylline/caffeine in plasma

The molar ratio of paraxanthine to caffeine concentrations was calculated 3 and 6 h after caffeine administration (Fuhr & Rost, 1994).

In addition to the administration of caffeine as described above, 100 mg of caffeine as citrate dissolved in 5 ml of water were administered orally in the morning prior to surgery. A 5 ml plasma sample was withdrawn during surgery when blood flow to the liver was interrupted or when resection was carried out if techniques without blood vessel ligation were used. In these samples, the molar paraxanthine/caffeine and theophylline/caffeine ratios were calculated. Since the former ratio increased in a linear way with time between administration of the test dose and sampling (Fuhr & Rost, 1994), it was transformed to clearance values as described to allow for differences in sampling times between patients. Likewise, the theophylline/caffeine ratio was divided by the time after caffeine administration.

Urinary ratios of caffeine and its metabolites

Four different molar urinary ratios were calculated from caffeine metabolite concentrations in the 6 h spot samples to estimate CYP1A2 activity *in vivo*:

Urinary ratio A: $\frac{17X}{137X}$

(Kadlubar *et al.*, 1990)

Urinary ratio B: $\frac{17X + 17U}{137X}$

(Butler *et al.*, 1992)

Urinary ratio C: $\frac{1X + 1U + AFMU}{17U}$

(Campbell *et al.*, 1987)

Urinary ratio D: $\frac{1X + 1U + AFMU + AAMU}{17U}$

(Kalow & Tang, 1991; modified)

Two molar urinary ratios were calculated for estimation of NAT2 activity *in vivo*:

Urinary ratio E: $\frac{AFMU}{1X}$

(Grant *et al.*, 1984)

Urinary ratio F: $\frac{AFMU + AAMU}{1X}$

(Tang *et al.*, 1987, modified)

Abbreviations: 17X, 1,7-dimethylxanthine (paraxanthine); 137X, 1,3,7-trimethylxanthine (caffeine); 17U, 1,7-dimethyluric acid; 1X, 1-methylxanthine; 1U, 1-methyluric acid; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; AAMU, 5-acetylamino-6-amino-3-methyluracil.

CHARACTERIZATION OF LIVER SAMPLES *IN VITRO*

Caffeine 3-demethylation

Preparation of liver microsomes was carried out using standard methods (see Fuhr *et al.*, 1990). The yield of microsomal suspensions was measured by several weighing steps during preparation. Caffeine (concentrations used: 62.5–2000 µM) was incubated in triplicate with the microsomes (Fuhr *et al.*, 1990). Enzyme constants were calculated by nonlinear regression assuming two caffeine binding sites using equation (1).

$$v = \frac{V_{max1} \cdot [S]}{K_{m1} + [S]} + \frac{V_{max2} \cdot [S]}{K_{m2} + [S]} \quad (1)$$

Binding site 1 is the high affinity site which was used for quantitation of CYP1A2 activity. The K_{m2} value was fixed at 30 mM (Tassaneeyakul *et al.*, 1992) since it could not be estimated in the caffeine concentration range used here. This procedure was validated for three liver samples (P11, P12, and P15), where additional incubations were carried out using caffeine concentrations of up to 32 mM. In these samples, enzyme constants for the high affinity site obtained when K_{m2} was allowed to float and data for caffeine concentrations of up to 32 mM were used and compared to those calculated using a fixed K_{m2} and caffeine concentrations of up to 2 mM.

Intrinsic clearance was calculated as V_{max}/K_m. Values were given for the amount of enzyme present in the whole liver which was estimated using microsome suspension yield and estimated functional liver volume.

Phenacetin O-deethylation

Incubations were carried out in 100 µl of 0.1 M potassium phosphate buffer, pH 7.6, containing 4 mM $MgCl_2$. The NADPH generating system consisted of 5 mM glucose-6-phosphate, 0.5 mM NADP and 2 mU ml^{-1} glucose-6-phosphate-dehydrogenase. Ten different concentrations of phenacetin in the range between 5 and 200 µM were used. The reaction was started by the addition of 10 µl of microsomal suspension and terminated after 20 min at 37°C by the addition of 100 µl methanol (4°C). Concentrations of the phenacetin metabolite, paracetamol, were measured by HPLC. After centrifugation of the sample, 50 µl of the supernatant were injected onto an ODS column (Beckman, 150 × 46 mm, 4 µm particle size). A gradient was used for separation. Solution A consisted of 94% of water: acetic acid:triethylamine 1980:20:1 (v/v/v), solution B corresponded to solution A but with water replaced by acetonitrile. From the start until 2.5 min of run time, the eluent was 94% of A and 6% of B. Then, a linear increase of the B fraction up to 80% was carried out until a run time of 3.5 min. This eluent composition was maintained for elution of phenacetin after which the initial conditions were re-established. The peaks of paracetamol and phenacetin, detected by UV absorption at 254 nm, were eluted after 2.4 min and 5.5 min, respectively. Enzyme parameters for the high affinity binding site of phenacetine were calculated using equation (1) (without the low affinity site). Only the linear part of the curve in the low concentration range was used. Intrinsic clearance and transformation of data to whole liver enzyme content was as described above.

Activity of N-*acetyltransferase NAT2*

The activity of NAT2 was determined as described (Grant *et al.*, 1990) with sulfamethazine (500 µM) as substrate. Enzymatic activity, given as pmol min^{-1} per mg cytosolic protein, was multiplied by functional liver volume for the *in vivo* vs *in vitro* comparison.

Immunoquantitation of relative CYP1A2 and CYP2E1 contents

CYP1A2 and CYP2E1 content in human liver microsomes were measured by immunoblots (Beaune *et al.*, 1985). Antibodies to recognize human CYP1A2 and CYP2E1 were generated as described in New Zealand female rabbits against purified human CYP1A2 and CYP2E1 expressed in *E. coli*. Anti-CYP1A2 recognized only the human cytochrome P450 isoforms 1A1 and 1A2, but not 3A4, 3A5, 2D6, 2C1, 2C8, 2C9, 2C18, or

2E1. This antibody detected only one band in all human liver microsomes comigrating with CYP1A2 (but not with CYP1A1) expressed in yeast. Anti-CYP2E1 recognized only the human cytochrome P450 isoform 2E1, but not 1A1, 1A2, 3A4, 3A5, 2D6, 2C1, 2C8, 2C9, or 2C18. It detected only one band in human liver microsomes comigrating with CYP2E1 expressed in yeast. Due to the lack of an absolute standard, the intensity of staining was given relative to that obtained by samples from patient no. 10, which was arbitrarily chosen as 100%. It was also checked that the staining was linear in the considered range.

