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Studies on the Stereoselective Metabolism of Citalopram by Human Liver Microsomes and cDNA-Expressed Cytochrome P450 Enzymes

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Key Words

Citalopram · Stereoselectivity · Metabolism · Cytochrome P450

Abstract

The involvement of CYP enzymes in the metabolism of citalopram was studied, inclusive the conversion of demethylcitalopram to didemethylcitalopram and the formation of citalopram N-oxide, which both have not been considered previously. Using human mixed liver microsomes and cDNA-expressed CYP enzymes, we confirmed that CYP3A4, 2C19 and 2D6 are involved in the first demethylation step of citalopram, all favouring conversion of the biologically active S-enantiomer. Inhibitor studies indicated that at therapeutic citalopram concentrations CYP3A4 was responsible for 40–50% of demethylcitalopram formation, while the contribution of CYP2C19 increased and that of CYP2D6 tended to decrease with increasing drug concentration. CYP2D6 exclusively mediated the second demethylation step, and citalopram N-oxide was also exclusively formed by CYP2D6. None of the studied CYP enzymes mediated deamination to the propionic acid derivative.

Citalopram (CITA) belongs to the group of selective serotonin reuptake inhibitors (SSRIs), used in the treatment of depression [1, 2]. CITA is marketed as a racemic mixture of the S-(+)-CITA and R-(–)-CITA enantiomers. The S-(+) enantiomer is most effective as inhibitor of serotonin reuptake and is considered to be of main importance for the clinical effects [3].

CITA is partly excreted unchanged or conjugated to glucuronic acid in the urine and partly metabolized in the liver [4]. The main metabolites are N-demethylcitalopram (DCITA), citalopram-N-oxide (CITA-NO), N-didemethylcitalopram (DDCITA) and citalo-

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pram propionic acid derivative (CITA-PROP) (fig. 1) [5, 6]. Two independent in vitro studies have dealt with the identification of the cytochrome P450 (CYP) enzymes involved in the first demethylation step of CITA to DCITA [7, 8]. The two studies agreed so far as both suggested that CYP3A4 and CYP2C19 play a role for the demethylation of citalopram but disagreed with respect to the relative importance of the two enzymes and a possible contribution of CYP2D6. Rochat et al. [8] found that CYP2D6 had a high affinity but a low capacity for DCITA formation, while Kobayashi et al. [7] was unable to demonstrate a significant contribution of the enzyme. Rochat et al. [8] also compared the enzyme kinetic constants for demethylation of the two enantiomers of CITA and found a pronounced stereoselectivity with respect to reaction velocity. The V_{max} of the CYPs 3A4 and 2C19 catalyzed DCITA formation of the S-enantiomer was about 1.5 times the V_{max} of the R-enantiomer. CYP2D6 showed an opposite stereoselectivity, the capacity for demethylation of the S-enantiomer being 0.7 times that of R-DCITA formation. In the above-mentioned studies the formation of CITA-NO, DDCITA and CITA-PROP was not considered, but recent investigations have shown that monoamine oxidases (MAO) and aldehyde oxidases presumably are of importance for CITA-PROP formation [9].

The purpose of the present study was to clarify the discrepancies of the above-mentioned studies and extend the knowledge on citalopram disposition. The focus was on the stereoselectivity of DCITA formation. Additionally, the second demethylation step of CITA to DD-CITA was examined in detail and finally the formation of CITA-NO, which both have not been studied previously. The present investigations were based both on a mixed human liver microsome preparation (HLM) and individual human cDNA-expressed CYP enzymes. As a part of the studies, the selectivities of traditionally used CYP inhibitors were considered.

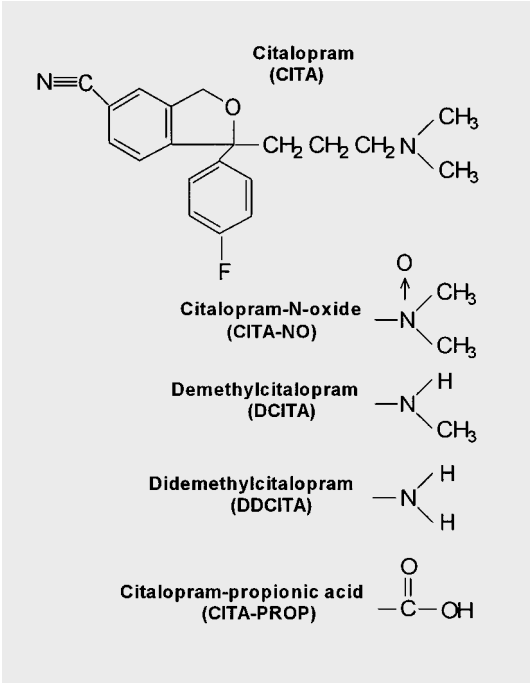


Fig. 1. Molecular structures of citalopram and metabolites.

