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Differential Effects of Fluvoxamine and Other Antidepressants on the Biotransformation of Melatonin

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Melatonin, the predominant product of the pineal gland, is involved in the maintenance of diurnal rhythms. Nocturnal blood concentrations of melatonin have been shown to be enhanced by fluvoxamine, but not by other serotonin reuptake inhibitors. Because fluvoxamine is an inhibitor of several cytochrome P450 (CYP) enzymes, the authors studied the biotransformation of melatonin and the effects of fluvoxamine on the metabolism of melatonin *in vitro* using human liver microsomes and recombinant human CYP isoenzymes. Melatonin was found to be almost exclusively metabolized by CYP1A2 to 6-hydroxymelatonin and *N*-acetylserotonin with a minimal contribution of CYP2C19. Both reactions were potently inhibited by fluvoxamine, with a K_i of 0.02 μM for the formation of 6-hydroxymelatonin and 0.05 μM for the formation of *N*-acetylserotonin. Other than fluvoxamine, fluoxetine, paroxetine, citalopram, imipramine, and desipramine were also tested at 2 and 20 μM . Among the other antidepressants, only paroxetine was able to affect the metabolism of melatonin at supratherapeutic concentrations of 20 μM , which did not reach by far the magnitude of the inhibitory potency of fluvoxamine. The authors concluded that fluvoxamine is a potent inhibitor of melatonin degradation. Because this inhibitory action is also found *in vivo*, fluvoxamine might be used as an enhancer of melatonin, which might offer new therapeutic possibilities of fluvoxamine. (J Clin Psychopharmacol 2001;21:167–174)

THE PINEAL HORMONE melatonin plays an important role in the maintenance of the light/dark cycle of vertebrates.¹ The enhanced secretion of melatonin at night is caused by increased activity of the key enzyme

in the melatonin formation, serotonin *N*-acetyl transferase.² The enzyme activity is regulated by the light/dark cycle via an adrenergic cAMP control of transcription and proteasomal proteolysis.³ Other than synchronization of biological functions, melatonin has been suggested to play a role in neurodegenerative diseases such as dementia,⁴ in cancer,⁵ in the regulation of the immune system,⁶ and in some forms of mental illness, especially affective disorders.^{7, 8} Although most of the proposed protective effects of melatonin are still questionable, the decrease in the melatonin serum concentration in depressed patients has been proven conclusively by several studies.^{7–9} Accordingly, the influence of antidepressant drugs on the melatonin serum levels has been studied intensively.^{10–12} A stimulating effect on the melatonin secretion was primarily found for desipramine¹² and (+)-oxaprotiline,¹³ both inhibiting predominantly the norepinephrine (NA) reuptake. Because the nocturnal increase of melatonin is triggered by an adrenergic-cAMP mechanism, the effect of the NA-reuptake blockers was attributed to an elevated NA stimulus on NA receptors in the pineal gland.¹³

The interpretation of reported effects of the selective serotonin reuptake inhibitors (SSRIs) was difficult because of conflicting results. Fluoxetine was found to negatively affect the melatonin secretion,¹⁴ but fluvoxamine markedly increases the melatonin blood concentrations.^{10, 12, 15} Although for the former, a reduced availability of the melatonin precursor serotonin in the cytoplasm was suggested,¹⁴ the positive effect of fluvoxamine remained poorly understood, so far.^{15, 16} Because fluvoxamine is known to inhibit several drug-metabolizing cytochrome P450 (CYP) isoenzymes, mainly CYP1A2¹⁷ and CYP2C19,¹⁸ a pharmacokinetic effect was discussed.¹² This suggestion was supported by the recent observations that the bioavailability of orally given melatonin was significantly increased by concomitant administration of fluvoxamine.¹⁹

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Melatonin is rapidly cleared after secretion by hepatic metabolism leading to its two main metabolites 6-hydroxymelatonin and *N*-acetylserotonin (Fig. 1).²⁰ Recent evidence showed that melatonin is almost exclusively metabolized by the CYP1A subfamily,²¹ which indicates a pharmacokinetic interaction between melatonin and fluvoxamine. Therefore, the aim of our study was to characterize the effects of fluvoxamine on the 6-hydroxylation and O-demethylation of melatonin *in vitro* in comparison to other antidepressants and the selective CYP1A2 inhibitor furafylline.