Determination of CYP1A1 *genotype*

Since CYP1A1 showed a high activity for caffeine 3-demethylation (Fuhr *et al.*, 1994, see Introduction), patients were *CYP1A1* genotyped to investigate whether the presence of mutant genes was related to peculiarities in caffeine metabolism. Two known *CYP1A1* mutations (m1 and m2) were identified as described (Drakoulis *et al.*, 1994).

CALCULATION AND STATISTICS

Not all data anticipated could be obtained for all patients for the following reasons: other important preoperative clinical examinations hindered its acquisition; liver tissue could not be obtained from all patients; or the amount of liver tissue available was not sufficient for all tests in some patients. The actual number of data pairs for *in vitro–in vivo* comparisons is therefore given in all Result listings.

All calculations were carried out using the statistical software package SYSTAT®, version 5.03, SYSTAT Inc., Evanston, Illinois, USA. Three explorative correlation matrices were calculated using linear regression analysis. Data pairs were weighted by the inverse of the dependent variables to avoid a strong influence of extremes on correlation coefficients (Sachs, 1992). One matrix each was calculated for interrelationship of enzyme parameters *in vitro* and for CYP1A2 and NAT pharmacokinetic parameters of caffeine *in vivo*. For any of these correlations, those variables thought best to reflect the enzyme were used as independent variables. The third correlation matrix shows the relationship between enzyme parameters *in vitro* as independent parameters and pharmacokinetic parameters of caffeine *in vivo* as dependent variables. Significance levels given for correlations are descriptive in all cases. For control, rank correlation coefficients according to Spearman were also calculated, which gave similar results, but were not used here, because this procedure does not allow the assessment of a quantitative relationship between two parameters.

Since the theophylline/caffeine ratio is a parameter that should be influenced by both CYP2E1 and CYP1A2, a multiple stepwise linear correlation (Armitage & Berry, 1987) was calculated using equations (2):

$$\textit{(Theophylline/caffeine ratio during surgery, divided by sampling time)} = a + b^{*}\ \textit{(CYP1A2 parameter)} + c^{*}\ \textit{(immunoreactive CYP2E1 amount)} \quad (2)$$

CYP1A2 parameters used in this equation were relative CYP1A2 amount, intrinsic clearance for caffeine 3-demethylation and for phenacetin *O*-deethylation (each given for whole liver), caffeine clearance derived from combined saliva and plasma samples and the paraxanthine/caffeine ratio (divided by sampling time) from the samples drawn during surgery.

Using the data obtained for *CYP1A1* genotyping, subjects were divided into groups with the presence or absence of *CYP1A1* mutations. All *in vivo* and *in vitro* parameters were compared between these groups using the Mann-Whitney U-test.

Results

Data obtained for the individual parameters obtained *in vivo* and *in vitro*, given as median and range, are shown in the left columns of Tables 2 and 3.

The method evaluation for determining enzyme constants of caffeine 3-demethylation at the microsomal high affinity site is shown in Table 4. The simplified procedure, which comprised fixing K_{m2} at 30 mM and using caffeine concentrations of 62.5–2000 μM, was compared to the results obtained when caffeine concentrations of 62.5–32000 μM were used and K_{m2} was estimated from the data. Only minor differences were observed for enzyme constants of the high affinity site between the two methods in samples of patients P11, P12 and P15, where K_{m2} values of 12 mM, 40 mM and 31 mM were found, respectively.

Between patients K_m for caffeine 3-demethylation at the high affinity site for all patients varied by approximately three-fold (Fig. 1), and V_{max} values by approximately nine-fold. The standard deviation of estimated enzyme parameters was below 10% of the parameter size in most cases. Derived intrinsic clearance, transformed to values for whole liver enzyme amount, ranged between 5.5 and 46.3 ml min^{-1}.

K_m for phenacetin *O*-deethylation varied seven-fold (Fig. 1) and V_{max} values of this metabolic pathway by a factor of approximately six. Intrinsic clearance for the whole liver ranged from 592 to 2950 ml min^{-1}.

The rate of sulfamethazine acetylation, which was apparently bimodally distributed, varied 26-fold between hepatic cytosol preparations (Fig. 2).

Table 2. Observed values and intercorrelation matrix of enzyme parameters measured *in vitro*. Upper right triangle of matrix: correlation coefficients, lower left triangle: number of data pairs available. Column variables were used as independent variables, row variables as dependent variables. Weighting was by the inverse of dependent variables

	Observed values		*Correlations:* r *values and case numbers*						
	n	*Median (range)*	*SMZ metabolism*	K_m *phenacetin*	V_{max} *phenacetin*	K_m *caffeine*	V_{max} *caffeine*	*CYP2E1 content*	*CYP1A2 content*
Sulfamethazine (SMZ, 500 μM) *N*-acetylation rate (pmol min^{-1} per mg protein)	20	39 (16–414)	–	−0.02	−0.04	−0.28	0.04	0.07	−0.12
K_m (μM) of phenacetin *O*-deethylation	19	18.7 (6.4–44.7)	$n = 19$	–	0.59**	0.07	0.39	0.16	0.20
V_{max} (pmol min^{-1} per mg protein) of phenacetin *O*-deethylation	19	710 (270–1696)	$n = 19$	$n = 19$	–	0.21	0.80****	0.17	0.46*
K_m (μM) of caffeine 3-demethylation	20	360 (202–655)	$n = 20$	$n = 19$	$n = 19$	–	0.40	0.50*	−0.18
V_{max} (pmol min^{-1} per mg protein) of caffeine 3-demethylation	20	120 (48–290)	$n = 20$	$n = 19$	$n = 19$	$n = 20$	–	0.36	0.58**
Immunoreactive CYP2E1 content per mg protein[a]	20	222 (100–309)	$n = 20$	$n = 19$	$n = 19$	$n = 20$	$n = 20$	–	0.02
Immunoreactive CYP1A2 content per mg protein[a]	20	132 (53–393)	$n = 20$	$n = 19$	$n = 19$	$n = 20$	$n = 20$	$n = 20$	–

[a]Relative contents in microsomal preparations were calculated as a percentage of the values obtained for patient 10.
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$.

