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Fluvoxamine is a Potent Inhibitor of the Metabolism of Caffeine *in vitro*

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Abstract: The selective serotonin re-uptake inhibitor, fluvoxamine, is a very potent inhibitor of CYP1A2, and accordingly causes pharmacokinetic interactions with drugs metabolised by CYP1A2, such as caffeine, theophylline, imipramine, tacrine and clozapine. Interaction between caffeine and fluvoxamine has been described *in vivo*, leading to lowering of total clearance of caffeine by 80% during fluvoxamine intake. The main purpose of the present study was to evaluate this interaction *in vitro* in human liver microsomes. A high-performance liquid chromatography method was developed in order to assay 1,3-dimethylxanthine, 1,7-dimethylxanthine, 3,7-dimethylxanthine and 1,3,7-trimethyluric acid formed from caffeine by human liver microsomes. The limit of detection was 0.06 nmol · mg protein^{-1} · hr^{-1}. As expected, fluvoxamine was a very potent inhibitor of the formation of the *N*-demethylated caffeine metabolites, displaying K_i values of 0.08–0.28 μM. The formation of 1,7-dimethylxanthine was virtually abolished by 10 μM of fluvoxamine, indicating that the *N*3-demethylation of caffeine is almost exclusively catalysed by CYP1A2. The CYP3A4 inhibitors, ketoconazole and bromocriptine, inhibited 1,3,7-trimethyluric acid formation with K_is of 0.75 μM and 5 μM, respectively, thus further supporting the involvement of CYP3A4 in the 8-hydroxylation of caffeine. The study shows that fluvoxamine, as expected, is a potent inhibitor of the metabolism of caffeine *in vitro*.

Fluvoxamine is an antidepressant that belongs to the group of selective serotonin re-uptake inhibitors. Fluvoxamine is oxidated by CYP1A2 and CYP2D6 (Spigset *et al.* 1995; Carrillo *et al.* 1996), and fluvoxamine is a very potent inhibitor of the former enzyme (Skjelbo & Brøsen 1992; Brøsen *et al.* 1993; Rasmussen *et al.* 1995). Accordingly fluvoxamine causes pharmacokinetic interactions with drugs metabolised by CYP1A2, such as theophylline (Sperber 1991; Diot *et al.* 1991), caffeine (Jeppesen *et al.* 1996), imipramine (Spina *et al.* 1992), clomipramine (Bertschy *et al.* 1991) and clozapine (Jerling *et al.* 1994; Hiemke *et al.* 1994).

Caffeine (1,3,7-trimethylxanthine) metabolism in humans is very complex and at least 14 metabolites have been identified. The main route of elimination is *N*3-demethylation to paraxanthine (1,7-dimethylxanthine), and this route accounts for more than 80% of the elimination of caffeine (Lelo *et al.* 1986). Further, caffeine is *N*1- and *N*7-demethylated to theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), and 8-hydroxylated to 1,3,7-trimethyluric acid. A minor amount of caffeine is excreted unchanged in the urine. All four primary caffeine metabolites are further demethylated and/or hydroxylated *in vivo*, but these metabolites are usually not seen *in vitro*. CYP1A2 has been shown to be the major enzyme catalysing the formation of 1,7-dimethylxanthine (Butler *et al.* 1989; Sesardic *et al.* 1990; Berthou *et al.* 1991), and to be partially involved in the formation of 3,7-dimethylxanthine and 1,3-dimethylxanthine (Grant *et al.* 1987; Berthou *et al.* 1991; Gu *et al.* 1992). The remainder of the formation of 3,7-dimethylxanthine and 1,3-dimethylxanthine is believed to be catalysed by CYP2E1 (Tassaneeyakul *et al.* 1994). At least three P450 enzymes, CYP1A2, CYP2E1 and CYP3A4 contribute to the formation of 1,3,7-trimethyluric acid (Gu *et al.* 1992).