Materials and Methods

Drugs and Chemicals

Lundbeck A/S (Copenhagen, Denmark) kindly supplied racemates and individual enantiomers of CITA, DCITA, DDCITA, CITA-NO and CITA-PROP. LU-10-2029, which corresponds to citalopram with chlorine substituted for fluor in the phenyl ring, amitriptyline, nortriptyline and E-10-OH-nortriptyline were also gifts from Lundbeck A/S. Fluvoxamine and ketoconazole were donated by Solvay Duphar B.V. (Weesp, the Netherlands) and Janssen Pharmaceutica N.V. (Beerse, Belgium), respectively. Caffeine and 1,7-dimethylxanthine (paraxanthine) were from Sigma Chemical Co. (St Louis, Mo., USA). (S)-mephe-

nytoin, 4′-hydroxymephenytoin, 4′hydroxydiclofenac, chlorzoxazone and 6-hydroxychlorzoxazone were purchased from Gentest Co. (Woburn, Mass., USA). Other chemicals were of analytical grade. An HLM, prepared as a mixture of microsomes from six different donors, and cDNA-expressed human CPYs 1A2, 3A4, 2C9, 2C19, 2E1 and 2D6, prepared from baculovirus-infected insect cells, were also obtained from Gentest Co. The microsomal preparations were received frozen and were stored at −80 °C until use. According to the supplier, the contents of the individual CYP enzymes in HLM have been determined by immunoquantitation. The amounts of the CYP enzymes investigated, expressed per mg protein, were 45 pmol CYP1A2, 108 pmol CYP3A4, 96 pmol CYP2C9, 19 pmol CYP2C19, 49 pmol 2E1 and 10 pmol CYP2D6. The activity of flavine containing monooxygenase (FMO) was given as 2.53 nmol/mg protein/min methyl *p*-tolyl sulfide oxidation.

Experimental Procedure

The enzymatic reactions were carried out in polypropylene tubes at 37 °C in a total volume of 300 µl of 0.1 mol/l phosphate buffer, at pH 7.4 as previously described [10]. To avoid unspecific inhibition no organic solvents were used for dissolving any of the compounds. The NADPH generating system consisted of 1 U isocitrate dehydrogenase, 5 mmol/l NADP, 5 mmol/l isocitrate and 5 mmol/l $MgCl_2$. The reaction was started by addition of 50–100 µg HLM protein or 2.5–5 pmol of the individual CYP enzymes and stopped after 7.5 min by addition of 0.5 ml ice-cold 1 mol/l acetic acid. Blank values were obtained by adding the stop reagent before enzymes. In order to qualitatively assess the CYP enzymes involved in the metabolism of CITA, incubation times of 60 min were used with substrate concentrations of 2, 10 and 200 µmol/l. Qualitative checks of the activities and identities of the individual CYP enzymes were carried out using established marker reactions. For CYP1A2 the ability to mediate the 3-demethylation of caffeine to paraxanthine was used [11]. Amitriptyline N-demethylation to nortriptyline served to demonstrate the activity of CYP3A4 [12]. CYP2C9 activity was checked by formation of 4′-hydroxydiclofenac [13]. CYP2C19 activity was assessed by the conversion of S-mephenytoin to 4′-hydroxy-mephenytoin [14]. The activity of CYP2D6 was evaluated by conversion of nortriptyline to E-10-hydroxynortriptyline [15]. Finally, the conversion of chlorzoxazone to 6-hydroxychlorzoxazone served as a check of CYP2E1 activity [16]. For all processes proportionality between the amount of enzyme added and the products formed was found.

Inhibitor Studies

In order to determine the quantitative contribution of the individual CYP enzymes to the total HLM catalyzed demethylation of CITA, inhibitors were added to the reaction medium. Ketoconazole in the concentration range 0.25–2.5 µmol/l was used to inhibit CYP3A4 activity, fluvoxamine in the concentration range 0.5–5 µmol/l served as inhibitor of CYP2C19, and quinidine in the concentration range 0.125–5 µmol/l was used as inhibitor of CYP2D6. The optimal concentration of an inhibitor was finally selected as a compromise between an acceptable high inhibitory effect on the target enzyme and a reasonably low inhibitory activity towards the other enzymes involved in DCITA formation as described in detail later.