Table 3. Observed values and intercorrelation matrix of pharmacokinetic parameters of caffeine measured *in vivo*. Upper right triangle of matrix: correlation coefficients, lower left triangle: number of data pairs available. Column variables were used as independent variables, row variables as dependent variables. Weighting was by the inverse of dependent variables

	Observed values		*Correlations:* r *values and case numbers*													
	n	*Median (range)*	*UR A*	*UR B*	*UR C*	*UR D*	*UR E*	*UR F*	*Cl in OP*	*Ratio 3h (S)*	*Ratio 3h (P)*	*Ratio 6h (S)*	*Ratio 6h (P)*	*Cl (P+S)*	*Cl (S)*	*Cl (P)*
Urinary ratio A (CYP1A2)	23	3.29 (0.2–14.3)	–	0.92 ****	0.62 ***	0.62 ***	0.35	0.24	0.60 *	0.52 *	0.57 **	0.51 *	0.68 ****	0.57 **	0.59 *	0.74 ****
Urinary ratio B (CYP1A2)	23	8.9 (0.8–49.0)	$n = 23$	–	0.50 *	0.50 *	0.23	0.15	0.46	0.55 **	0.55 **	0.53 *	0.65 ***	0.53 *	0.56 *	0.68 ***
Urinary ratio C (CYP1A2)	23	5.1 (2.7–10.5)	$n = 23$	$n = 23$	–	0.99 ****	−0.08	−0.16	0.69 ***	0.74 ****	0.82 ****	0.74 ****	0.75 ****	0.66 ****	0.78 ****	0.46
Urinary ratio D (CYP1A2)	23	6.0 (3.1–11.6)	$n = 23$	$n = 23$	$n = 23$	–	−0.03	−0.11	0.67 **	0.75 ****	0.82 ****	0.74 ****	0.75 ****	0.66 ****	0.71 ***	0.47
Urinary ratio E (NAT2)	23	0.50 (0.16–2.45)	$n = 23$	$n = 23$	$n = 23$	$n = 23$	–	0.93 ****	−0.20	−0.12	−0.22	−0.17	−0.21	−0.23	−0.22	−0.10
Urinary ratio F (NAT2)	23	0.96 (0.25–3.50)	$n = 23$	$n = 23$	$n = 23$	$n = 23$	$n = 23$	–	−0.28	−0.21	−0.34	−0.28	−0.35	−0.32	−0.31	−0.20
Caffeine clearance derived from ratio paraxanthine/ caffeine during surgery	18	2.28 (0.71–4.74)	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 16$	–	0.83 ****	0.87 ****	0.86 ****	0.84 ****	0.90 ****	0.86 ****	0.87 ****
Ratio paraxanthine/caffeine in saliva 3 h postdose	23	0.38 (0.11–0.81)	$n = 21$	$n = 21$	$n = 21$	$n = 21$	$n = 21$	$n = 21$	$n = 17$	–	0.97 ****	0.98 ****	0.95 ****	0.92 ****	0.92 ****	0.85 ****
Ratio paraxanthine/caffeine in plasma 3 h postdose	24	0.48 (0.15–0.91)	$n = 22$	$n = 22$	$n = 22$	$n = 22$	$n = 22$	$n = 22$	$n = 17$	$n = 23$	–	0.92 ****	0.98 ****	0.93 ****	0.93 ****	0.84 ****
Ratio paraxanthine/caffeine in saliva 6 h postdose	21	0.72 (0.16–1.42)	$n = 19$	$n = 19$	$n = 19$	$n = 19$	$n = 19$	$n = 19$	$n = 16$	$n = 21$	$n = 21$	–	0.95 ****	0.96 ****	0.99 ****	0.97 ****
Ratio paraxanthine/caffeine in plasma 6 h postdose	23	0.83 (0.16–1.69)	$n = 21$	$n = 21$	$n = 21$	$n = 21$	$n = 21$	$n = 21$	$n = 17$	$n = 22$	$n = 23$	$n = 21$	–	0.94 ****	0.94 ****	0.89 ****
Caffeine clearance (ml min^{-1} kg^{-1}) from combined plasma + saliva	24	1.27 (0.33–3.02)	$n = 22$	$n = 22$	$n = 22$	$n = 22$	$n = 22$	$n = 22$	$n = 17$	$n = 23$	$n = 24$	$n = 21$	$n = 23$	–	0.94 ****	0.88 ****
Caffeine clearance (ml min^{-1} kg^{-1}) from saliva concentrations	20	1.31 (0.33–2.91)	$n = 18$	$n = 18$	$n = 18$	$n = 18$	$n = 18$	$n = 18$	$n = 14$	$n = 20$	$n = 20$	$n = 19$	$n = 19$	$n = 19$	–	0.99 ****
Caffeine clearance (ml min^{-1} kg^{-1}) from plasma concentrations	19	1.38 (0.77–3.06)	$n = 17$	$n = 17$	$n = 17$	$n = 17$	$n = 17$	$n = 17$	$n = 14$	$n = 19$	$n = 19$	$n = 18$	$n = 19$	$n = 19$	$n = 16$	–

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$.