Methods

Chemicals

Melatonin, 6-hydroxymelatonin, and *N*-acetylserotonin were purchased from Sigma Chemical Company (St. Louis, MO), furafylline from RBI (Natick, MA), and nicotinamide adenine dinucleotide phosphate (reduced form) from Roche (Mannheim, Germany). All other chemicals were at least of analytical grade and were obtained from Merck (Darmstadt, Germany). Fluvoxamine was kindly provided by Solvay-Duphar (Hannover, Germany), paroxetine by SmithKline Beecham (Munich, Germany), fluoxetine by Lilly (Gießen, Germany), citalopram by Promonta-Lundbeck (Hamburg, Germany), and imipramine and desipramine by Novartis (Basel, Switzerland).

Human liver samples and preparation of microsomes

Liver samples were obtained from three organ donors (HL1–HL3) who had given their consent for the scientific use of their livers. The use of the liver samples was approved by the local ethics committee. Microsomes were prepared by differential ultracentrifugation as reported elsewhere for the preparation of rat liver microsomes.²²

For kinetic analyses and inhibition experiments with fluvoxamine, we used microsomes from all three livers samples. To study inhibitory properties of different antidepressants on the melatonin metabolism under comparable conditions, we used only microsomes prepared from the first liver, HL1. The microsomes were tested for possible CYP2C19 or CYP2D6 deficiencies by use of diazepam N-demethylation and dextromethorphan O-demethylation as probe reaction. All three livers were able to form remarkable amounts of nordiazepam and dextroprhan. The formation of nordiazepam was further inhibited to more than 70% by 50 μ M tranylcypramine (CYP2C19 inhibitor), whereas the formation of temazepam remained unaffected. The formation of dextroprhan was completely abolished by 10 μ M quinidine (CYP2D6 inhibitor). Thus, there were obviously no phenotypic CYP2C19 and CYP2D6 deficiencies in our liver samples.

Well-characterized pooled microsomes from six different livers (Cat. No. H161, Lot 4; Gentest Corporation, Woburn, MA) were used to ensure the enzymatic activity of our microsomal preparation with regard to the melatonin 6-hydroxylation and O-demethylation.

Recombinant Human Cytochrome P450s

Recombinant human CYP1A2 and CYP3A4 were prepared at the Biomedical Research Center at Ninewells Hospital (Dundee, Scotland). CYPs were coexpressed with human reductase (reductase activity between 300 and 1000 nmol of cytochrome c reduced/min \times mg-protein) in *Escherichia coli* using the 17 α -expression construct for 3A4.²³ Catalytic activity of these recombinant human CYPs is reported elsewhere.²⁴ Recombinant CYP2C19 plus P450 reductase (activity: 650 nmol of cytochrome c reduced/min \times mg-protein) SupersomesTM were purchased from Gentest Corporation (Woburn, MA).

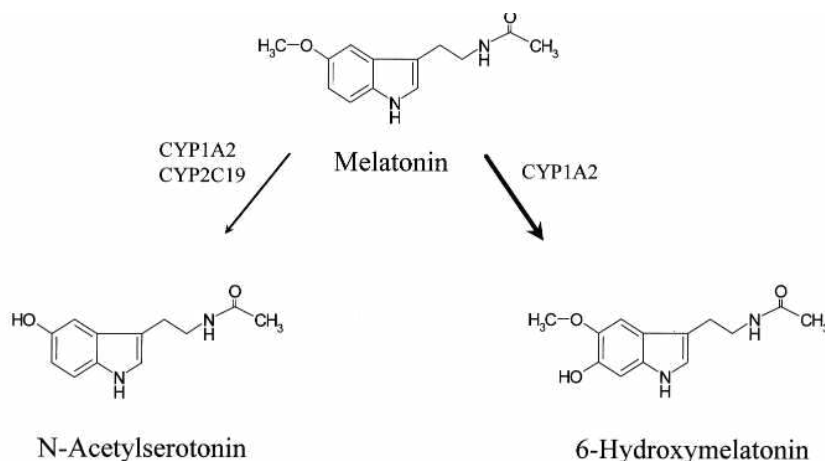


FIG. 1. Metabolic fate of melatonin to its main metabolites 6-hydroxymelatonin and *N*-acetylserotonin and involved cytochrome P450 enzymes suggested from data of this study. The predominant metabolic route (6-hydroxylation) is depicted by the boldfaced arrow.

