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Stereoselective Biotransformation of the Selective Serotonin Reuptake Inhibitor Citalopram and Its Demethylated Metabolites by Monoamine Oxidases in Human Liver

Bertrand Rochat, Markus Kosel,* Gilles Boss,† Bernard Testa,† Michel Gillet‡ and Pierre Baumann*§*

*Unité de biochimie et psychopharmacologie clinique, Département universitaire de psychiatrie adulte, CH-1008 Prilly-Lausanne; †Institut de Chimie Thérapeutique, Ecole de Pharmacie, Université de Lausanne, CH-1015 Lausanne-Dorigny; and ‡Service de Chirurgie, CHUV, CH-1011 Lausanne, Switzerland

ABSTRACT. Citalopram (CIT) is an antidepressive drug of the group of selective serotonin reuptake inhibitors (SSRIs). The tertiary amine CIT is given as a racemic drug, but its pharmacological activity resides mainly in *S*-CIT. CIT is metabolised by cytochrome P450 (CYP) to N-demethylcitalopram (DCIT) and N-didemethylcitalopram (DDCIT). The citalopram propionic acid derivative (CIT-PROP) is another, but pharmacologically inactive, metabolite, the formation of which has been poorly characterised but is postulated to occur by deamination of CIT, DCIT and/or DDCIT. The aim of the present investigation was to study the formation of the enantiomers of CIT-PROP from CIT and its two N-demethylated metabolites, DCIT and DDCIT, in an *in vitro* incubation system (microsomal and cytosolic fractions) obtained from human livers. The production of CIT-PROP was measured by a stereospecific HPLC method. Incubation of *rac*-CIT, *rac*-DCIT and *rac*-DDCIT (500 μM each, separately) in the presence (or absence) of NADP showed that CIT-PROP formation was substrate-dependent and essentially NADP-independent. Monoamine oxidases (MAO) type A and B and aldehyde oxidase were identified as the probable enzymes involved in the formation of CIT-PROP from CIT, DCIT and DDCIT. Indeed, the irreversible monoamine oxidase type A inhibitor clorgyline and the irreversible monoamine oxidase type B inhibitor selegiline (both at 0.5 μM in the incubation mixture) inhibited CIT-PROP formation, depending on the substrate, up to 70% and 88%, respectively. The participation of aldehyde oxidase in the subsequent step is suggested by the inhibition caused by menadione (50 μM) in CIT-PROP formation. Preliminary experiments suggest the presence of four unknown metabolites, probably products of deamination, which were detected in plasma and urine samples of patients treated with CIT as well as in *in vitro* biotransformations. Their presence confirms the importance of deamination in the biotransformation of CIT and its demethylated metabolites, especially in the brain where, in contrast to the liver, the role of cytochrome P450 appears to be low. BIOCHEM PHARMACOL **56**;1:15–23, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. human liver, microsomes, SSRI, citalopram, enantiomer, monoamine oxidase.

The antidepressant CIT‖ is a racemic drug and a very potent SSRI. Its biotransformation occurs partially by N-demethylation to DCIT and DDCIT, but also by deamination to a propionic acid derivative CIT-PROP and to a limited degree by N-oxygenation to CIT-N-oxide (Fig. 1) [1]. Its serotonergic activity resides mainly in its *S*-isomer and to some extent in *S*-DCIT [2], but CIT-PROP is not considered to have any 5-HT reuptake inhibiting activity [3]. In 29 patients routinely treated with CIT, the plasma concentrations of the *S*-enantiomers of CIT and DCIT were lower than those of their antipodes with a mean *S*/*R* ratio of 0.56 and 0.69, respectively [4]. In contrast, biotransformation to CIT-PROP showed a mean *S*/*R* ratio of 3.6 in plasma [5]. After administration of a single intravenous dose of 20 mg of CIT, the concentrations of CIT-PROP reached only ca. 2%–20% of the parent compound, but DCIT and DDCIT were not detected under these conditions [6]. After an oral treatment of depressive patients with CIT (40 mg/day) for 2 weeks, mean plasma concentrations of CIT, DCIT, DDCIT and CITPROP were 86 ng/mL, 35 ng/mL, 7 ng/mL and 31 ng/mL, respectively [7]. Other biochemical path-

§ Corresponding author: Prof. P. Baumann, Ph.D., Unité de biochimie et psychopharmacologie clinique, Département universitaire de psychiatrie adulte, CH-1008 Prilly-Lausanne, Switzerland. Tel. +4121 643 64 34; FAX + 4121 643 64 44; E-mail: Pierre.Baumann@inst.hospvd.ch.

‖ *Abbreviations:* ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; AO, aldehyde oxidase; CIT, citalopram; CIT-PROP, citalopram propionic acid; CYP, cytochrome P450; DCIT, N-demethylcitalopram; DDCIT, N-didemethylcitalopram; FMO, flavin monooxygenase; IS, internal standard; Km, Michaelis constant; MAO, monoamine oxidase; MAOI, MAO inhibitor; S-flurbi, S-flurbiprofen; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressants; UK, unknown metabolite; and XO, xanthine oxidase.