Table 4. Comparison of methods to estimate enzyme constants for caffeine 3-demethylation at the high affinity site in human liver microsomes. Results in microsomes from patients 11, 12 and 15 were estimated from measured metabolic rates by nonlinear regression analysis, using either caffeine concentrations of up to 2 mM and a K_{m2} fixed at 30 mM, or caffeine concentrations of up to 32 mM and estimation of K_{m2}. Maximal metabolite formation rates and intrinsic clearance values are given for the whole liver

	K_{m2} *fixed to 30 mM*			K_{m2} *estimated*		
Patient no.	K_{m1} *(μM)*	V_{max1} *(nmol/min)*	*Intrinsic clearance (ml min^{-1})*	K_{m1} μM	V_{max1} *(nmol min^{-1})*	*Intrinsic clearance (ml/min)*
11	370	6462	17.5	358	6189	17.3
12	321	5324	16.6	309	5246	17.0
15	655	3595	5.5	638	3446	5.4

The intercorrelation of *in vitro* parameters is given in Table 2. The best correlations were observed between V_{max} values of caffeine 3-demethylation and of phenacetin *O*-deethylation ($p < 0.001$), and between caffeine V_{max} values and CYP1A2 content ($p < 0.01$). No significant correlation was observed between K_m values for caffeine 3-demethylation and phenacetin *O*-deethylation at the respective high affinity site (Fig. 1).

A variability in the same order of magnitude was also found in *in vivo* parameters (Table 3). CYP1A2 parameters were intercorrelated significantly in most cases. The highest correlation coefficients were observed for caffeine clearance values and for the paraxanthine to caffeine ratios, less valid correlations were found for urinary metabolite ratios. No significant correlations were observed between CYP1A2 and NAT2 parameters.

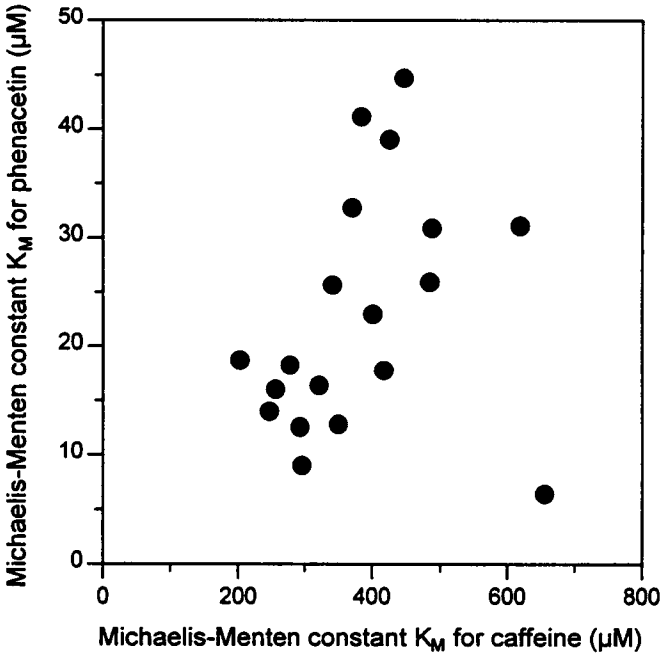

Fig. 1. Lack of correlation between K_M values for caffeine 3-demethylation and phenacetin *O*-deethylation at their high affinity sites in human liver microsomes.

None of the *in vivo* parameters was significantly correlated to the estimated functional liver volume (Table 5).

The results of the *in vivo* vs *in vitro* comparisons are given in Table 5. Caffeine clearance values (normalized to body weight) and the paraxanthine to caffeine ratios were closely correlated to intrinsic clearance of caffeine 3-demethylation at the high affinity site and to CYP1A2 content (as an example, see Fig. 3). Similar correlations were found for absolute clearance data (data not shown), where the slope of the regression lines approximating a value of 3 suggests that *in vivo* biotransformation of caffeine is several times faster than its *in vitro* metabolism. No correlation was observed for these parameters to sulfamethazine *N*-acetylation, to intrinsic clearance for phenacetin *O*-deethylation, or to CYP2E1 content (Fig. 4). Only those urinary CYP1A2 parameters that are based on paraxanthine metabolism showed a significant correlation to intrinsic clearance of caffeine 3-demethylation.

The allocation of patients to the slow or rapid *N*-acetylator phenotype by the cytosolic sulfamethazine acetylation rate was in all cases reflected by the urinary NAT2 ratios (Fig. 2). Ratio E was < 0.55 (Grant *et al.*, 1990), ratio *F* was < 1.03 for all patients identified as slow acetylators by sulfamethazin acetylation rates of less than 136 pmol min^{-1} per mg cytosolic protein. For rapid acetylators according to the sulfamethazine activity *in vitro*, the above limits for the urinary ratios were surpassed in all cases.

In the stepwise multiple linear regression analysis, carried out to estimate a possible influence of CYP2E1 activity on the ratio theophylline/caffeine in blood samples drawn during surgery, all *in vivo* and *in vitro* CYP1A2 parameters included in the equation (see Methods section), with the exception of the intrinsic clearance for phenacetin, correlated significantly ($p < 0.05$) with that metabolic ratio. In contrast, no significant correlation was observed between CYP2E1 content and the ratio in any of the calculations ($p > 0.05$).