Protein and cytochrome P450 concentration

Total CYP concentration in human liver and recombinant CYP microsomes was measured by the method used by Omura and Sato.²⁵ The protein concentration was estimated by the method described by Bradford²⁶ using bovine serum albumin as standard (Bio-Rad, Munich, Germany).

Microsomal incubations, extraction procedure, and high-performance liquid chromatography

The formation of 6-hydroxymelatonin and *N*-acetylserotonin showed a linear increase with time for up to 30 minutes and up to 0.5 mg/mL of human liver microsomal protein. Incubations were performed at 37°C in a total volume of 0.5 mL of a 0.1 M phosphate buffer (pH 7.4) containing 5 mM NADPH, 0.5 mg/mL of human liver microsomal protein, and different concentrations of melatonin (0.5–10 μ M). Each concentration was assayed in duplicate. After a 2-minute preincubation, the reaction was started by the addition of the microsomes and stopped after 30 minutes by the addition of 0.5 mL of ethylacetate and rapid cooling on ice.

The mixture was vigorously shaken for 2 minutes, centrifuged for 5 minutes at 10,000 *g*, and transferred to an ice bath consisting of solid CO₂ and methanol. The organic supernatant was evaporated to dryness, and the residue was dissolved in 250 μ L of the high-performance liquid chromatography (HPLC) eluent. Each series was accompanied by five calibration samples in a concentration ranging between 2 and 100 ng/mL for 6-hydroxymelatonin and between 1 and 30 ng/mL for *N*-acetylserotonin. Calibration samples were prepared and processed exactly the same way as the incubations, but no substrate was added and the reaction was stopped using ethylacetate and transferred on ice immediately after the addition of the microsomes.

The concentration of the metabolites 6-hydroxymelatonin and *N*-acetylserotonin was assayed by means of a slightly modified reversed-phase HPLC method with electrochemical detection.²⁷ In brief, the analytical eluent consisted of 11.5% acetonitrile in a phosphate-citrate buffer (pH 7.4) and was delivered by a flow rate of 0.6 mL/min. Separation of the analytes were performed on a Hypersil ODS column (125 \times 3 mm) with a particle size of 3 μ m (MZ Analyventtechnik, Mainz, Germany). Analytes were detected using an ESA Coulochem (Bischoff, Leonberg, Germany) detector with detector 1 set at -0.05 V, detector 2 set at +0.4 V, and the guard cell set at 1.0 V. The quantification limit was found to be 2 ng/mL (8 nM) for 6-hydroxymelatonin and 1 ng/mL (4 nM) for *N*-acetylserotonin.

Calibration curves were constructed by unweighted linear regression of the calibration points. The correla-

tion coefficient (R^2) was always greater than 0.99. The mean coefficient of variation (from 10 different days) for the calibration points in the range between 2 and 100 ng/mL for 6-hydroxymelatonin and 1 to 30 ng/mL for *N*-acetylserotonin amounted to 20.9% for 6-hydroxymelatonin and 19.6% for *N*-acetylserotonin, respectively.

Inhibition experiments

Furafylline was used in a final concentration of 20 μ M. When the mechanism-based inhibitor furafylline was applied, a 10-minute preincubation was carried out, and the reaction was started by the addition of the substrate (1 μ M). Fluvoxamine was used either at five different concentrations between 0.01 and 0.2 μ M or at five different concentrations between 0.2 and 10 μ M. Citalopram, fluoxetine, desipramine, and imipramine were used at two different concentrations, 2 and 20 μ M. All inhibition experiments were performed using either 1 or 10 μ M melatonin.

Incubations using recombinant CYP2C19 and CYP3A4

Because the formation of melatonin metabolites was not always linear within 30 minutes of incubation for all recombinant CYPs, recombinant CYPs were incubated for only 10 minutes. During this time, the reaction was found to be in the linear range. Based on the amount of total P450 determined in the microsomes of the liver used (250 pmol/mg-protein = 125 pmol per assay), the respective amounts of the recombinant CYPs were used according to their abundance in human livers.²⁸ The amount of P450 per assay was 4 pmol for CYP2C19 and 36 pmol for CYP3A4. Thus, we were able to directly compare the formation rates by use of either human liver microsomes or recombinant CYPs.

Analysis of kinetic data

The velocities of the enzyme reactions (*v*) were determined from the time-dependent formation of the products 6-hydroxymelatonin and *N*-acetylserotonin after incubation of melatonin in a concentration ranging between 0.5 and 10 μ M. The substrate concentration at half maximal velocity (K_m) and the maximal velocity (V_{max}) were determined by means of a nonlinear regression analysis using the GraFit program (version 4.03, Erithacus Software Ltd., Staines, United Kingdom).