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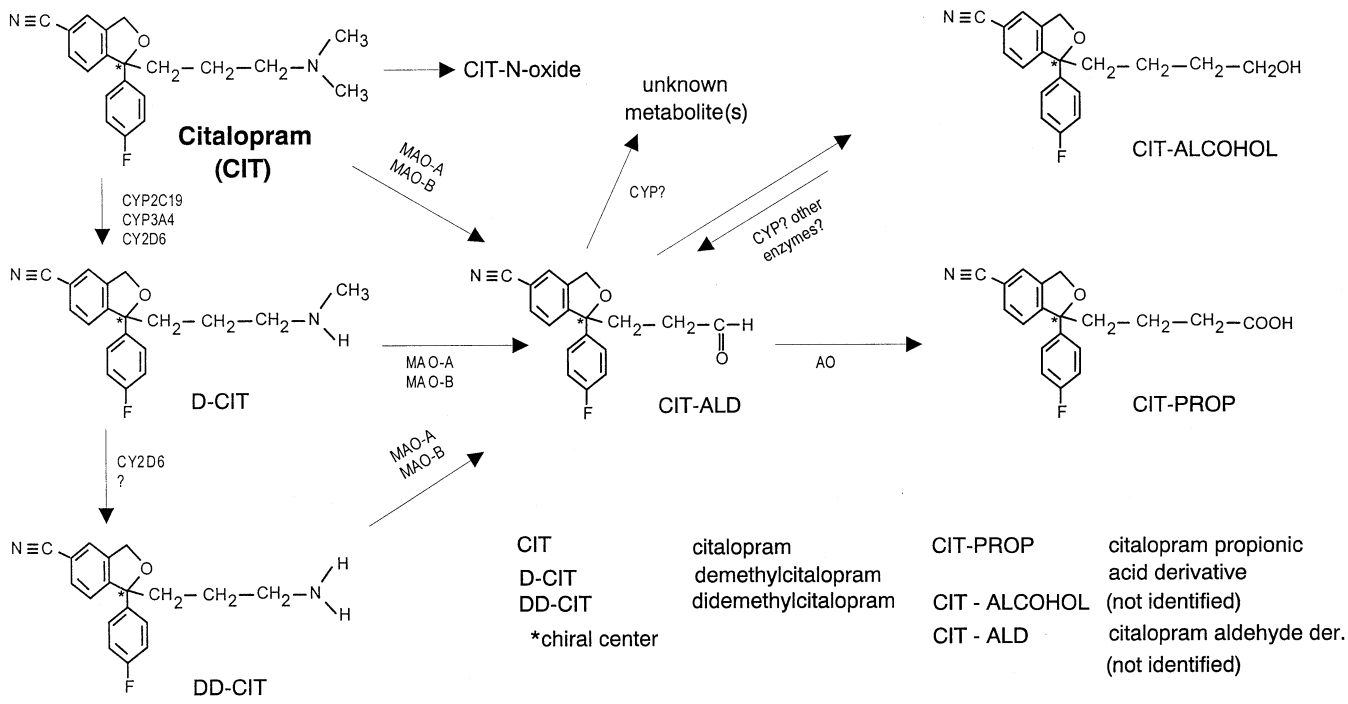

FIG. 1. Chemical structure of CIT and its metabolites, with the pathways and enzymes postulated to lead to the propionic acid derivative. For explanations, see list of abbreviations.

ways and routes of elimination have not yet been characterised in humans [8]. For instance, only ca. 46% of a daily dose (40 mg) administered for 4 weeks was detected in urine as CIT, DCIT and DDCIT [8]. Nevertheless, it has recently been shown that CIT and DDCIT are highly excreted as glucuroconjugates in human urine. In fact, 100% ± 10% of a single dose of radiolabelled CIT given to 4 healthy volunteers was recovered in urine as CIT, DCIT, DDCIT, CIT-PROP, CIT-N-oxides and glucuronides, whereas trace amounts of unknown metabolites were detected (Dalgaard *et al.*, unpublished data).

The present metabolic study was focused on the deamination of CIT, DCIT and DDCIT to CIT-PROP. Deamination of xenobiotic amines and the formation of carboxylic acids may be mediated in a two-step sequence—in the first metabolic step, by CYP (EC 1.1.14.1.) and/or MAOs (EC 1.4.3.4.) and, in the second step, by CYP and/or dehydrogenases [9, 10]. From the amines, aldehydes are formed in the first step as intermediate metabolites, and in the second step aldehydes are often biotransformed by oxidoreduction to the corresponding alcohol and carboxylic acid [11, 12] (Fig. 1). Generally, the great majority of aldehydes are metabolised to carboxylic acids because the Km values are much higher for the formation of alcohols and also because the reduction of aldehydes to alcohols is readily reversible in contrast to the oxidation of aldehydes to carboxylic acids [12].