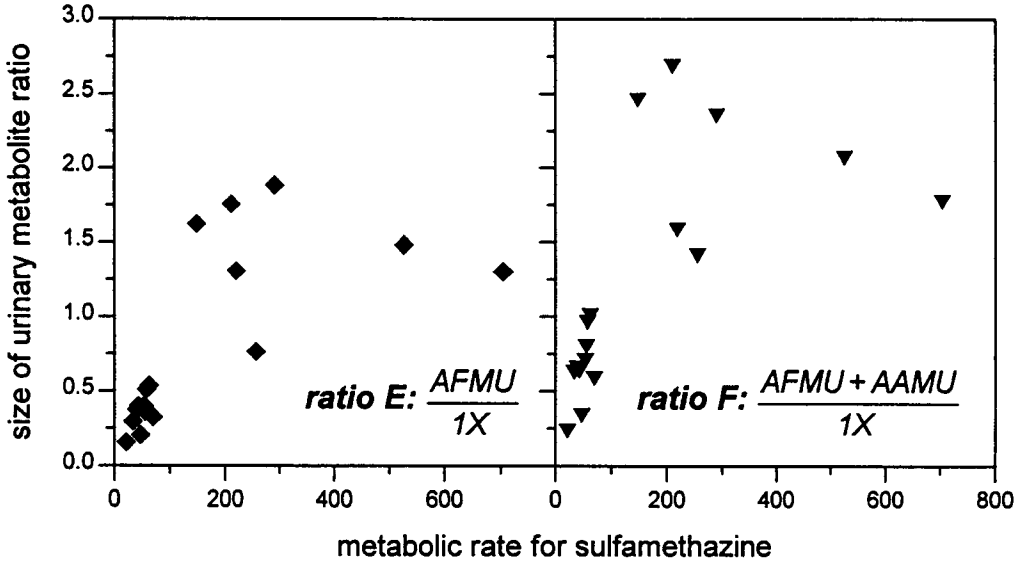

Fig. 2. Relationship of sulfamethazine (500 μM) *N*-acetylation rate in human liver cytosol to urinary caffeine metabolite ratios. Measured metabolic rates of sulfamethazine (nmol per min and per mg cytosolic protein) were multiplied by functional liver volume (ml). For identification of urinary metabolites, see Methods section.

Three out of the 20 patients tested had one *CYP1A1* allele with an m1 mutation and one wild type allele, two had one m2 allele and one wild type allele, while two further patients had one allele with both mutations and one wild type allele. With one exception (lower sulfamethazine *N*-acetylation rate in patients without the m2 mutation), no differences in any of the parameters were observed between patients with and without the m1, the m2 or both mutations in the *CYP1A1* gene.

Discussion

The objective of the study was to evaluate the use of caffeine kinetics for estimation of CYP1A2, NAT2, and CYP2E1 activity *in vivo*. To this end, multiple explorative correlation coefficients were calculated to compare between parameters describing enzyme content and activity *in vitro* and *in vivo*. Since significant values may be achieved by chance (type II error), and because of the lack of formal adjustment of significance levels due to the explorative character of the study, any interpretation must be cautious and is valid only if a group of observations provides similar and plausible results. For these reasons, we also calculated correlation coefficients between unrelated enzyme activities, which may serve as a control.

INTERCORRELATION OF *IN VITRO* ENZYME PARAMETERS

As expected from the involvement of CYP1A2 in caffeine 3-demethylation and phenacetin *O*-deethylation, there was a close correlation between maximal formation rate of paraxanthine at the high affinity site and both maximal phenacetin metabolic rate and CYP1A2 content. A similar correlation between caffeine and phenacetine metabolism has been described (Sesardic *et al.*, 1988). The lack of correlation between caffeine and phenacetin affinity to the enzyme (Fig. 1, Table 2), however, provides evidence for one of the following explanations:

(a) Microsomal enzymes other than CYP1A2 make a relevant contribution to caffeine 3-demethylation or phenacetin *O*-deethylation at the high affinity site. This cannot be excluded definitely, since other cytochrome P450 enzymes are also capable of metabolizing caffeine (see Introduction), but several lines of evidence support the high degree of specificity of these reactions for human CYP1A2 in hepatic microsomes (Sesardic *et al.*, 1988; Butler *et al.*, 1989; Fuhr *et al.*, 1992a; Tassaneeyakul *et al.*, 1992).

(b) These two substrates may bind to different parts of the enzyme binding site. Variability in affinity, which clearly exceeds the experimental error, may be caused by CYP1A2 mutants, which have only minor effects on enzyme function, but do not result in a uniform change of affinity for phenacetin and caffeine.

The absence of a correlation between CYP1A2 and CYP2E1 content corresponds to other observations (Lucas *et al.*, 1993).

INTERCORRELATION OF CAFFEINE-BASED *IN VIVO* ENZYME PARAMETERS

As to be expected from numerous observations (see

Table 5. Matrix of correlation between enzyme parameters measured *in vitro* and pharmacokinetic parameters of caffeine measured *in vivo*. Correlation coefficients *r* and number of data pairs available (in parentheses) are given. Column variables were used as independent variables, row variables as dependent variables. Weighting was by the inverse of dependent variables

	Estimated functional liver volume	*Sulfamethazine (500 μM) N-acetylation rate estimated for whole liver*	*Intrinsic clearance of phenacetin O-deethylation*[a]	*Intrinsic clearance of caffeine 3-demethylation*[a]	*Immunoreactive CYP2E1 liver amount*	*Immunoreactive CYP1A2 liver amount*
Urinary ratio A (CYP1A2)	0.327 (n = 19)	0.17 (n = 17)	0.07 (n = 16)	0.44 (n = 17)	0.07 (n = 17)	0.22 (n = 17)
Urinary ratio B (CYP1A2)	0.232 (n = 19)	0.13 (n = 17)	−0.12 (n = 16)	0.39 (n = 17)	0.01 (n = 17)	0.31 (n = 17)
Urinary ratio C (CYP1A2)	0.343 (n = 19)	0.02 (n = 17)	0.36 (n = 16)	0.56 (n = 17)*	0.48 (n = 17)*	0.34 (n = 17)
Urinary ratio D (CYP1A2)	0.348 (n = 19)	0.06 (n = 17)	0.34 (n = 16)	0.53 (n = 17)*	0.48 (n = 17)*	0.32 (n = 17)
Urinary ratio E (NAT2)	0.003 (n = 19)	0.75 (n = 17)****	0.36 (n = 16)	0.36 (n = 17)	0.17 (n = 17)	0.36 (n = 17)
Urinary ratio F (NAT2)	0.021 (n = 19)	0.72 (n = 17)***	0.47 (n = 16)	0.45 (n = 17)	0.26 (n = 17)	0.40 (n = 17)
Caffeine clearance derived from ratio paraxanthine/caffeine during surgery	−0.262 (n = 17)	−0.08 (n = 16)	0.37 (n = 15)	0.68 (n = 16)**	−0.16 (n = 16)	0.45 (n = 16)
Ratio paraxanthine/caffeine in saliva 3 h postdose	0.126 (n = 19)	0.00 (n = 17)	0.19 (n = 16)	0.70 (n = 17)***	0.03 (n = 17)	0.65 (n = 17)***
Ratio paraxanthine/caffeine in plasma 3 h postdose	0.312 (n = 20)	0.03 (n = 18)	0.32 (n = 17)	0.70 (n = 18)***	0.24 (n = 18)	0.58 (n = 18)*
Ratio paraxanthine/caffeine in saliva 6 h postdose	0.209 (n = 18)	−0.03 (n = 17)	0.25 (n = 16)	0.74 (n = 17)****	0.01 (n = 17)	0.67 (n = 17)***
Ratio paraxanthine/caffeine in plasma 6 h postdose	0.340 (n = 20)	−0.03 (n = 18)	0.33 (n = 17)	0.78 (n = 18)****	0.10 (n = 18)	0.66 (n = 18)***
Caffeine clearance (ml min^{-1} kg^{-1}) from combined plasma + saliva	0.298 (n = 20)	−0.04 (n = 18)	0.46 (n = 17)	0.80 (n = 18)****	0.04 (n = 18)	0.76 (n = 18)****
Caffeine clearance (ml min^{-1} kg^{-1}) from saliva concentrations	0.199 (n = 17)	−0.09 (n = 16)	0.40 (n = 15)	0.82 (n = 16)****	0.19 (n = 16)	0.82 (n = 16)****
Caffeine clearance (ml min^{-1} kg^{-1}) from plasma concentrations	0.207 (n = 16)	0.09 (n = 14)	0.39 (n = 14)	0.74 (n = 14)***	0.12 (n = 14)	0.77 (n = 14)***