Assay of DCITA, DDCITA, CITA-NO and CITA-PROP

DCITA and DDCITA in the incubate were determined essentially as previously described [17]. In short, after addition of 0.5 ml 1.6 mol/l sodium carbonate/bicarbonate buffer, pH 10 and internal standard (LU-10-2029), the compounds were extracted into 7.5 ml heptane-isoamylalcohol (98:2, v/v) by shaking. After centrifugation the analytes were back-extracted from the organic phase into 125 µl phosphoric acid 250 mmol/l, of which 65 µl was injected into the HPLC apparatus. After extraction of DCITA and DDCITA from the incubate, amitriptyline was added as a new internal standard, and the more polar CITA-NO was extracted into 7.5 ml heptane-isoamylalcohol (90:10, v/v) and back-extracted into 125 µl phosphoric acid as described above. The acid CITA-PROP was extracted directly from the incubate after addition of 0.5 ml 1 mol/l acetic acid also using 10% isoamylalcohol in heptane. The organic phase was evaporated to dryness, and the residue was dissolved in 125 µl mobile phase, of which 65 µl was injected into the HPLC apparatus. The compounds were separated with an acetonitrile-44 mmol/l phosphate buffer, pH 2.5-mobile phase containing 10 mmol/l TEA on a Hypersil BDS C_{18} column and measured by UV detection at 240 nm. The lower levels of quantification were 1.8, 2.5, 3.3 and 3.8 pmol for DCITA, DDCITA, CITA-NO and CITA-PROP, respectively. The interday CV for DCITA and DDCITA determination was $<8\%$, and for CITA-NO and CITA-PROP the CV was $<10\%$.

Data Analysis

Reaction velocities (V) were expressed as nmol of product formed per hour per mg protein, when HLM was used, and as mol of product formed per hour per

Table 1. Kinetic parameter values for the metabolism of citalopram enantiomers

Reaction	Parameters	Microsomes		CYP2C19		CYP2D6	
		(+)S	(–)R	(+)S	(–)R	(+)S	(–)R
N-demethylation	K_m, µmol/l	95 (12)	76 (11)	148 (25)	135 (18)	7.3 (0.6)[b]	24 (1.6)
	V_{max}, nmol h^{-1} mg^{-1} protein	26 (1.0)[b]	18 (0.7)				
	V_{max}, mol h^{-1} mol^{-1} CYP			1490 (88)[b]	890 (42)	626 (11)[b]	360 (7.2)
N-oxidation	K_m, µmol/l	13 (3.9)	33 (11)			7.0 (1.5)[a]	18 (3.7)
	V_{max}, nmol h^{-1} mg^{-1} protein	0.63 (0.05)[b]	0.16 (0.02)				
	V_{max}, mol h^{-1} mol^{-1} CYP					110 (5.0)[a]	131 (7.5)
N-didemethylation	K_m, µmol/l					7.3 (0.9)	6.0 (0.6)[1]
	V_{max}, mol h^{-1} mol^{-1} CYP					82 (2.4)[b]	99 (2.0)[1]

Values are means with SEs in parentheses based on 2–3 replicates at 6–8 different substrate concentrations.
S-enantiomer versus R-enantiomer: [a] $p < 0.05$; [b] $p < 0.01$.
[1] K_m and V_{max} calculated using one-binding site.

mol of CYP enzyme, when the CYP enzyme activities were measured. V as a function of increasing substrate concentrations (S) was fitted by nonlinear regression according to the Michaelis-Menten equation, using GraphPad Prism software (San Diego, Calif., USA). The software allows a statistical comparison between a one-site binding fit and a two-site binding fit by means of an F test. V_{max} and K_m were determined on the basis of the mean of two or three replicates of V measurements using 6–8 levels of substrate concentration. Standard errors for the parameter estimates V_{max} and K_m were derived from the scatter of the individual points around the estimated curve on the basis of a least-squares computation principle [18]. In addition to the saturation curves, Eadie-Hofstee plots were also displayed. In this linearisation of the Michaelis-Menten equation, V is plotted against V/S, and the slope and intercept at the y-axis, determined by linear regression, correspond to $-K_m$ and V_{max}, respectively [19].

Results

The estimated K_m and V_{max} values for the HLM-mediated N-demethylation and N-oxidation of S- and R-CITA are shown in table 1. It appears that the rate of N-oxidation was small compared to the rate for N-demethylation. The apparent V_{max} values for both DCITA and CITA-NO formation were significantly highest for the S-enantiomer. The apparent K_m values of the two enantiomers were not significantly different for the two processes (table 1).

Initial screening of the CYP enzymes 1A2, 3A4, 2C9, 2C19, 2D6 and 2E1 for activity towards citalopram showed that CYPs 3A4, 2C19 and 2D6 displayed significant N-demethylation activity. The formations of S- and R-DCITA as a function of incubation time are shown in figure 2. The figure shows that the reaction rate generally decreased with time, and for CYP3A4 the DCITA formation ceased completely after about 10 min. Therefore, an incubation time of 7.5 min was used throughout in the quantitative studies. Only CYP2D6 mediated formation of measurable amounts of CITA-NO, and CYP2D6 was also the only of the CYP enzymes tested, which mediated the second demethylation of CITA, the conversion of DCITA to DDCITA. Saturation plots for CYP2C19- and CYP2D6-mediated reactions and the corresponding Eadie Hofstee plots are shown in figure 3, and the measured K_m and V_{max} values are given in

table 1. CYP2D6 exhibited the highest affinity, while CYP2C19 had the highest capacity for conversion of CITA to DCITA.