MAO is a flavin-containing enzyme located mainly in the outer membrane of mitochondria. Two isozymes, MAO-A and MAO-B, have been identified. MAO is capable, without a NADPH-regenerating system, to oxidatively deaminate neurotransmitters and xenobiotic amines [10, 13, 14]. For instance, oxidative deamination of sumatriptan by MAO-A was observed without CYP participation [15]. In contrast, CYP has been shown to be able to deaminate diltiazem to the active carboxylic acid metabolite via an aldehyde intermediate and without implication of MAO [16]. Therefore, two pathways have been hypothesised for the deamination of CIT and its demethylated metabolites. These are the biochemical processes involving the CYP NADP-dependent system, and/or MAO. In the second step of acid formation, various enzymes could be involved such as: CYP, AO (EC 1.2.3.1.), ALDH) (EC 1.2.1.3.), ADH (EC 1.1.1.), FMO (EC 1.14.13.8.) and XO (EC 1.1.3.22.).

Recently, three cases of serotonin syndrome were described after overdoses of CIT and the MAO-A inhibitor, moclobemide [17]. The occurrence of a serotonin syndrome may be explained by the concurrent effect of CIT by 5-HT uptake inhibition and by inhibition of 5-HT metabolism by moclobemide. It is hypothesised that the risk of a serotonin syndrome may be increased by MAO inhibitors decreasing the metabolism of CIT.

This study provides evidence that MAO and AO are able, in a probable two-step reaction, to stereoselectively deaminate CIT, DCIT and DDCIT and to form the acid metabolite, CIT-PROP.

MATERIAL AND METHODS

Chemicals

The following drugs were gifts: The racemates and enantiomers (purity >99%) of CIT hydrobromide or oxalate, DCIT hydrochloride or fumarate, DDCIT L-tartrate or

fumarate, citalopram N-oxide (CIT-N-oxide) and CIT-PROP (Lundbeck AS, Copenhagen, Denmark), the IS *S*-Flurbi (Boots Limited, Nottingham, U.K.), ketoconazole (Prof. P. Dayer, Geneva, Switzerland), and omeprazole (Astra, Dietikon, Switzerland).

CIT, its metabolites and *S*-Flurbi were dissolved in methanol at a concentration of 1 mg/mL of base (racemates, enantiomers or internal standards) as stock solutions stored at −20° and in 0.01 M of HCl at 1 ng/mL of base as working solutions. CIT-PROP (at 1 ng/mL) was freshly prepared from stock solutions by dilution in 0.002 M of NaOH.

Methyliodide (Fluka, Buchs, Switzerland) was used as the derivatising agent for carboxylic acids. All other reagents were of analytical or HPLC grade. Other specific inhibitors were purchased from Sigma (Basel, Switzerland) except furafylline and sulfaphenazole (Ultrafine, Manchester, U.K.) and selegiline (i.e. *R*-(−)-deprenyl; Research Biochemicals Inc., Natick, MA, U.S.A.).

Human Livers, In Vitro *System Preparations and Rat Brain Mitochondria*

The use of material from liver biopsies was approved by the Ethical Committee of the Département universitaire de psychiatrie, Lausanne (Switzerland). None of the patients who underwent surgical interventions for different reasons had abnormal liver histology or a CYP2D6 deficiency. A few grams of 4 healthy biopsy samples were taken for the preparation of incubations and directly frozen as small pieces in liquid nitrogen before storage at −80°. Microsomal and cytosolic fractions were prepared as previously described [18] and stored at −80° until use. These microsomal fractions also contain mitochondria [15]. Rat brain mitochondria were prepared according to Walther *et al.* (cf. [19]). Sprague-Dawley rats were anesthesised with CO_2 and decapitated. Blood vessels and pial membranes were removed, the brain homogenised and, after final centrifugation (at 11,000 g for 20 min at 4°), the mitochondrial pellets were washed and rehomogenised. Protein was determined in liver and brain preparations according to [20].

Incubations

Incubations were performed in duplicate with at least two different liver preparations. Means of at least two experiments were calculated. One hundred μg of human liver microsomes or/and cytosolic proteins were incubated at 37° in a final volume of 250 μL of 0.1 mM of KH_2PO_4 (pH = 7.4) as previously described [18], and they contained 500 μM of CIT, DCIT or DDCIT as racemates or pure enantiomers. Several incubations were performed with some mg of liver enzymes and CIT, DCIT and DDCIT in order to produce large amounts of unknown metabolites for their identification. Incubations were performed with or without an NADPH-regenerating system and in plastic Eppendorf vials to prevent adsorption on glassware, started by addition of liver proteins and stopped after 45 min by addition of 750 μL of 0.25 N HCl at 4°. In some cases, NAD^+ instead of $NADP^+$ was added to the regenerating system. Linearity conditions of *S*-CIT-PROP production in the incubation system were tested relative to duration of incubation and protein concentration.

Measurement of Rat Brain MAO Inhibition by CIT, DCIT and DDCIT

The study of linearity during the kynuramine deamination was performed according to [21]. Incubations were carried out at pH = 7.4 and 37°. The mitochondria suspensions were preincubated at 37° for 5 min with either 250 nM of clorgyline (completely blocking MAO-A) or 250 nM of selegiline (completely blocking MAO-B). The nonselective substrate kynuramine was then added to a concentration equal to its Km (90 μM for MAO-A and 60 μM for MAO-B). The deamination of kynuramine by MAO led to the production of 4-hydroxyquinoline, which was monitored continuously at 314 nm for 6 min with a Kontron Uvikon 941 spectrophotometer (Kontron, Zurich, Switzerland).