[a]intrinsic clearance (ml min^{-1}) was calculated as $V_{max/Km}$, where V_{max} is given for the estimated enzyme amount in the whole liver.
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$.

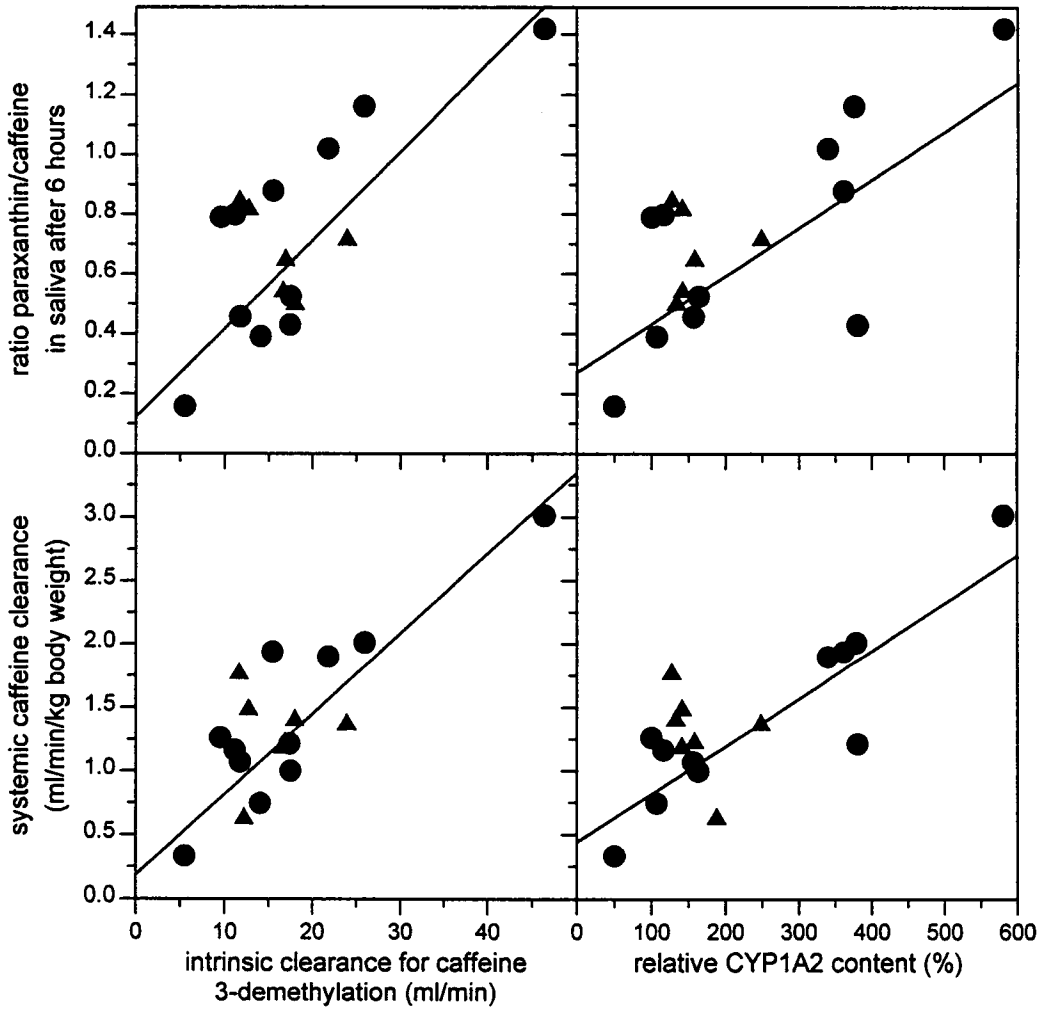

Fig. 3. Relationship of intrinsic clearance for caffeine 3-demethylation and relative amount of CYP1A2 in human liver microsomes to pharmacokinetic parameters of caffeine *in vivo*. Points are measured values, lines represent the weighted regression lines. Circles represent patients without, triangles those with *CYP1A1* mutations. *In vitro* parameters are presented as values for the amount of enzyme present in the whole liver (see Methods section). CYP1A2 amounts are given in arbitrary units and were calculated relative to those of patient 10 (= 100%). Systemic caffeine clearance was derived from both plasma and saliva concentrations. The ratio paraxanthine/caffeine was measured in saliva withdrawn 6 h after administration of the 165 mg caffeine test dose.

Kalow & Tang, 1993; Fuhr & Rost, 1994), CYP1A2 parameters were closely correlated to each other. Caffeine clearance values derived from plasma and/or saliva concentrations and the paraxanthine/caffeine ratios had coefficients for intercorrelation of 0.83 and more, suggesting that all these parameters measure (almost) the same and are interchangeable. Whether the less valid but still significant correlations of these parameters to urinary CYP1A2 metabolite ratios are caused primarily by the use of spot urine samples instead of samples from fixed intervals (Kalow & Tang, 1993) or by other known confounding factors including urinary flow and CYP2A6 activity (Tang *et al.*, 1994b; Tucker *et al.*, 1995) cannot be derived from the data.