With regard to CYP3A4, it was not possible to determine K_m and V_{max}, because at high substrate concentration irreproducible results were obtained with respect to DCITA formation. However, an almost linear relationship between substrate concentrations and DCITA formation was found, when the CITA concentration was kept below 20 µmol/l. The relations (mean and SE) between the reaction rate

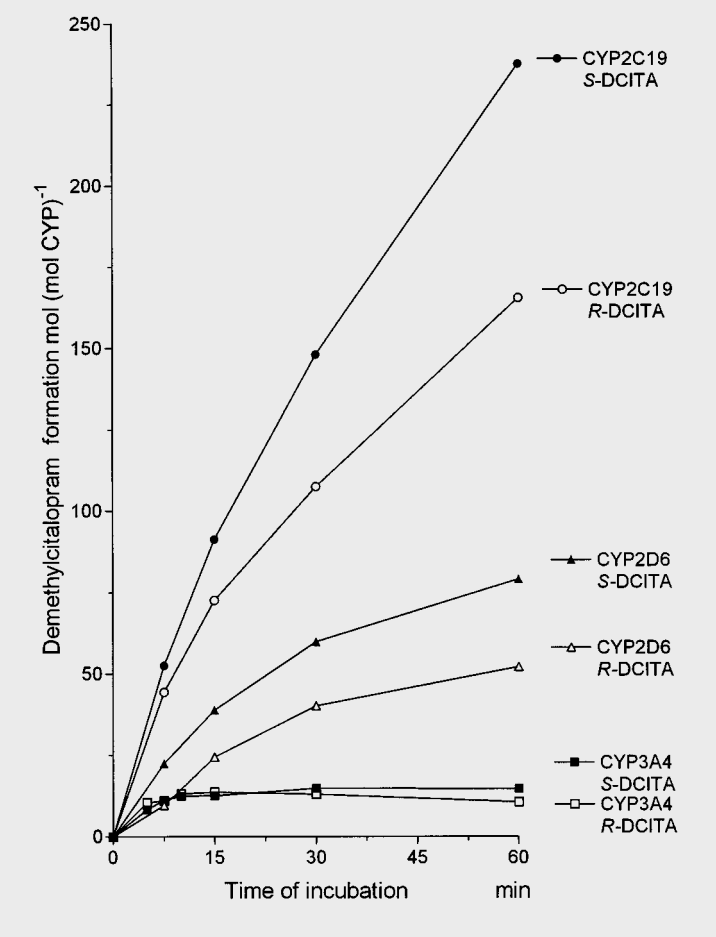
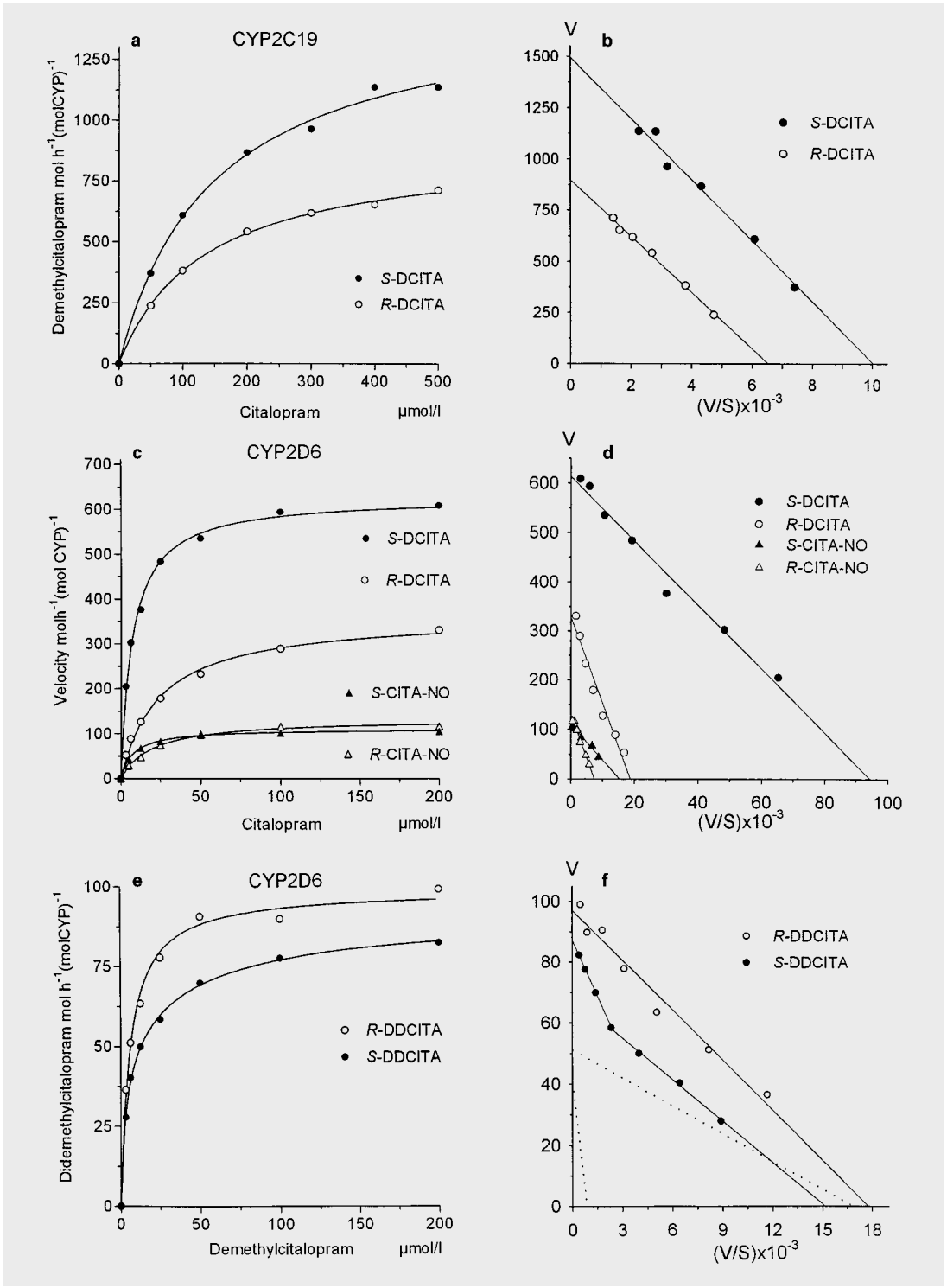