The interaction between caffeine and fluvoxamine has been thoroughly investigated *in vivo* by Jeppesen *et al.* (1996), who showed that the total clearance of caffeine decreased by 80% during concomitant fluvoxamine intake in eight healthy volunteers, but the interaction has never been evaluated *in vitro*.

The present study was undertaken in order to confirm that fluvoxamine inhibits caffeine metabolism *in vitro*. The aim of the study was to further support that caffeine is metabolised by CYP1A2, and although fluvoxamine also inhibits CYP2C19 (Jeppesen *et al.* 1997; Rasmussen *et al.* 1998), to substantiate that fluvoxamine is a useful tool for assessment of CYP1A2 in drug metabolism. Thus, an HPLC-method for the determination of the primary caffeine metabolites, 1,3-dimethylxanthine, 1,7-dimethylxanthine, 3,7-dimethylxanthine and 1,3,7-trimethyluric acid in human liver microsomes was developed in order to perform inhibition studies with fluvoxamine and other drugs.

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Materials and Methods

Chemicals and reagents. The drugs used were kindly donated by the following companies: fluvoxamine: Duphar B. V. (Weesp, Holland), paroxetine: Novo Nordisk Farmaka A/S (Copenhagen, Denmark),

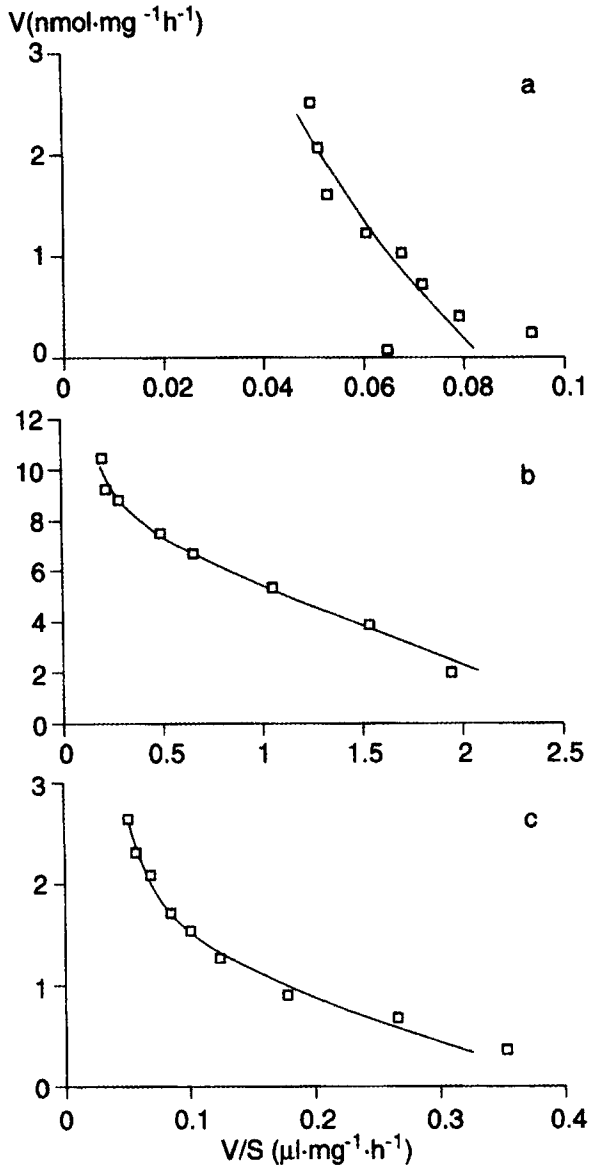

Fig. 1. Eadie-Hofstee plots of the formation rate (V) of a) 1,3-dimethylxanthine, b) 1,7-dimethylxanthine and c) 3,7-dimethylxanthine after incubating microsomes from HL2 with caffeine in concentrations (S) from 1.0 to 50 mM. Each point represents the mean of dublicate determinations and the lines represent the best fits according to Equation 1.