Data were transformed by use of the Eadie-Hofstee plot. Curved linear plots that pointed to the involvement of multiple enzymes were further analyzed assuming two distinct enzymes as follows:

$$v = V_{max_1} \times [S]/(K_{m_1} + [S]) + V_{max_2} \times [S]/(K_{m_2} + [S])$$

either graphically using the method of Rosenthal²⁹ or by use of the GraFit program.

K_m and V_{max} were calculated from the mean formation rates of five replicated incubations; each substrate concentration was analyzed in duplicate. Using pooled microsomes, kinetic analyses were conducted once for each substrate concentration in duplicate.

The inhibition constants were estimated from Dixon plots and from the secondary plots of the slope or y-axis intercept obtained from Lineweaver-Burk plots versus the inhibitor concentration. The x-axis intercept of each graph gives the K_i .

IC_{50} values were calculated by use of the GraFit program applying a two parameter equation:

$$y = 100\% / (1 + [x / IC_{50}]^s)$$

with s indicating the slope factor and x indicating the inhibitor concentration.

Results

Kinetic analysis

Melatonin was converted *in vitro* by human liver microsomes to 6-hydroxymelatonin and, to a minor extent, to *N*-acetylserotonin. From inspection of the Eadie-Hofstee plot constructed from the mean formation rates of five independent experiments, the formation of 6-hydroxymelatonin seemed to be catalyzed by a single enzyme at the concentration range applied (Fig. 2A). A linear regression analysis revealed a correlation coefficient (R^2) of 0.92, and the residuals were randomly distributed around zero. Assuming a two-enzyme Michaelis-Menten equation, the F -test showed a significant ($p < 0.05$) reduction in the χ^2 value, where χ^2 is calculated as:

$$\chi^2 = \sum (\Delta y_n \times \sigma_n^{-1})^2$$

where Δy_n is the residual of the n^{th} data point and σ_n is the variance of the n^{th} data point. The mean (\pm SD) K_m and V_{max} were $6.3 \pm 3.6 \mu\text{M}$ and $15.98 \pm 8.9 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$, respectively ($N = 5$ incubations using three different livers) for the single enzyme model.

The O-demethylation revealed biphasic kinetics (Fig. 2B) indicating at least two different enzymes catalyzing the formation of *N*-acetylserotonin. The mean K_{m1} and V_{max1} for the high-affinity enzyme were $0.47 \pm 0.1 \mu\text{M}$ and $1.0 \pm 0.22 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$, respectively, and for the low-affinity enzyme, K_{m2} and V_{max2} were $8.8 \pm 3.2 \mu\text{M}$ and $3.5 \pm 1.0 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$, respectively. Using pooled liver microsomes, similar K_m and V_{max} values were found. For the formation of 6-hydroxymelatonin, also best described by single-enzyme Michaelis-Menten kinetics, the K_m and V_{max} values were $4.0 \mu\text{M}$ and 18.2