CORRELATIONS BETWEEN *IN VIVO* AND *IN VITRO* PARAMETERS

Correlations are not causative. Therefore, it was necessary to exclude at least two situations where the amount of the respective enzyme in the human body depends on unspecific mechanisms. These are a direct relationship of enzyme parameters *in vivo* to either liver size or to overall microsomal protein expression. The lack of correlation of any of the *in vivo* parameters to functional liver volume and of *in vivo* parameters for one enzyme to

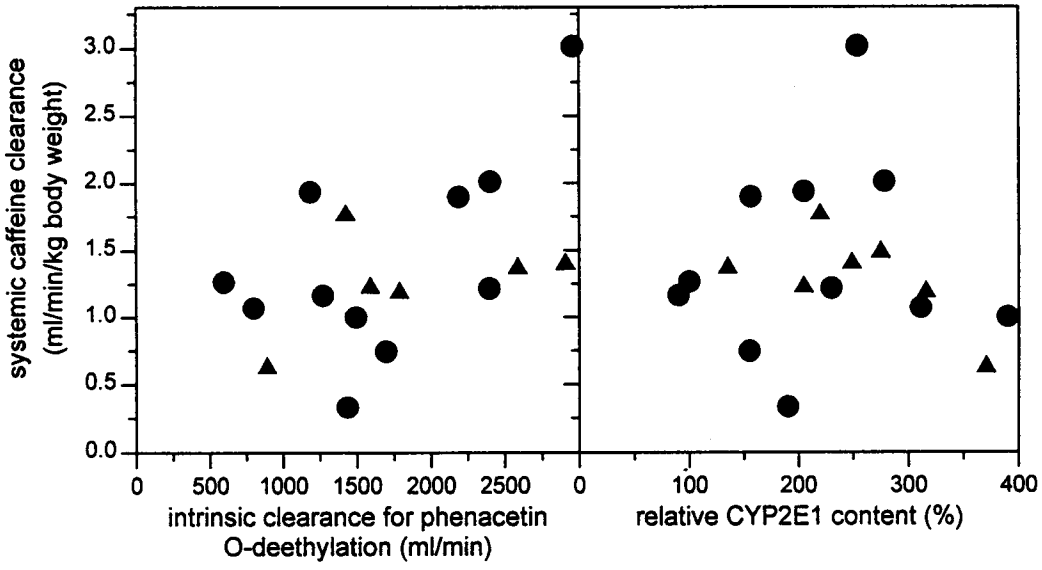

Fig. 4. Relationship of intrinsic clearance for phenacetin *O*-deethylation and relative amount of CYP2E1 in human liver microsomes to systemic caffeine clearance *in vivo*. Points are measured values, regression lines are not shown since correlations were not significant. Circles represent patients without, triangles those with *CYP1A1* mutations. *In vitro* parameters are presented as values for the amount of enzyme present in the whole liver (see Methods section). CYP2E1 amounts are given in arbitrary units and were calculated relative to those of patient 10 (= 100%). Systemic caffeine clearance was derived from both plasma and saliva concentrations.

the *in vitro* parameters of another enzyme, however, suggest that unspecific mechanisms are of minor importance.

CYP1A2

Intrinsic clearance was used to describe enzyme activity *in vitro*, since this parameter takes both affinity and metabolic rate into account and therefore is most suitable for *in vivo* vs *in vitro* comparisons (Kroemer *et al.*, 1992).

Several lines of evidence for the use of caffeine clearance to estimate CYP1A2 activity existed prior to this study. In addition to previous knowledge of caffeine metabolism *in vitro* (see Introduction), there were two important results available. These are the almost identical patterns of metabolic pathways for cDNA expressed human CYP1A2 and for caffeine metabolism *in vivo* (Fuhr *et al.*, 1992a) and the almost complete inhibition of caffeine elimination by furafylline (Tarrus *et al.*, 1987), a specific mechanism-based CYP1A2 inhibitor (Clarke *et al.*, 1994; Tassaneeykul *et al.*, 1994b). The close correlations between CYP1A2 parameters *in vivo* based on plasma or saliva concentrations and CYP1A2 mediated caffeine 3-demethylation and/or CYP1A2 content *in vitro* found in this study support the view that CYP1A2 activity is the major determinant of these parameters (Table 5). A fraction of approximately r^2 = 0.45–0.65 of the variability of these *in vivo* parameters is explained by interindividual diversity of CYP1A2. Thus, they provide a valid and specific estimation of the activity of this enzyme in the patients tested. The study confirms the use of systemic caffeine clearance as a 'standard of convenience' (Kalow & Tang, 1993) for CYP1A2 phenotyping. Due to the simplicity of sampling and analytical methods, the non-invasiveness and excellent correlation to CYP1A2 parameters *in vitro*, the parameter which seems most appropriate for the estimation of CYP1A2 activity *in vivo* is the paraxanthine to caffeine ratio in saliva 6 h postdose.

Lacking correlations of CYP1A2 parameters *in vivo* to phenacetin *O*-deethylation *in vitro* should be caused by the use of intrinsic clearance, which includes affinity of the substrate. K_m values for phenacetin, however, did not correlate to those for caffeine (see 'Intercorrelations of *in vitro* parameters').

As described above, less valid correlations of urinary metabolite ratios to CYP1A2 parameters *in vitro* may in part be due to the experimental conditions. From both *in vivo* intercorrelations and the *in vivo* vs *in vitro* comparisons, however, it seems that those parameters based on paraxanthine metabolism (urinary ratios C and D) provide better estimations of CYP1A2 activity than those based on caffeine 3-demethylation (ratios A and B). This corresponds to the observations of Tang *et al.* (1994b) in healthy volunteers.