Fig. 2. Demethylation of citalopram enantiomers mediated by CYP2C19, CYP2D6 and CYP3A4 as a function of time.

Fig. 3. Saturation curves and the corresponding Eadie-Hofstee plots for demethylation of citalopram enantiomers by CYP2C19 (**a**, **b**), CYP2D6 (**c**, **d**) and demethylation of DCITA enantiomers by CYP2D6 (**e**, **f**). The slope and intercept of the dotted lines in figure 5f represent the calculated $-K_m$ and V_{max} values using a two-binding-site equation. Each point represents the mean of 2 or 3 determinations of V.



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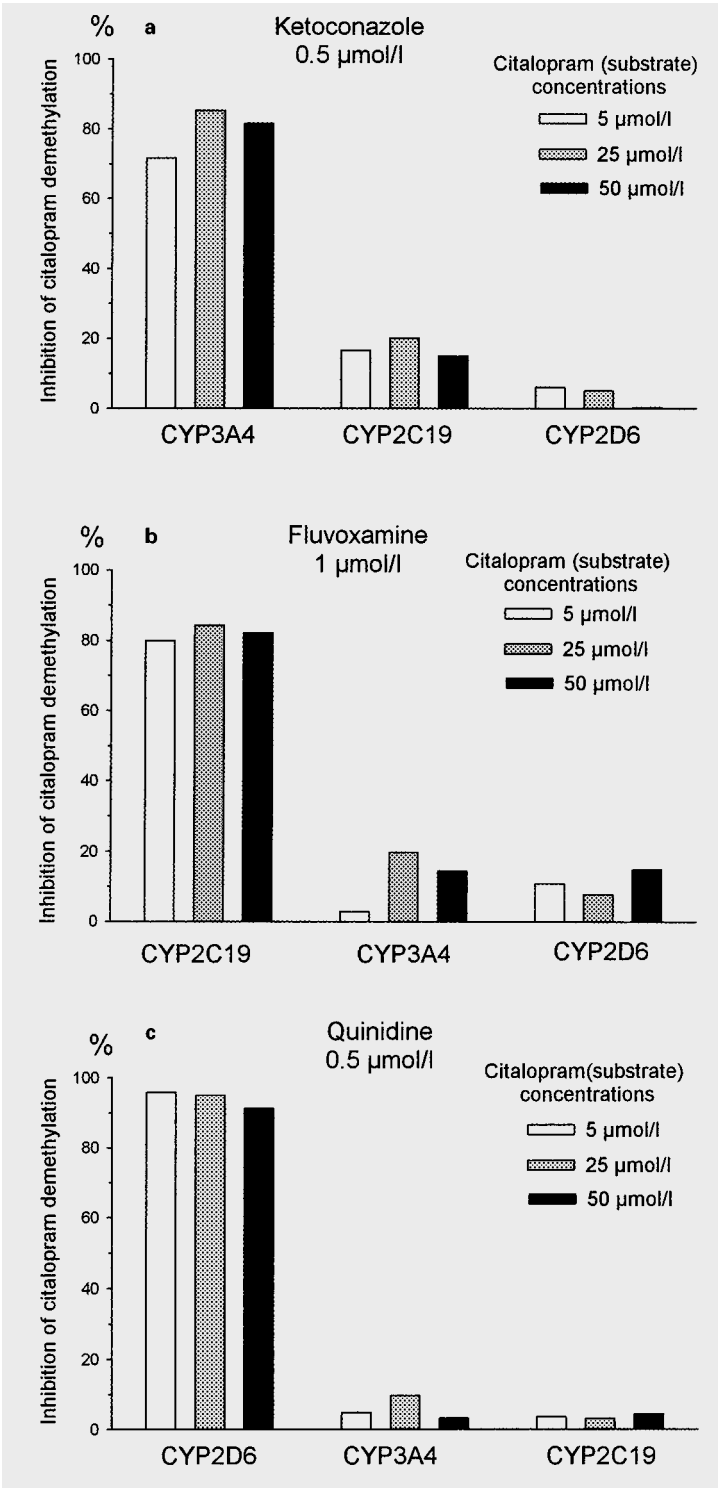