fluoxetine: Eli Lilly A/S (Copenhagen, Denmark), citalopram: Lundbeck A/S (Copenhagen, Denmark), 8-methoxypsoralen: Nycomed DAK A/S (Copenhagen, Denmark), bromocriptine: Sandoz Ltd (Basel, Switzerland), ketoconazole: Janssen Pharma (Beerse, Belgium). Caffeine, 1,3-dimethylxanthine, 1,7-dimethylxanthine, 3,7-dimethylxanthine, 1,3,7-trimethyluric acid, β-hydroxyethyltheophylline, diethyldithiocarbamate, α-naphthoflavone and quinidine were purchased from Sigma (Missouri, USA). All drugs were dissolved in distilled water, exept for 8-methoxypsoralen, which was dissolved in 2.5 mM HCl, and bromocriptine and α-naphthoflavone which were dissolved in 5% methanol. Other chemicals and solvents were of analytical grade and were obtained by either Sigma or Merck (Darmstadt, Germany).

Liver microsomes. Three human livers (HL1, HL2 and HL3) were obtained from kidney donors shortly after circulatory arrest. The livers were immediately cut into slices, frozen in dry ice and stored at −80°. Microsomes were prepared by a standard technique (Meier *et al.* 1983) and the microsomal protein concentration was measured according to Lowry *et al.* (1951). Approval was obtained from the regional Ethics Committee. Microsomes from HL1, HL2 and HL3 were used for determining initial estimates of V_{max} and K_m and microsomes from HL1 were used in the inhibition studies.

Assay conditions. Microsomes were incubated in a final volume of 500 µl in a sodium-phosphate buffer (0.1 M, pH 7.4) using 500 µg microsomal protein. The reaction was started by the addition of 50 µl of an NADPH-generating system (concentrations in microsomal suspensions: $NADPNa_2$, 1 mM; isocitrate, 5 mM; isocitrate dehydrogenase 1 U/ml, $MgCl_2$, 5 mM). Incubations were carried out at 37° in a shaking water bath, and the reactions were stopped after 40 min. by the addition of 350 µl ice-cold zinc sulphate (2% w/v) followed by 25 µl of the internal standard, β-hydroxyethyltheophylline (300 µM). The extraction procedure and the subsequent analysis of the metabolites formed were performed by modifications of a previously published method for determining theophylline and metabolites in human plasma and urine (Rasmussen & Brøsen 1996). Briefly, the solution was acidified by 25 µl HCl (2 M), and the compounds were extracted by ethylacetate/2-propanol (90:10 v/v). The samples were analysed by HPLC at 273 nm using a mobile phase that consisted of sodium acetate (pH 4.0)/methanol (91:9 v/v), and a flow gradient system with a flow-rate of 1 ml/min. from 0–11.5 min., 1.5 ml/min. from 11.6–18.0 min. and 2.5 ml/min. from 18.1–33.0 min. The intra-day precision of the assay was 1 to 8% and the inter-day reproducibility was 3 to 5% (coefficients of variation). The limit of determination was 0.06 nmol · mg protein^{-1} · hr^{-1}. The reactions were linear with incubation time from 10 to 90 min. and with a protein content from 100 to 1000 µg.

The commercially available caffeine used in the assay was contaminated with small amounts of its metabolites. These impurities were taken into account by subtracting samples containing non-metabolised caffeine from the tests samples at all concentrations of caffeine used.

Kinetic analysis of data. In order to estimate initial values of K_m and V_{max} for the use in the inhibition studies, the velocities of formation of 1,3-dimethylxanthine, 1,7-dimethylxanthine, 3,7-dimethylxanthine and 1,3,7-trimethyluric acid were investigated with microsomes from three livers. In a pilot study, the K_m values found for the formation of the caffeine metabolites ranged between 1–3 mM. Therefore, caffeine was used in 9 final concentrations ranging from 1 to 50 mM. For 1,3-dimethylxanthine, 1,7-dimethylxanthine and 3,7-dimethylxanthine the relation between velocity and the ratio of velocity to substrate concentration was curvilinear (Fig. 1, results for HL2), indicating that at least two distinct enzymes are responsible for the formation of each of the three metabolites. Hence, an equation that describes a two-enzyme model was fitted to the data:

$$V = V_{max}\frac{[S]}{K_m+[S]} + L\cdot[S] \qquad \text{(Eq. 1)}$$

This model assumes that each of the metabolites is formed in parallel by a high affinity enzyme (low K_m) and a low affinity enzyme (high K_m). K_m and V_{max} are the Michaelis constant and the maximal formation rate, respectively, of the high affinity enzyme, and L is the ratio between V_{max} and K_m for the low affinity enzyme(s) and is hence a constant that relates the caffeine concentration (S) to formation rate (V) via a low affinity enzyme.

Equation 1 was fitted to data by means of an iterative curve-fitting program based on non-linear regression analysis (Holford 1990). Further, a one-enzyme model and a model including two saturable enzymes and a low linear affinity enzyme were fitted to the data. Goodness-of-fit was evaluated by comparison of loglikelihood

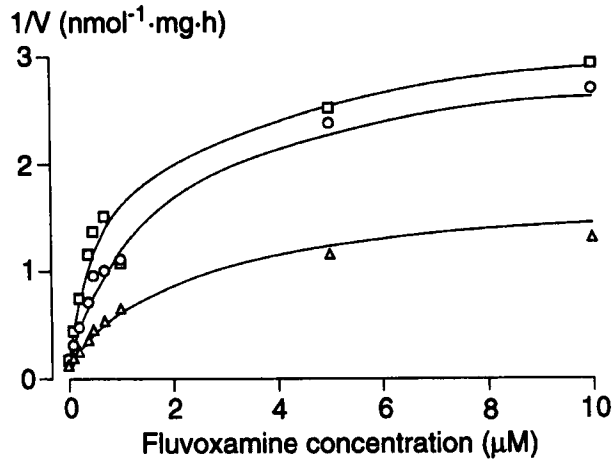

Fig. 2. The effect of fluvoxamine on the formation of 1,7-dimethylxanthine in human liver microsomes from HL1 (Dixon plot). Caffeine concentrations: (□) 0.5 mM, (○) 1.0 mM and (△) 2.0 mM. Each point represents the mean of dublicate determinations and the lines represent the best fits according to Equation 2.

values. No acceptable model could be fitted to the data for the formation of 1,3,7-trimethyluric acid from caffeine. In this case, initial values for V_{max} and K_m for use in the inhibition studies were estimated from Lineweaver-Burke plots.

For the inhibition studies, caffeine was incubated in a final concentration of 1 mM, and a number of drugs in final concentrations of 0, 0.1, 1, 10, 50 and 100 μM were screened for their ability to inhibit caffeine metabolism in microsomes from HL1. Inhibition studies with microsomes from HL1 only were considered to be sufficient as the enzyme kinetic parametres determined for HL1, HL2, and HL3 were approximately similar. Subsequently, inhibition studies were carried out at three caffeine concentrations around K_m for the formation of the three demethylated metabolites, that is 0.5 mM, 1.0 mM and 2.0 mM, and at 10 different inhibitor concentrations around the putative inhibitor constant, K_i. A two-enzyme model was fitted to the data:

$$V = V_{max}\frac{[S]}{[S]+K_m(\frac{[I]}{K_i}+1)} + L\cdot[S] \qquad \text{(Eq. 2)}$$

According to this model, the formation of the three metabolites proceeds in parallel via a high affinity enzyme showing competitive and a low affinity enzyme showing linear kinetics in the concentration range tested. I is the inhibitor concentration and K_i is the inhibitor constant for inhibition of the high affinity site. The equation was fitted to the data using an iterative method (Holford 1990).