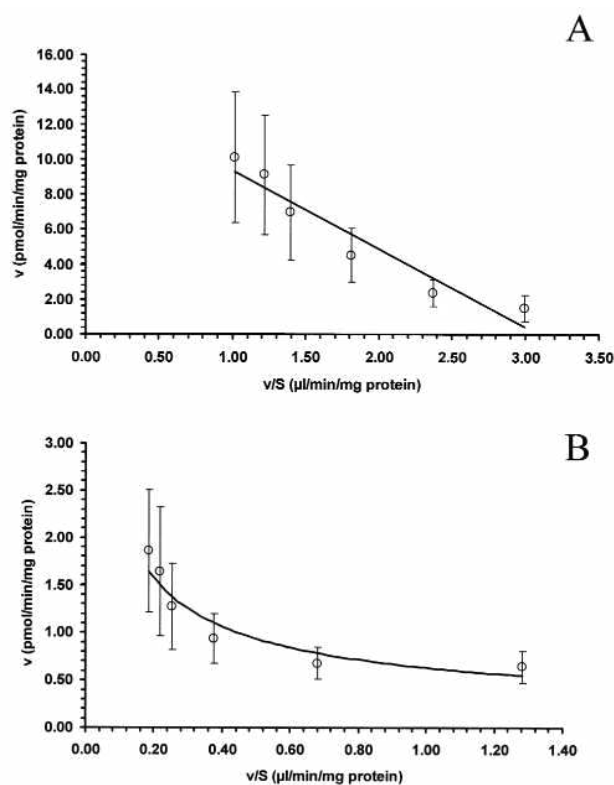


FIG. 2. Eadie-Hofstee plots for the formation of 6-hydroxymelatonin (A) and *N*-acetylserotonin (B) after a 30-minute incubation of microsomes ($0.5 \text{ mg} \cdot \text{protein} / \text{mL}$) with different concentrations of melatonin (0.5 – $10 \mu\text{M}$). Given are the means \pm SD of five different series using three different livers. Each concentration was performed in duplicate for each series.

$\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$, respectively. The formation of *N*-acetylserotonin followed a two-enzyme Michaelis-Menten equation with K_m and V_{max} of the high-affinity enzyme amounting to $0.16 \mu\text{M}$ and $0.81 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$ and $3.2 \mu\text{M}$ and $2.9 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$ for the low-affinity enzyme, respectively.

Inhibition by fluvoxamine

At fluvoxamine concentrations exceeding $0.2 \mu\text{M}$, a formation of 6-hydroxymelatonin could no longer be found after incubation of either 1 or $10 \mu\text{M}$ melatonin. The formation of *N*-acetylserotonin was also reduced by more than 90% at fluvoxamine concentrations of $1.0 \mu\text{M}$. However, even at $2 \mu\text{M}$ fluvoxamine, *N*-acetylserotonin was still detectable, using either 1 or $10 \mu\text{M}$ melatonin.

For assessing the K_i and IC_{50} , lower fluvoxamine concentrations (0.01 – $0.2 \mu\text{M}$) were applied. The resulting K_i values were $0.02 \mu\text{M}$ fluvoxamine for the inhibition of 6-hydroxymelatonin formation and $0.05 \mu\text{M}$ for the inhibition of *N*-acetylserotonin formation (Fig. 3A and B).

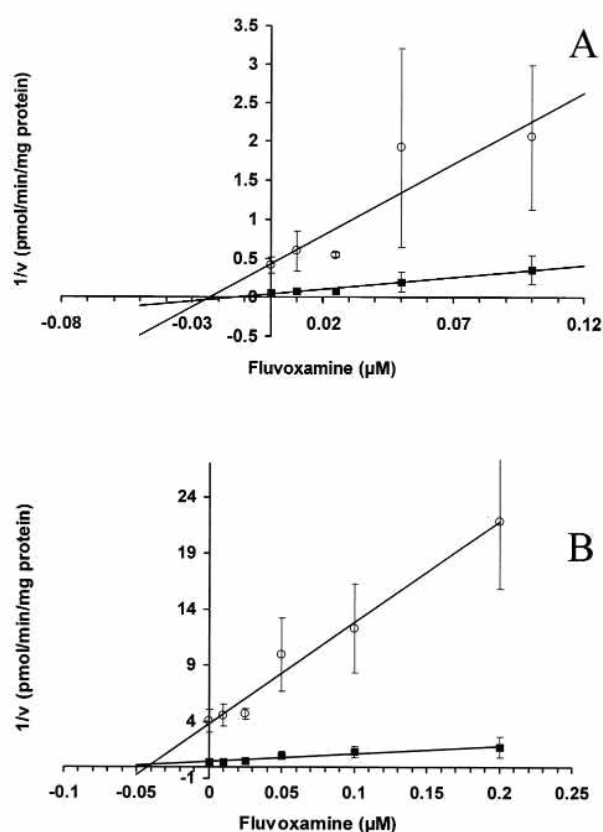


FIG. 3. Dixon plots representing the inhibition of the formation of 6-hydroxymelatonin (A) and *N*-acetylserotonin (B) after concomitant incubation of 1 (□) or 10 (○) µM melatonin with fluvoxamine in five (A) or six (B) different concentrations between 0 and 0.2 µM. Values given are the means \pm SD of four different series using three different livers. Each concentration was assayed in duplicate for each series.

From the construction of IC_{50} curves, a complete inhibition of 6-hydroxylation at fluvoxamine concentrations greater than 0.1 µM could be shown (resulting IC_{50} = 0.035 and 0.087 µM at 1 or 10 µM melatonin, respectively). In contrast, the IC_{50} curves uncovered partial inhibition of the O-demethylation by fluvoxamine with IC_{50} of 0.039 and 0.047 µM (Fig. 4A–D).