Plasma and Urine Samples of CIT-treated Patients

Plasma and urine samples from 3 depressive patients (2 females, 44 and 62 years old; one male, 44 years old) receiving CIT orally (20 to 60 mg daily) for more than one week were collected for CIT-PROP analysis at least 12 hr after the final medication. O-Glucuroconjugated compounds in urine and plasma samples were hydrolysed by an enzymatic treatment with β-glucuronidase-sulfatase [21].

Extraction Procedure

For the determination of CIT-PROP, 50 ng of *S*-Flurbi (IS), 1 mL of 0.6% KH_2PO_4 (pH = 3) and 150 μL of 1 N HCl were added to 1 mL of heparinised plasma, urine or incubation mixture. The acidified plasma, urine or incubation mixtures were used for the solid-phase extraction of CIT-PROP and IS [22]. CIT, DCIT and DDCIT are not extracted by this procedure. Derivatisation was performed with methyliodide [22]. After liquid-liquid extraction, the dried sample was reconstituted in 120 μL of mobile phase, (hexane-isopropanol 50:50 v/v). CIT-N-oxide enantiomers did not coelute with the other compounds and the concentrations of CIT-N-oxide were, in most cases, below the limit of detection [5].

Instrumentation and Analytical Method

Analyses were carried out by HPLC (model 112 and 340, Beckman Instruments, Nyon, Switzerland) using an automatic injector with a 100-μL loop (Kontron MSI 660) [5]. For the separation and quantification of the derivatized CIT-PROP enantiomers, a chiral analytical column, Chira-

TABLE 1. The effect of NADP on the production and S/R ratio of CIT-PROP by incubations of the racemates of CIT, DCIT or DDCIT in human liver microsomes*

	Production of CIT-PROP (ng/45 min/0.1 mg protein in 0.25 mL incubation mixture)				S/R ratio of CIT-PROP			
Substrate	with NADP mean ± SD (N = 4)		without NADP mean ± SD (N = 4)		with NADP mean ± SD (N = 4)		without NADP mean ± SD (N = 4)	
rac-CIT	47.6(a)	6.6	56.2(d)	5.0	2.3 (g)	0.31	1.11 (j)	0.09
rac-DCIT	137.7(b)	32.5	202.0(e)	79.4	3.90(h)	0.79	3.15(k)	0.72
rac-DDCIT	95.0(c)	20.8	133.0 (f)	45.6	10.4 (i)	2.15	9.20 (l)	1.63

*The substrates (500 μM) were incubated for 45 min at 37° in human liver microsomal preparations Student's *t*-test.
$P < 0.01$: a vs b; b vs c; a vs c; g vs j; h vs k.
$P < 0.05$: a vs d; d vs e; e vs f; d vs f.

cel OD (particle size: 10 μm, 0.46 × 25 cm) from Daicel (Ph. Stehelin, Basel, Switzerland) was coupled with a LC 240 fluorimetric detector (Perkin-Elmer, Le-Mont-sur-Lausanne, Switzerland) set at 240 nm and 296 nm for excitation and emission wavelengths, respectively. The mobile phase was hexane-isopropanol (50:50 v/v) and the column flow rate was 0.65 mL/min. Chromatograms were analysed with a "System gold" software (Beckman) running on an IBM PS/230.

RESULTS

Production of CIT-PROP from CIT, DCIT and DDCIT

In preliminary experiments, S-enantiomers of CIT, DCIT and DDCIT were incubated for 120 min in the presence of an NADPH-regenerating system (NADP). CIT-PROP formation by the human liver microsomal fraction was observed for all substrates. *S*-CIT-PROP production was linear at least until 60 min and with microsomal protein concentrations ranging from 50 to 200 μg with or without cytosolic proteins (100 μg) in the incubation mixture (data not shown). The cytosolic fraction alone was unable to produce CIT-PROP (data not shown). In contrast, addition of the cytosolic fraction to the microsomal fraction led to an increased production of CIT-PROP enantiomers, which reached 59%, 53% and 55% for S-CIT, S-DCIT and S-DDCIT, respectively. This suggests that some cytosolic enzymes could be involved in the production of this metabolite. Therefore, the following incubations were carried out for 45 min, in a mixture of cytosolic and microsomal fractions (100 μg each).

Identification of the Enzymes Involved in the Production of CIT-PROP

In order to characterise the possible role of CYP, MAO and other enzymes in CIT-PROP production, the consequences of a presence or absence of NADP and the effect of specific inhibitors were tested in incubation mixtures containing 500 μM of *rac*-CIT, *rac*-DCIT or *rac*-DDCIT.

Role of NADP

In the presence of NADP, the production of CIT-PROP was 2.9 and 2.0 times greater with DCIT and DDCIT than with CIT, respectively (Table 1). Moreover, the formation of CIT-PROP enantiomers was more stereoselective for the secondary amines and especially for the primary amines. Indeed, the mean (N = 4 liver microsomal preparations) of the *S*/*R* ratios of CIT-PROP produced from *rac*-CIT, *rac*-DCIT or *rac*-DDCIT, in the presence of NADP in the incubation system, were 2.3, 3.9 and 10.4 (Table 1).