Results

Eadie-Hofstee plots showed that the formation of 1,3-dimethylxanthine, 1,7-dimethylxanthine and 3,7-dimethylxanthine exhibited biphasic kinetics with microsomes from HL1, HL2 and HL3 (Fig. 1, results for HL2). For the formation of the three metabolites the two-enzyme model fitted the data best. The loglikelihoods of the estimations using the biphasic model ranged from 12.9 to 14.7, 2.0 to 6.4, and 11.8 to 21.3 for 1,3-dimethylxanthine, 1,7-dimethylxanthine and 3,7-dimethylxanthine, respectively as compared with 11.3 to 11.6, -8.8 to 3.5 and –1.4 to 7.7 using the equation for a one-enzyme model. No further improve-

Table 1.

The effects of putative inhibitors on the formation of 1,3-dimethylxanthine (13DMX), 1,7-dimethylxanthine (17DMX), 3,7-dimethylxanthine (37DMX) and 1,3,7-trimethyluric acid (137TMU) from caffeine in microsomes from human liver HL1. The K_i values are given as means of determinations at three caffeine concentrations of 0.5, 1.0 and 2.0 mM.

	13DMX	17DMX	37DMX	137TMU
	% of control activity (caffeine concentration 1.0 mM)			
Fluvoxamine, 10 μM	37	0	25	78
8-methoxypsoralen, 10 μM	–[a]	1	22	–[a]
α-Naphthoflavone, 10 μM	0	0	0	70
Diethyldithiocarbamate, 50 μM	18	34	28	56
Ketoconazole, 10 μM	65	89	92	37
Bromocriptine, 100 μM	41	44	25	47
Citalopram, 100 μM	105	101	99	93
Fluoxetine, 100 μM	90	90	84	67
Paroxetine, 100 μM	101	87	92	96
Quinidine, 100 μM	100	94	85	–[a]
	Inhibitor constant, K_i (μM)			
Fluvoxamine	0.28	0.08	0.08	4.4
8-methoxypsoralen	–[a]	0.27	0.32	–[a]
α-Naphthoflavone	0.05[b]	0.05[b]	0.05[b]	>100[b]
Diethyldithiocarbamate	10	24	13	29
Ketoconazole	9.3	20	13	0.75
Bromocriptine	21	20	20	5.0
Citalopram	>100	>100	>100	>100
Fluoxetine	>100	>100	>100	>100
Paroxetine	>100	>100	>100	>100
Quinidine	>100	>100	>100	–[a]

[a] Values could not be determined due to interfering peaks in the chromatogram.
[b] IC_{50} value determined at a caffeine concentration of 1mM.

ment of the goodness-of-fit was obtained when using a model with two saturable enzymes and a low affinity enzyme. Due to the high concentrations used (1–50 mM) compared to previously published K_m values below 1 mM (Campbell *et al.* 1987; Grant *et al.* 1987; Tassaneeyakul *et al.* 1992), V_{max}, K_m and L were only used as initial estimates in the inhibition studies.