Inhibition by furafylline and incubations using recombinant CYPs

When 1 µM melatonin was coincubated with 20 µM furafylline, a selective mechanism-based inhibitor of CYP1A2 activity, the formation of 6-hydroxymelatonin was completely blocked. The formation of *N*-acetylserotonin was reduced to approximately 10% of the control without addition of furafylline. Neither recombinant CYP2C19 nor CYP3A4 was able to hydroxylate melatonin. On the other hand, remarkable amounts of *N*-acetylserotonin were found when recombinant

CYP2C19 was used. At the low melatonin concentration of 1 µM, only approximately 14% of the total *N*-acetylserotonin formation rate in microsomes were found, but at 10 µM melatonin, the *N*-acetylserotonin found after incubation with recombinant CYP2C19 amounted to approximately 50% of *N*-acetylserotonin formed when human liver microsomes were applied.

Effects of other antidepressants

Paroxetine was the only antidepressant tested, with which effects on either the 6-hydroxylation and the O-demethylation were found. However, the significant effects were restricted to the high concentration of paroxetine, resulting in approximately 90% decrease in the formation of 6-hydroxymelatonin and approximately 75% reduction in the formation of *N*-acetylserotonin (Fig. 5A and B). Besides paroxetine, 6-hydroxylation was only affected (–47%) by imipramine, but only when 20 µM imipramine and 1 µM melatonin were coincubated (Fig. 5A).

Discussion

This study confirmed and extended previous results concerning the biotransformation of melatonin and the effect of fluvoxamine on the metabolism of melatonin. As suggested recently, melatonin is almost exclusively metabolized by CYP1A2.²¹ Because that study did not differentiate between the two main metabolites 6-hydroxymelatonin and *N*-acetylserotonin, the authors missed the influence of another enzyme on the O-demethylation of melatonin, CYP2C19, which became visible in our study. It cannot be excluded that other CYPs contribute to melatonin O-demethylation. Other enzymes besides CYP1A2, however, are unlikely to play a major role in the degradation of melatonin because the formation of *N*-acetylserotonin amounted to only 10% of the metabolite formation and was blocked to approximately 90% by furafylline. Melatonin seems therefore likely to be a rather selective substrate of CYP1A2.

Consistent with the suggested predominant role of CYP1A2 in the metabolism of melatonin, this study demonstrated a powerful blockade of the melatonin metabolism by the addition of fluvoxamine. Although fluvoxamine is already known as a potent inhibitor of CYP1A2,¹⁷ the K_i and IC_{50} reported here are approximately a magnitude lower than those reported for inhibition of the metabolism of phenacetin,³⁰ theophylline,³¹ or imipramine.³² Assuming therapeutic fluvoxamine blood concentrations ranging between 0.1 and 1 µM,³² the biotransformation of melatonin should be markedly inhibited *in vivo*.

In this regard, none of the other tested antidepressants was similar to fluvoxamine. Only paroxetine at a concentration of 20 µM affected the 6-hydroxylation