In the absence of *NADP*, the production of *S*- and *R*-CIT-PROP was increased compared to similar incubations performed with NADP but, probably due to the limited number of experiments, the increase was only statistically significant for CIT (Table 1). This result indicates that the enzymes involved in the formation of CIT-PROP are essentially NADP-independent. In contrast, the formation of CIT-PROP shows a lower stereoselectivity in the absence of NADP because the means (N = 4) of the *S*/*R* ratios of CIT-PROP produced from *rac*-CIT, *rac*-DCIT and *rac*-DDCIT were 1.1, 3.2 and 9.2, respectively (Table 1). For instance, with *rac*-CIT as substrate, the absence of NADP in the incubation mixture resulted in a lack of stereoselectivity of CIT-PROP production, the *S*/*R* ratios of CIT-PROP being 2.3 and 1.1 in the presence and absence of NADP in the incubation mixture, respectively. This tends to underline the likelihood that NADP-dependent enzymes (e.g. CYP) are also implicated, with a preference for the *R*-enantiomer biotransformation but by another pathway than CIT-PROP formation (Fig. 1). Thus, rac-CIT or rac-DCIT may be partly demethylated before deamination, in the presence of NADP.

In contrast, using *S*-CIT, *S*-DCIT or *S*-DDCIT as substrates, no modification of the production of *S*-CIT-PROP was observed in the presence or absence of an NAD-regenerating system (data not shown). This suggests that no NAD^+-dependent enzyme (e.g. ALDH) is involved in this pathway.

Effect of Specific Inhibitors

The formation of CIT-PROP was monitored in the presence of NADP and proadifen, a nonselective inhibitor of

TABLE 2. Effect of MAO inhibitors on the production of CIT-PROP

Substrate	Inhibitors	Inhibition (%) of CIT-PROP production				S/R ratio of CIT-PROP	
		R-CIT-PRO mean ± SD		S-CIT-PROP mean ± SD		mean ± SD	
rac-CIT	None	0.0	0.0	0.0	0.0	1.10	0.14
	Clorgyline	87.0	1.6	55.0	14.1	3.80	1.12
	Selegiline	10.9	13.8	35.5	16.9	0.78	0.02
rac-DCIT	None	0.0	0.0	0.0	0.0	3.44	1.13
	Clorgyline	64.5	10.2	19.4	11.0	8.04	2.56
	Selegiline	16.1	23.6	67.4	22.5	1.15	0.13
rac-DDCIT	None	0.0	0.0	0.0	0.0	10.46	3.35
	Clorgyline	27.4	4.0	−0.5	9.0	14.16	3.16
	Selegiline	48.8	17.6	92.7	5.0	1.27	0.03

Inhibition (% of control activity) of the production of CIT-PROP by clorgyline (0.5 μM) and selegiline (0.5 μM) during incubations of the racemates of CIT, DCIT or DDCIT (500 μM) in human liver microsomes in the absence of NADP. Means ± SD; N = 3 different livers.

CYP isozymes, or in the presence of inhibitors of MAO, AO, XO, ALDH, FMO and ADH but without NADP in the incubation mixtures. The nonselective MAOI phenelzine (50 μM) strongly decreased CIT-PROP production from the three racemic amines (inhibition >90%; data not shown). Selective inhibitors of MAO-A and MAO-B, clorgyline and selegiline, respectively, were added to incubation mixtures. Inhibitory effects of clorgyline and selegiline on the formation of *S*-CIT-PROP from *S*-CIT and *S*-DCIT were observed at different concentrations and confirm the main role of MAO in this pathway (inhibition ranging from 50% to 90% for both MAOI at 10 μM; data not shown). The most interesting results were obtained at a 0.5-μM concentration of these MAO inhibitors (Table 2). Interestingly, the deamination of the enantiomers of CIT was more strongly inhibited by the MAOI-A clorgyline, whereas that of the enantiomers of DDCIT was more strongly inhibited by the MAOI-B selegiline (Table 2). Taking this stereoselectivity into account, selegiline inhibited the formation of *S*-CIT-PROP from the three amines more strongly than that of its antipode. In contrast, clorgyline more strongly inhibited the formation of *R*-CIT-PROP. These results suggest that, at a substrate concentration of 500 μM, the *S*-enantiomers of CIT, DCIT and DDCIT would be preferentially metabolized by MAO-B, and their antipodes by MAO-A.

In comparison to controls, the presence of the nonselective CYP inhibitor proadifen (SKF-525A) (which is also known to be a weak AO and MAO inhibitor [23, 24]) at 75 μM in an NADP-containing incubation mixture slightly decreased *S*-CIT-PROP formation to 79.8% ± 79.0%, 74.3% ± 7.4% and 65.6% ± 4.4% with *S*-CIT, *S*-DCIT and *S*-DDCIT as substrates, respectively (n = 3). Interestingly, *R*-CIT-PROP formation was strongly increased: 262% ± 24.5%, 163% ± 12% and 100% ± 7.7% with the substrates *R*-CIT, *R*-DCIT and *R*-DDCIT, respectively. These results suggest that CYP plays a role in a biochemical pathway other than deamination, leading to CIT-PROP. The effect of proadifen is in accordance with that obtained in the absence of NADP.