A number of drugs were screened for their ability to inhibit the metabolism of caffeine in human liver microsomes from HL1. Six of the drugs were found to inhibit the metabolism of caffeine, and the V_{max}, K_m and L values (mean (range), n=6) determined together with the K_i values were for V_{max} 4.9 (4.3–5.8), 15 (12–16), 2.5 (1.9–3.0) and 8.8 (6.1–11) nmol · mg protein^{-1} · hr^{-1}, for K_m 7.6 (7.2–8.4), 1.2 (0.9–1.7), 1.3 (0.9–1.9) and 2.6 (1.7–3.7) μM and for L 0.2 (0.04–0.5), 0.5 (0.03–0.7), 0.2 (0.1–0.4) and 1.0 (0.5–2.0) μl · mg protein · hr^{-1} for 1,3-dimethylxanthine, 1,7-dimethylxanthine, 3,7-dimethylxanthine and 1,3,7-trimethyluric acid, respectively. As expected, fluvoxamine had marked inhibitory effect on the formation of all primary caffeine metabolites. Thus, the formation of 1,3-dimethylxanthine, 1,7-dimethylxanthine and 3,7-dimethylxanthine and 1,3,7-trimethyluric acid was decreased to 37%, 0%, 25% and 78%, respectively. Dixon plots of fluvoxamine inhibition kinetics confirmed the biphasic model (fig. 2, results for 1,7-dimethylxanthine) and the Dixon plots were consistent with competitive inhibition. The K_i values for fluvoxamine inhibition of the high affinity enzyme were 0.28 μM, 0.08 μM, 0.08 μM and 4.4 μM, respectively. Two other inhibitors of CYP1A2, α-naphthoflavone and 8-methoxypsoralen, showed marked inhibition of the formation of caffeine metabolites with apparent K_i values below 1 μM (table 1). Ketoconazole and bromocriptine were moderate inhibitors of the *N*-demethylations but rather potent inhibitors of the 8-hydroxylation of caffeine with K_i values of 0.75 μM and 5.0 μM, respectively, for the inhibition of 1,3,7-trimethyluric acid formation. Diethyldithiocarbamate was a moderate inhibitor, whereas citalopram, fluoxetine, paroxetine and quinidine showed weak or no inhibition of the formation of caffeine metabolites in the concentration range tested.

Discussion

The study shows that as expected fluvoxamine is a potent inhibitor of the formation of 1,3-dimethylxanthine, 1,7-dimethylxanthine, 3,7-dimethylxanthine and 1,3,7-trimethyluric acid from caffeine. The formation of 1,7-dimethylxanthine was completely blocked during fluvoxamine at a caffeine concentration of 1 mM, confirming that CYP1A2 almost exclusively catalyses the *N*3-demethylation of caffeine, whereas CYP1A2 is only partly involved in the metabolism of 1,3-dimethylxanthine, 3,7-dimethylxanthine and 1,3,7-trimethyluric acid. The K_i values reported here were similar to published values of 0.12–0.24 μM reported for the *O*-deethylation of phenacetin (Brøsen *et al.* 1993), 0.14 μM for the *N*-demethylation of imipramine (Skjelbo & Brøsen 1992), and 0.07–0.24 μM for the metabolism of theophylline (Rasmussen *et al.* 1995). Fluvoxamine has recently been shown also to be an effective inhibitor of CYP2C19, the source of the mephenytoin oxidation polymorphism (Goldstein *et al.* 1994), both *in vivo* (Jeppesen *et al.* 1997) and *in vitro* (Rasmussen *et al.* 1998). However, there have been no reports indicating that CYP2C19 should be involved in the metabolism of caffeine, so most likely the interaction is due to inhibition of CYP1A2. Fluvoxamine is also a weak inhibitor of CYP2D6 (Skjelbo & Brøsen 1992), but the lack of inhibition by quinidine in the present study eliminates the role of CYP2D6 in caffeine metabolism. The result is in agreement with the previous *in vivo* study (Jeppesen *et al.* 1996), where caffeine metabolism was markedly impaired during fluvoxamine intake. Both the present *in vitro* data and the previous *in vivo* study (Jeppesen *et al.* 1996) point to the possibility of caffeine intoxication during fluvoxamine treatment. It is, therefore, recommended that patients during fluvoxamine treatment restrict their intake of caffeine-contaning beverages, such as coffee, tea and cola. Three other selective serotonin re-uptake inhibitors, paroxetine, fluoxetine and citalopram did not show any inhibition of the caffeine metabolism, indicating they do not inhibit CYP1A2.