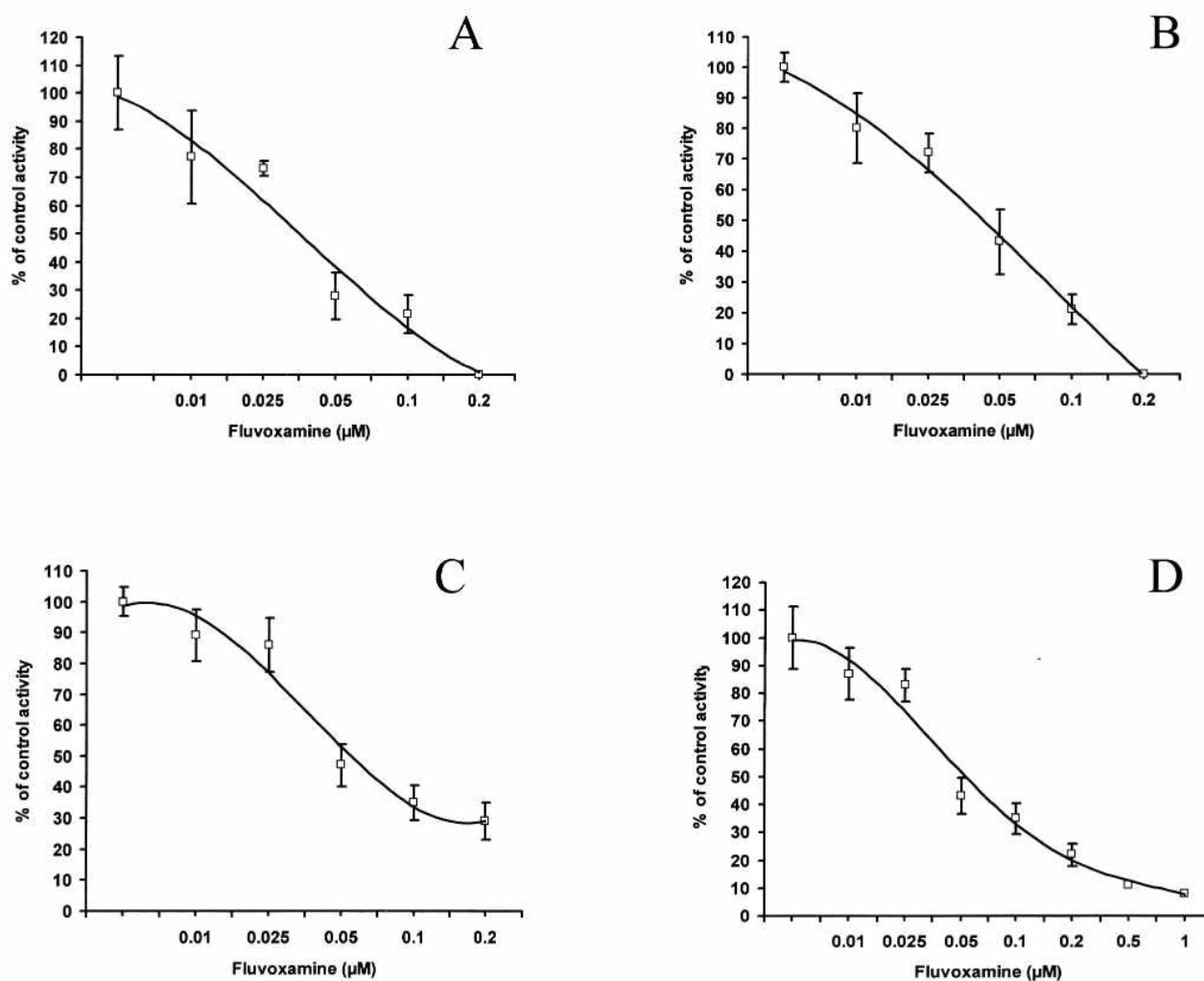


FIG. 4. IC_{50} curves representing the inhibition of the formation of 6-hydroxymelatonin after incubation of at least six different concentrations of fluvoxamine (0–0.2 μ M) with 1 μ M melatonin (A) and 10 μ M melatonin (B). Inhibition of the formation of *N*-acetylserotonin after coincubation of fluvoxamine with 1 and 10 μ M melatonin is shown in the lower plots (C) and (D), respectively. Given are the means \pm SD of four different series using three different livers. Each concentration was assayed in duplicate for each series. The y-axis (% of control activity) indicates the percentage of control activity of either noninhibited 6-hydroxymelatonin or *N*-acetylserotonin formation.

and O-demethylation of melatonin. This concentration, however, far exceeds the paroxetine blood concentrations in patients receiving common therapeutic dosages of paroxetine, and no effect on melatonin serum concentrations was found in patients treated with 20 mg of paroxetine.³³ Despite the inhibitory effect of high paroxetine concentrations *in vitro*, the effect of fluvoxamine on the metabolism of the endogenous substrate melatonin seems unique among the SSRIs and even among other psychotropic drugs. This finding indicates that a psychotropic drug interacts not only with the metabolism of other xenobiotics, but also with the biotransformation of an endogenous substrate.

The pharmacodynamic consequences of these effects are not fully clear. However, it should be emphasized that fluvoxamine was efficiently used to increase the bioavailability of orally administered melatonin, resulting in sufficient sleep induction in a patient with therapy-resistant sleep disturbances.³⁴ The therapeutic use of melatonin is compromised so far partially because of its low oral bioavailability and rapid metabolism.³⁵ Interactions with fluvoxamine might be used as an enhancer strategy to improve the therapeutic effectiveness of melatonin. Because CYP1A2 significantly contributes to the hydroxylation of estrogens,³⁶ fluvoxamine probably interferes with the metabolism of several endogenous compounds.