Fig. 4. Inhibition of CYP3A4, 2C19 or 2D6 catalyzed demethylation of racemic citalopram. Bars represent means of triplicate determinations of the percentage inhibition of control reactions without inhibitors.

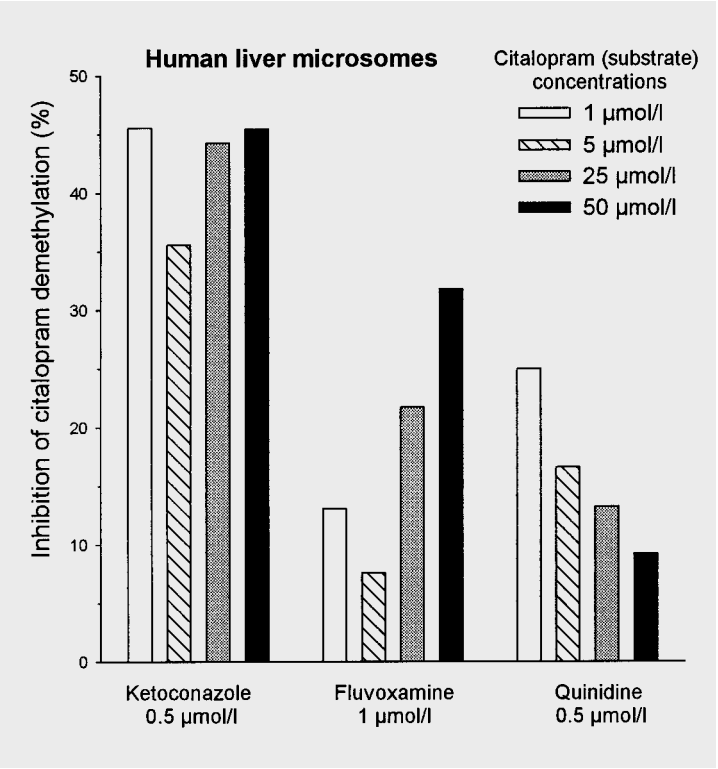

Fig. 5. Inhibition of HLM-mediated demethylation of racemic citalopram by ketoconazole, fluvoxamine and quinidine. Bars represent mean of triplicate determinations of percentage inhibition of control reaction without inhibitors.

(V) expressed as mol h^{-1} mol CYP and the substrate concentration (S) expressed in µmol/l were $V = S \times 0.82 \pm 0.017$ (n = 10) and $V = S \times 0.55 \pm 0.015$ (n = 8) for S-CITA and R-CITA, respectively. The difference between the slopes was significant ($p < 0.01$), indicating that for all the three enzymes involved in the demethylation of CITA, the velocity for demethylation of the S-enantiomer was higher than for the R-enantiomer.

The second demethylation of CITA to DDCITA was exclusively mediated by CYP2D6 (fig. 3e, f). A significantly better fit ($p < 0.001$) was obtained when a two-binding-site model was used for S-DDCITA formation. K_m was 3.0 µmol/l and K_{m2} was 44 µmol/l, and the corresponding V_{max} values were 51 and 40 mol h^{-1} (mol CYP2D6)$^{-1}$, respectively (fig. 4f). The recorded K_m and V_{max} values, provided that a one-binding-site equation was used for the CYP2D6-mediated DDCITA formation, are given in table 1.