Another CYP1A2 inhibitor α-naphthoflavone (Burke *et al.* 1977; Grant *et al.* 1987; Tassaneeyakul *et al.* 1993), virtually abolished the formation of the three *N*-demethylated metabolites, whereas the formation of 1,3,7-trimethyluric acid could be reduced to 70% of the control value. IC_{50} values of 0.05 μM were in agreement with previous published values for α-naphthoflavone inhibition of CYP1A2-mediated phenacetin metabolism (Tassaneeyakul *et al.* 1993), ethoxyresorufin *O*-deethylation (Burke *et al.* 1977) and caffeine metabolism (Grant *et al.* 1987; Tassaneeyakul *et al.* 1992). The fact that the formation of 1,3-dimethylxanthine and 3,7-dimethylxanthine, which are only partly catalysed by CYP1A2, is totally blocked by α-naphthoflavone suggests that the compound may possess some unspecific inhibitory potential towards P450s other than CYP1A2.

Acute administration of 8-methoxypsoralen markedly impaired caffeine (Mays *et al.* 1987) and theophylline (Apseloff *et al.* 1990) metabolism *in vivo* and *in vitro* (Tassaneeyakul *et al.* 1992) where the *N*3-demethylation of caffeine was abolished by a concentration of 5 μM 8-methoxypsoralen. The interaction was confirmed in the present study where 8-methoxypsoralen was a potent inhibitor of the *N*3-demethylation with a K_i value of 0.27 μM, suggesting that 8-methoxypsoralen is a potent inhibitor of CYP1A2. Previously, 8-methoxypsoralen has been found also to be a very potent inhibitor other forms of P450, in particular of the CYP2A6-catalysed 7-hydroxylation of coumarin with an IC_{50} of 0.3 μM (Mäenpää *et al.* 1994), so the drug can not be considered to be a specific inhibitor of CYP1A2.

Even at high concentrations, fluvoxamine and α-naphthoflavone only reduced 1,3,7-trimethyluric acid formation to 78% and 70%, respectively. The residual activity is mediated by P450s other than CYP1A2, and the present study

confirms that CYP3A4 plays a major role. A concentration of 10 μM ketokonazole reduced the formation of 1,3,7-trimethyluric acid to 37%. A K_i value of 0.75 μM could be determined, and although the value is somewhat higher than previously published K_is for ketoconazole inhibition of CYP3A4 (0.006–0.1 μM) (Gascon & Dayer 1991; von Moltke *et al.* 1996; Bourrie *et al.* 1996), it is considered consistent with CYP3A4 inhibition. The K_is obtained for the *N*-demethylations were markedly higher (table 1), which suggests that CYP3A4 plays a minor role in the formation of 1,3-dimethylxanthine, 1,7-dimethylxanthine and 3,7-dimethylxanthine. The effect of ketokonazole on these metabolites is probably due to unspecific inhibition of other P450 by ketoconazole. The results are supported by the fact that another CYP3A4 inhibitor bromocriptine, inhibited 1,3,7-trimethyluric acid formation with a K_i of 5 μM, comparable to those previously published of 0.2–8 μM (Pichard *et al.* 1990; Nielsen *et al.* 1998), whereas bromocriptine was a moderate to weak inhibitor of the formation of 1,3-dimethylxanthine, 1,7-dimethylxanthine and 3,7-dimethylxanthine.

At a concentration of 50 μM, diethyldithiocarbamate reduced the formation of all four metabolites (table 1). Diethyldithiocarbamate is known to inhibit CYP2E1 with K_i values close to those found for the inhibition of the formation of 1,3-dimethylxanthine and 3,7-dimethylxanthine (10–13 μM) (Guengerich *et al.* 1991; Tassaneeyakul *et al.* 1994). The specificity of diethyldithiocarbamate has been questioned, but the results give some support to earlier results demonstrating that CYP2E1 is involved in the catalysis of the *N*7- and *N*1-demethylations of caffeine (Tassaneeyakul *et al.* 1994) in parallel with CYP1A2.

Quinidine did not inhibit formation of the four caffeine metabolites, confirming that CYP2D6 is not involved in the metabolism of caffeine.

In conclusion, the present study shows that as expected, fluvoxamine is a potent inhibitor of the metabolism of caffeine *in vitro*. Thus, the possibility of caffeine intoxication exists and patients are recommended to restrict the intake of coffee, tea and cola during fluvoxamine treatment.

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