Specific CYP inhibitors [25, 26], such as furafylline (50 μM), quinidine (10 μM), coumarin (10 μM), 4-methylpyrazole (10 μM), sulfaphenazole (10 μM), troleandomycin (20 μM) and ketoconazole (2.5 μM), *rac*-omeprazole (100 μM) and *rac*-mephenytoin (100 μM) for CYP1A2, 2D6, 2B6, 2E1, 2C9, 3A4 and 2C19, respectively, did not significantly modify (<10%, data not shown) the production of *S*-CIT-PROP from *S*-CIT or *S*-DCIT.

Menadione (50 μM) and hydralazine (50 μM), thiobenzamide (100 μM) and cysteamine (100 μM), allopurinol (50 μM), disulfiram (100 μM) and pyrazole (100 μM) were used as specific inhibitors of AO, FMO, XO, ALDH and ADH, respectively, which could be involved in the second step of CIT-PROP formation. Only menadione and hydralazine were able to significantly inhibit the production of CIT-PROP enantiomers (>10%). Inhibition by menadione was slightly stronger than by hydralazine and was (n = 3) 31.3%, 59.4% and 66.4% for *rac*-CIT, *rac*-DCIT and *rac*-DDCIT as substrates, respectively (Table 3). On the other hand, menadione decreased the *S*/*R* ratios of CIT-PROP enantiomers (Table 3).

Effect of CIT, DCIT and DDCIT on Rat MAO Activity

A simple screening assay was performed in order to evaluate CIT, DCIT and DDCIT as competitors of kynuramine deamination. The addition of the racemates of the tested compounds at 100 μM decreased apparent MAO-B activity by only 23%, 24% and 22% for CIT, DCIT and DDCIT. No significant decrease in MAO-A was detected (<5%).

Unknown Metabolites: Detection in In Vivo *and* In Vitro *Biotransformations*

In the presence of NADP with *rac*-CIT, *rac*-DCIT or *rac*-DDCIT, four unknown peaks (UK) were detected in HPLC chromatograms after incubation in human liver microsomes (Fig. 2A). Moreover, urine and plasma samples of 3 patients routinely treated with CIT were extracted, after treatment with CB-glucuronidase. During extraction,

TABLE 3. Effect of menadione on the production of CIT-PROP

Substrate	Inhibition (%) of CIT-PROP production mean ± SD		S/R ratio of CIT-PROP without menadione mean ± SD		S/R ratio of CIT-PROP with menadione mean ± SD	
rac-CIT	31.3	6.1	1.07	0.08	1.00	0.12
rac-DCIT	59.4	10.9	3.34	0.68	1.83	0.17
rac-DDCIT	66.4	9.8	9.20	0.93	4.08	0.85

Inhibition (% of control activity) of the production of CIT-PROP by menadione (50 μM) during incubations of the racemates of CIT, DOT or DDCIT (500 μM) in human liver microsomes. Means ± SD; N = 3 different livers.

the internal standard *S*-flurbiprofen was not added to avoid its coelution with the unknown peaks. Four unknown compounds were also detected in these samples, as shown in Fig. 2B and C. Similar results were obtained in the other two patients. The unknown peaks were identified by their capacity factor as UK1, UK2, UK3 and UK4.

Identification of these unknown compounds was attempted using HPLC coupled to a mass spectrometer (MS). The organic mobile phase used in the analytical separation being incompatible with a good MS detection, the unknown peaks were collected, dried and resuspended in an aqueous phase before direct injection into the MS. Chemical ionisation (CI) and electronic impact (EI) were performed in order to identify the structure of these compounds. Unfortunately, no mass was unequivocally identified and only the observation of a similar fragment by MS-EI (m/z: 238) for UK3, UK4, CIT-PROP, CIT, DCIT and DDCIT (data not shown) suggests that UK3 and UK4 could be metabolites of CIT. Further studies are needed to identify the chemical structure of these products.

DISCUSSION

Several clinical pharmacokinetic studies showed the presence of CIT-PROP in the blood of patients treated with CIT (for a review, cf. [8, 27]). This study demonstrates the stereoselective *in vitro* formation of CIT-PROP from the three racemic amines CIT, DCIT and DDCIT in human liver extracts (Table 1). CIT-PROP production was highest using a mixture of microsomal and cytosolic fractions. In the presence of NADP, deamination was observed with substrate selectivity and stereoselectivity, the *S*/*R* ratio of CIT-PROP was highest using DDCIT as a substrate, and production of CIT-PROP relative to that from CIT (=1.0) was 2.9 and 2.0 for DCIT and DDCIT, respectively (Table 1). The prediction of *in vivo S*/*R* ratios of CIT-PROP from *in vitro* data was calculated to be 3.7, taking into account the racemic concentrations of CIT, DCIT and DDCIT in plasma samples of treated patients at steady state (ca. 180 nM, 60 nM and 15 nM, respectively) [8]. The calculated *S*/*R* ratio is close to the mean *S*/*R* ratio of CIT-PROP observed in the plasma samples of CIT-treated patients in two different studies, i.e. 3.6 [5] and 4.2 [28]. This underlines that the *in vitro* results, even if obtained with a substrate concentration of 500 μM, seem to have significance regarding the *in vivo* conditions where the *rac*-CIT concentrations are approximately 2,500 times lower in plasma samples.