The individual contribution of CYPs 3A4, 2C19 and 2D6 to the demethylation of racemic CITA mediated by the mixed human liver microsome preparation was determined by addition of inhibitors to the incubation medium. Reasonable compromises with respect to inhibitor concentrations are shown in figure 4. Ketoconazole (0.5 µmol/l) reduced the mean CYP3A4 control (uninhibited) activity by 80 ± 7% (SD), while the activities of CYP2C19 and CYP2D6 were reduced by 17 ± 3 and 4 ± 3%, respectively (fig. 4a). Fluvoxamine (1 µmol/l) reduced the CYP2C19 activity by 82 ± 2% and the activities of

CYP3A4 and CYP2D6 by 12 ± 9 and 11 ± 4%, respectively (fig. 4b). Finally, the CYP2D6 catalyzed control activity was reduced by 94 ± 2% by quinidine (0.5 μmol/l), while CYP2C19 and CYP3A4 activities were diminished by 6 ± 2% and 4 ± 1%, respectively (fig. 4c). Addition of ketoconazole inhibited HLM catalyzed DCITA formation by 36–46% independent of the substrate concentration, indicating that CYP3A4 could be held responsible for 40–50% of DCITA formation (fig. 5). The contribution of CYP2C19, measured by fluvoxamine inhibition, increased significantly ($p < 0.05$) from 13 to 30% with increasing substrate concentration, while the contribution of CYP2D6, measured by quinidine inhibition, tended to decrease when the substrate concentration was increased from 1 to 50 μmol/l.

The ability of HLM to catalyze CITA-PROP formation was tested with CITA, DCITA, DDCITA and CITA-NO, respectively, as substrates. Using an incubation time of 60 min and a substrate concentration of 100 μmol/l, DCITA was the only one of the compounds tested which gave rise to a measurable amount of CITA-PROP formation. However, only about 0.1 nmol/mg protein of CITA-PROP was formed during 1 h, and at lower substrate concentrations the amount of metabolite formed was below the level of quantification.

Discussion

Concerning the first demethylation step of citalopram, our results support the findings of Rochat et al. [8]. Both inhibition studies of HLM-mediated demethylation and cDNA-expressed CYP-mediated demethylation of citalopram show that CYP3A4, 2C19 and 2D6 are involved. Compared to the study by Rochat et al. [8], we found a somewhat higher V_{max} for CYP2D6 and also the same stereoselective pattern for this enzyme as for the other two. According to our study, all enzymes favour a more rapid demethylation of the S-enantiomer, which agrees with the fact that the mean plasma concentration of the S-enantiomer in patients in steady state only amounts to 35–37% of the total concentration of CITA [20, 21].

To judge about the relative contribution in vivo of the three enzymes from in vitro studies, estimates of drug concentrations in hepatic tissue are necessary. The plasma concentrations of racemic CITA in patients treated with standard doses of 20–40 mg/24 h are in the range 30–500 nmol/l, and about 80% is protein bound [6]. Rochat et al. [8] used the unbound fraction multiplied by a liver-water partition coefficient of 20 as a measure of a likely concentration of CITA at the CYP receptor site. Using this calculation principle, the concentrations of CITA in liver tissue will be in the range 0.1–2 μmol/l. If protein binding is not taken into account, the CITA concentration in liver would be in the range 0.6–10 μmol/l. Therefore, it seems reasonable to suggest that during human steady-state treatment with CITA the concentration in liver tissue hardly exceeds 10 μmol/l. According to the measured kinetic parameters of the pure CYPs 2C19 and 2D6 and an activity of CYP3A4, which is proportional to the substrate concentration when kept below 20 μmol/l, each of the three CYP enzymes should significantly contribute to the metabolism of CITA. The content of the CYPs 3A4, 2C19 and 2D6 in the HLM was, according to the supplier, determined to be 108, 19 and 10 pmol per mg protein. Using the kinetic parameters obtained from the experiments with pure CYP enzymes, the simulated contribution of the individual CYP enzymes to the demethylation of CITA can be calculated. For CYP2C19 and 2D6 the DCITA formation