Our results were obtained despite a highly heterogeneous study population, the conduction of the caffeine test and liver surgery on different days, multiple comedications during the caffeine test and during surgery, and the limited precision of functional liver volume estimation. The true relationship between caffeine based CYP1A2 parameters *in vivo* and CYP1A2 activity may therefore be even closer in the absence of such confounding factors.

The results should apply also to subjects exposed to cytochrome P450 inducing chemicals. Since caffeine plasma concentrations in most patients did not exceed 30 μM, primary caffeine demethylations should be predominantly due to activity of the high affinity site, which is CYP1A2 (Tassaneeyakul *et al.*, 1992, 1994a), even if caffeine concentrations at the enzyme may be higher than those in plasma. Induction of CYP2E1, the enzyme that presumably mediates the low affinity demethylations of caffeine (Tassaneeyakul *et al.*, 1994a), should therefore have only a minor influence on overall primary caffeine metabolism. Likewise, an increased activity of CYP3A enzymes should have a minor effect on overall caffeine metabolism because caffeine 8-hydroxylation, the pathway mediated by this enzyme subfamily (Tassaneeyakul *et al.*, 1994a), plays only a minor role *in vivo* (Arnaud & Welsch, 1982).

The only other human enzyme that is able to metabolize caffeine with high affinity and a similar pattern of metabolites as CYP1A2 is CYP1A1 (Eugster *et al.*, 1993; Tassaneeyakul *et al.*, 1993; Fuhr *et al.*, 1994). However, CYP1A1 is expressed at very low levels in the liver, even in smokers (Schweikl *et al.*, 1993, and references therein), and caffeine metabolism usually is strictly hepatic since caffeine clearance in patients with liver cirrhosis is reduced to a small fraction of the normal value, given as 0.9 ml min^{-1} per kg body weight (McDonagh *et al.*, 1991). A pronounced extra-hepatic induction of CYP1A1 due to exposure to xenobiotics appears as the only plausible mechanism to reach a relevant contribution of CYP1A1 to caffeine metabolism *in vivo*. An isolated CYP1A1 induction, however, is not probable due to common regulation mechanisms for CYP1A1 and CYP1A2 (Schweikl *et al.*, 1993; Quattrochi *et al.*, 1994). Finally, we did not find differences in caffeine metabolism *in vivo* or *in vitro* between the *CYP1A1* genotypes tested. This is evidence against a major role of CYP1A1 in caffeine metabolism, since both different enzymatic activities for the gene products (Crofts *et al.*, 1994; Landi *et al.*, 1994) and differences in cancer susceptibility between genotypes (Drakoulis *et al.*, 1994), probably brought about by a different handling of carcinogens, have been reported. In summary, current knowledge suggests that caffeine metabolism by CYP1A1 *in vivo* is negligible.

An open question remains why intrinsic clearance for caffeine 3-demethylation, upscaled to whole liver size, was several times lower than caffeine clearance *in vivo* despite the exclusively hepatic elimination of this low clearance drug. That metabolic rates for caffeine *in vitro* are indeed very low has been found by many researchers (e.g. Grant *et al.*, 1987; Berthou *et al.*, 1988). In contrast to our data, recent investigations on the high clearance drug midazolam, comparing 1′-hydroxylation of this CYP3A substrate *in vitro* with midazolam clearance *in vivo* by using the well-stirred model of hepatic clearance (Thummel *et al.*, 1994), and on the low clearance drug aminopyrine (G. Engel, personal communication) provided almost identical values for *in vivo* clearance and upscaled intrinsic clearance. However, this may be a chance finding due to several factors that affect clearance values, including: (i) different conditions for the enzymatic reaction *in vivo* and *in vitro*, including pH, small ions, co-factors, osmolarity etc.; (ii) processes modifying the concentration of the substrate at the enzyme *in vivo*, e.g. intracellular compartmentalization or active extrusion by p-glycoprotein; and (iii) the non-uniform distribution of cytochrome P450s within liver lobules (Ratanasavanh *et al.*, 1991). This suggests that much more still needs to be known and that a considerable refinement of hepatic clearance models is also needed before a truly quantitative upscaling of metabolic *in vitro* data can be calculated in the absence of an empirical *in vivo* vs *in vitro* comparison.

NAT2

The concordant identification of NAT2 phenotypes by both caffeine-based urinary NAT2 ratios and the metabolic rate for sulfamethazine *N*-acetylation confirms that the urinary ratios are appropriate to distinguish between the two NAT2 phenotypes (Grant *et al.*, 1984). A very similar result had been reported in patients undergoing diagnostic liver biopsy (Grant *et al.*, 1990). However, the obvious lack of a correlation between *in vivo* and *in vitro* parameters within the two phenotypes (Fig. 2) suggests that factors confounding the relationship between *in vivo* and *in vitro* activity are also present. These may include the use of a single substrate concentration instead of intrinsic clearance in the standard *in vitro* test applied, the existence of NAT2 mutations with minor effects on enzyme activity (Grant *et al.*, 1994), and a possible contaminating effect of CYP1A2 activity on the urinary metabolite ratios used to estimate NAT2 activity *in vivo* (Tucker *et al.*, 1995; Tucker GT, personal communication).

CYP2E1

The limited data available in this study suggest that CYP1A2 rather than CYP2E1 mediates theophylline

formation from caffeine *in vivo*. This may be different in persons consuming high amounts of ethanol on a regular basis (Tang *et al.*, 1994a).

Conclusions

In conclusion, this study extends previous investigations by others and shows strong *in vivo/in vitro* correlations between the levels and activities of CYP1A2 and NAT2 and the kinetics of caffeine, confirming that indeed the main determinator of caffeine clearance and some derived pharmacokinetic parameters is hepatic CYP1A2 activity, and that differentiation between slow and extensive acetylators using caffeine *in vivo* is in concordance with the results obtained in hepatic cytosol using sulfamethazine as a substrate. Thus, the use of caffeine as an innocuous probe drug provides valid data for the assessment of individual *in vivo* activities of these two enzymes. The parameter which seems most appropriate for the estimation of CYP1A2 activity *in vivo* was the molar concentration ratio of paraxanthine to caffeine in a saliva sample taken 6 h after caffeine intake.

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