DCIT appeared to be a better substrate than DDCIT: structure-metabolism relations for MAO substrates are far from being fully understood. Whereas earlier work indicated selectivity for primary amines [29, 30], evidence from a variety of xenobiotics, i.e. neurotoxins such as MPTP and numerous mechanism-based inhibitors, proved the good capacity of MAO enzymes to also oxidize secondary and tertiary amines [10; 31]. Enantiospecificity is also documented for MAO [10; 32], but no general understanding has yet emerged from the scattered data available. The present results thus add to the body of existing evidence and may ultimately contribute to the elaboration of predictive rules.

The chromatograms presented in Fig. 2 suggest the formation of unknown metabolites of CIT but, before their chemical structure is known, their position as intermediate or final metabolites in the probable two-step formation of CIT-PROP can only be hypothesised. Nevertheless, the biochemical experiments yielded some evidence that CIT and its N-demethylated metabolites are first deaminated to aldehyde intermediates by MAO isozymes before CIT-PROP is produced by AO (Fig. 1).

Involvement of MAO Isozymes

In the presence of NADP, the *S*/*R* ratio of CIT-PROP was significantly increased for CIT and DCIT but not for DDCIT as compared to similar incubations performed in the absence of NADP (Table 1). These results suggest the presence of an aldehyde intermediate and its biotransformation to unknown metabolites with a stereoselective preference for the *R*-enantiomer. The increased production of CIT-PROP in the presence of the cytosolic fraction suggests the presence of free enzymes or cofactors which could be involved in the production of the acid from the aldehyde intermediate.

The lack of a decrease in CIT-PROP production in the absence of NADP suggests that CYP isozymes are not involved in its formation (Table 1). On the other hand, proadifen at 75 μM, a nonselective CYP inhibitor, was able to slightly inhibit the formation of the *S*-CIT-PROP (up to 35%). It is likely that proadifen at 75 μM is also able to

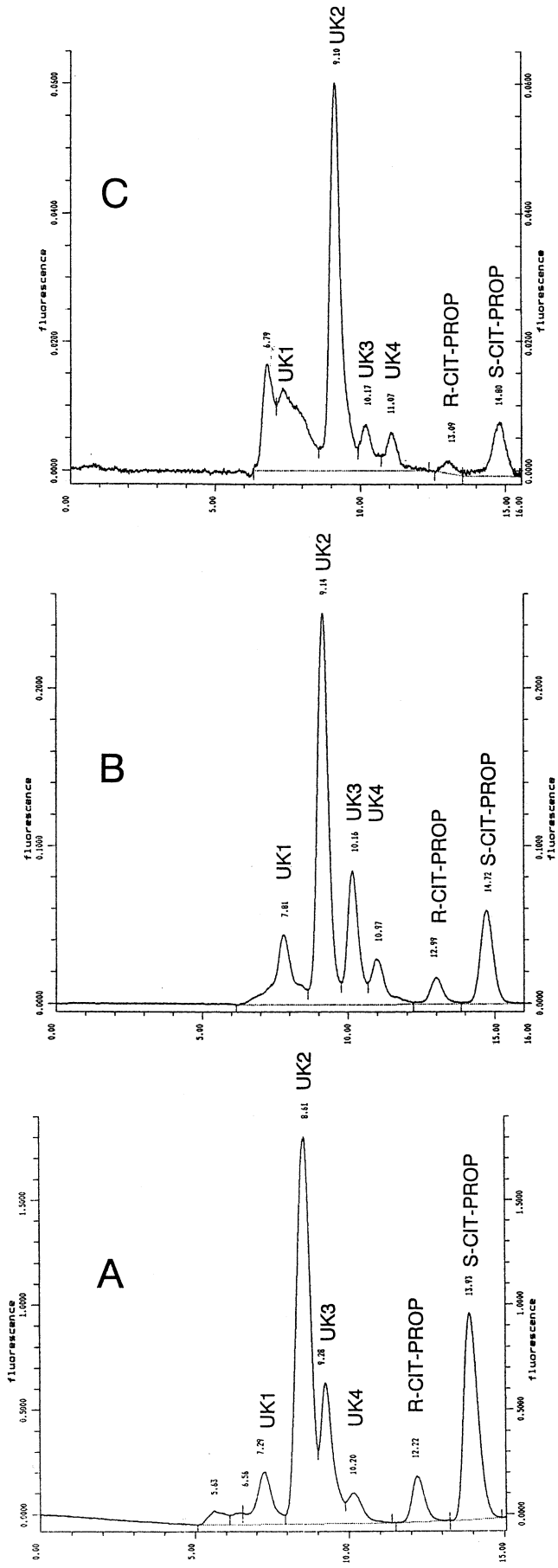

FIG. 2. HPLC chromatograms showing four unknown metabolites (UK1, 2, 3 and 4) and the enantiomers of CIT-PROP. Samples were obtained from (A) an incubation of the racemates of CIT, DCIT and DDCIT in human liver microsomes; (B) urine sample of a patient orally treated with CIT for a few weeks; and (C) a plasma sample from the same patient. Urine and plasma samples were taken the same day, 12 hrs after the last drug intake, and extracted after treatment with β-glucuronidase.