(V), expressed as pmol h^{-1} (mg protein)$^{-1}$ is $V = V_{max} \times S/(K_m + S)$ multiplied by the number of pmol CYP per mg HLM. For CYP3A4, which showed atypical kinetic properties corresponding to a linear relationship between substrate concentration and DCITA formation at substrate concentrations below 20 μmol/l, the corresponding DCITA formation is $V = 0.69 \times S$ multiplied by 108. The simulated percentage contribution of CYP3A4 to DCITA formation using substrate concentrations in the range 1–50 μmol/l increases from 13 to 28%, the contribution of CYP2C19 increases from 26 to 43% and the contribution of CYP2D6 decreases from 61 to 29%. Thus, compared to the inhibitor study of the HLM-mediated DCITA formation, a simulation study highly overestimates the contribution of CYP2D6 and underestimates the contribution of CYP3A4 to the total DCITA production. As both quinidine and ketoconazole in the used concentrations can be regarded as relatively specific inhibitors of CYP2D6 and 3A4, respectively, it seems reasonable to suggest that the contributions found by the inhibitor study is more close to in vivo conditions. We used fluvoxamine as inhibitor of CYP2C19 knowing that fluvoxamine is an even stronger inhibitor of CYP1A2 and therefore only can be used as inhibitor of CYP2C19 in the absence of CYP1A2 activity [22, 23]. At higher fluvoxamine concentrations, the activities of CYP2D6 and CYP3A4 are also inhibited [24]. This explains the finding of Rochat et al. [8] that fluvoxamine in a concentration of 100 μmol/l in the incubation medium inhibited HLM catalyzed DCITA formation by 64%.

CYP2D6 was found to be exclusively responsible for the second demethylation of CITA to DDCITA and it seems in agreement with in vivo findings. Studies on panels of poor and extensive metabolizers with regard to CYP2D6 and 2C19 suggested that 2D6 solely played a role for the second demethylation step and 2C19 was partially responsible for the first demethylation step [25]. The latter is in agreement with our in vitro findings. Contradictory results have been obtained with respect to the role of CYP2D6 for DCITA formation in humans. Administration of a single dose of methotrimeprazine, a phenothiazine derivative known as inhibitor of CYP2D6, to human volunteers given CITA increased the plasma concentration of DCITA but not the half-life of CITA [26]. However, long-term coadministration of phenothiazines to patients in steady-state treatment with CITA led to a 30% increase of the mean plasma CITA concentration [27]. Concerning a role of CYP3A4 for the DCITA formation in humans, it was found that among benzodiazepines only alprazolam, known as a rather specific substrate for CYP3A4, increased the plasma CITA concentration [28]. The finding that concomitant treatment with carbamazepine lowered the plasma CITA concentration may also suggest a role for CYP3A4 [29]. Finally, comedication with the multiple CYP enzyme inhibitors cimetidine and fluvoxamine has led to increase of CITA in plasma [30, 31].

Only CYP2D6 mediated a measurable N-oxidation of CITA, and both our experiments using pure enzyme and HLM suggest a minor role of this pathway for the metabolism of CITA. The concentration of CITA-NO in plasma is negligible, and the excretion of the metabolite in urine amounts to only 7% of the ingested dose [4, 5]. Finally, our results indicate that CYP enzymes play an insignificant role for the deamination of CITA, DCITA, DDCITA or CITA-NO to CITA-PROP. A recent study indicates that deamination of CITA, DCITA, and DDCITA are mediated by monoamine oxidases (MAO) and aldehyde oxidases [9].

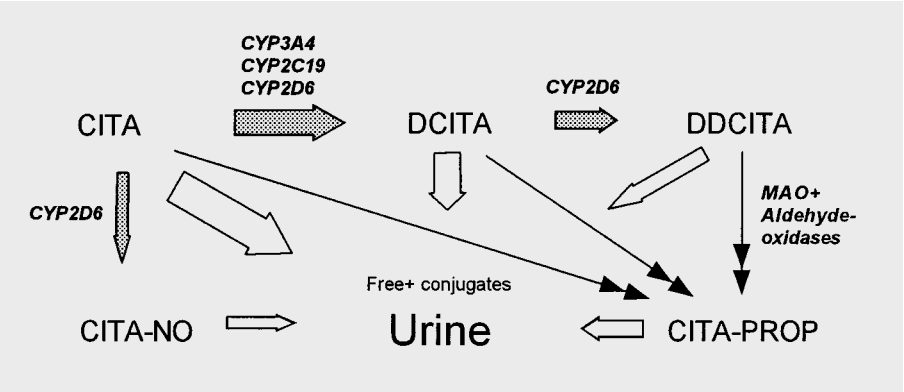

Fig. 6. Schematic drawing of the metabolic pathways and excretion of citalopram.

The main metabolic pathways and the total elimination of CITA in humans seem almost fully elucidated (fig. 6). About 38% of an ingested dose is excreted in the urine either unchanged or conjugated to glucuronic acid and about 19% as free DCITA in the urine [4]. About 15% of a dose is excreted as free and conjugated DDCITA and about 7% as CITA-NO. Finally, CITA, DCITA, DCCITA and CITA-NO may be deaminated to and excreted as CITA-PROP in amounts corresponding to about 8% of the dose.

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