inhibit the activity of MAO and AO [22, 23]. In contrast, the formation of *R*-CIT-PROP was strongly increased (up to 2.5-fold): CYP may interact with a biochemical pathway other than deamination but also leading to R-CIT-PROP. Nevertheless, participation of MAO in the deamination of CIT, DCIT and DDCIT was confirmed by potent and specific MAO inhibitors. The nonselective MAO inhibitor phenelzine (at 50 μM) was able to strongly inhibit the production of CIT-PROP. This also confirms the predominant role of MAO in the deamination of CIT, DCIT and DDCIT. In the case of diltiazem deamination, the absence of NADP as well as the presence of proadifen in the *in vitro* incubation mixture led to a very strong decrease in the formation of its aldehyde and acidic metabolites and, in contrast, 100 μM of MAOI pargyline or iproniazid did not produce any inhibition [16]. These results strongly suggest that diltiazem deamination is under the control of CYP. In contrast, it has been shown that deamination of sumatriptan is mainly mediated by MAO, because the absence of NADP has no effect in this pathway [15]. These authors also used human liver microsomes in their study and demonstrated the presence of mitochrondria in their microsomal fractions. In the present study, it appears that CIT, DCIT and DDCIT are mainly deaminated by MAO isozymes in the microsomal fractions obtained by a similar method.

The K_i of clorgyline and selegiline are 0.05 μM and 58 μM for MAO-A and 76 μM and 0.33 μM for MAO-B, respectively [13]. The use of small concentrations (0.5 μM) of selegiline and clorgyline showed that in contrast to CIT, the primary amine DDCIT seems to be preferentially metabolised by MAO-B. Moreover, deamination of the *S*-enantiomers of CIT, DCIT and DDCIT appears to be controlled by MAO-B, in contrast to that of the *R*-enantiomers, which are carried out preferentially with MAO-A (Table 2).

Involvement of AO

Inhibition of CIT-PROP production by menadione and hydralazine suggests the involvement of AO and supports the notion that an aldehyde intermediate would be transformed to CIT-PROP in a second step (Table 3). On the other hand, ALDH, an NAD^+-dependent enzyme, would not be involved in this second step since disulfiram or an NAD^+ regenerating system did not modify the formation of CIT-PROP.

Unknown Metabolites: Detection in In Vivo *and* In Vitro *Biotransformations*

Four unknown compounds were detected in plasma and urine samples of routinely treated patients as well as in *in vitro* incubations performed with *rac*-CIT, *rac*-DCIT or *rac*-DDCIT (Fig. 2). Their chemical structures could not be characterised by MS. Taking into account that amine compounds could not be recovered, these unknown compounds are probably deaminated metabolites. This underlines the possible pharmacokinetic importance of deamination in chronic CIT treatment. In contrast, Dalgaard and coworkers have shown that 100% ± 10 of a radiolabelled CIT dose were excreted in human urine as CIT, DCIT, DDCIT, CIT-PROP, CIT-N-oxide and glucuroconjugates of some of these compounds [8, and in preparation]. Only 7% ± 3 of the radiolabelled CIT dose was identified as unknown compounds. Nevertheless, a possible underevaluation of these unknown compounds could occur from a single dose biotransformation, whereas CIT-PROP could be mainly produced in a chronic treatment.

OVERVIEW AND PERSPECTIVES

Taking into account that CYP content in human brain is very low [33–35], *in situ* deamination of CIT and its demethylated metabolites by MAO should be the main biotransformation in this target organ. It could be very interesting to investigate more intensively this biochemical pathway from a pharmacokinetic as well as pharmacodynamic point of view.

No data are available concerning CIT brain concentrations under therapeutic conditions. It is, however, of interest that chronic oral administration of CIT (80 mg/kg for 2 weeks) in epileptic mice decreased MAO-A activity by more than 50% in brain tissue preparations as measured by the production of 5-hydroxyindolacetic acid (5-HIAA) [36]. Their plasma levels of CIT (538 nM) were comparable to those found in routinely treated depressive patients. For this reason, inhibition of MAO-A and MAO-B by CIT and its demethylated metabolites was measured using an *in vitro* screening test performed with rat mitochondrial preparations [19]. We observed only a slight decrease (ca. 20–25%) of MAO-B activity after addition of CIT and its N-demethylated metabolites. Nevertheless, it has recently been shown that fluoxetine and its demethylated metabolite, norfluoxetine, were able to inhibit MAO activity in a time-dependent manner [37].

In conclusion, these *in vitro* experiments showed a stereoselective production of CIT-PROP from CIT, DCIT and DDCIT. The results suggest that CIT-PROP formation may involve a two-step sequence: 1) deamination mediated by MAO-A and MAO-13 leading to the formation of an aldehyde as intermediate, and 2) oxidation of this intermediate, mediated by AO, leading to the formation of CIT-PROP. Preliminary data suggest the presence of four unknown metabolites, which, however, remain to be characterised. Apparently, metabolism by MAO has not yet been described for any other antidepressant or antipsychotic drug of the amine type.

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