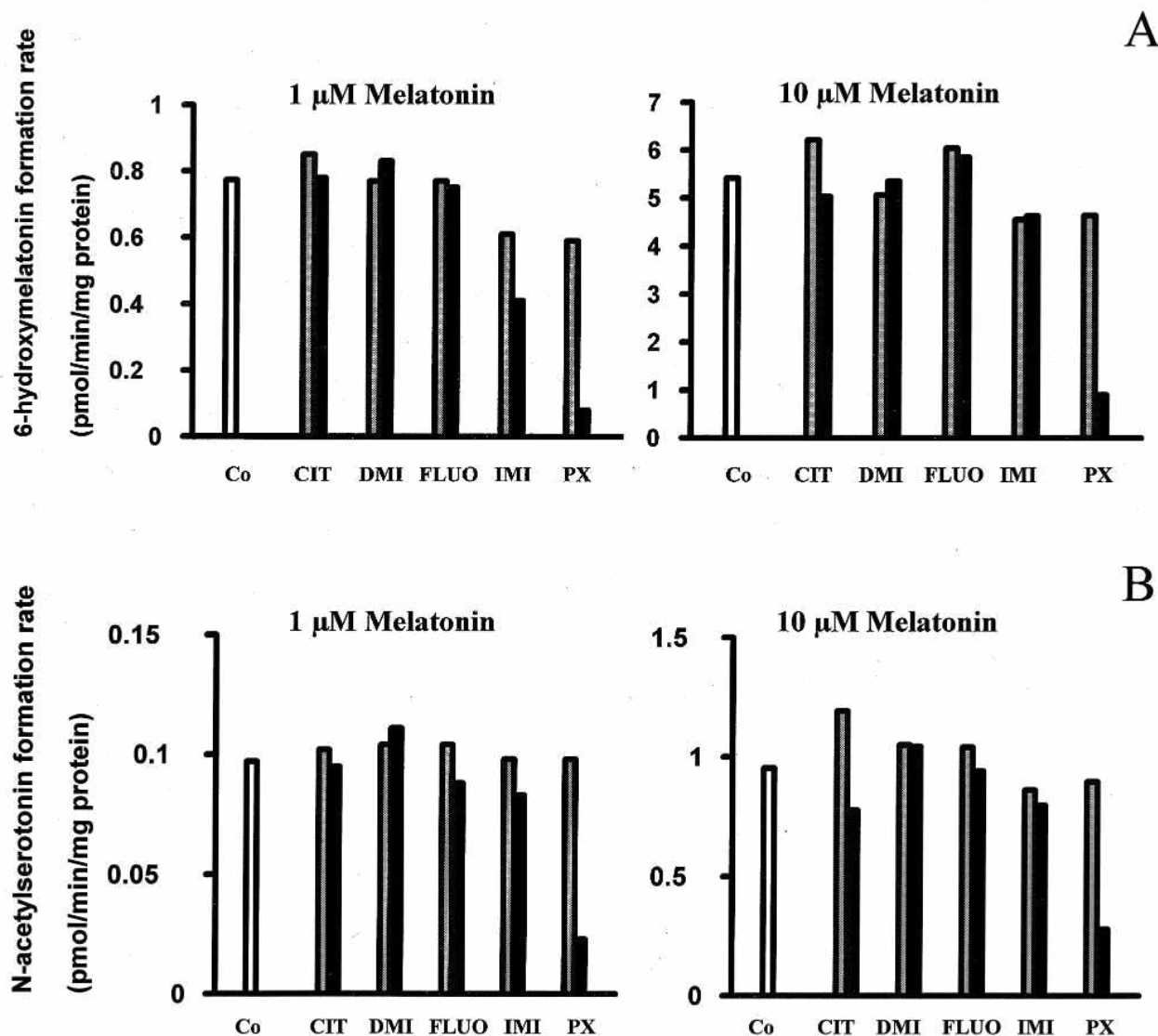


FIG. 5. Influence of different antidepressants on the formation of 6-hydroxymelatonin (A) or *N*-acetylserotonin (B) after coincubation of 1 or 10 μ M melatonin with 2 μ M (shaded bars) or 20 μ M (black bars) of each antidepressant. The control (Co), without addition of any antidepressant, is given in each plot as a white bar. CIT, citalopram; DMI, desipramine; Fluo, fluoxetine; IMI, imipramine; PX, paroxetine. Microsomes from only one liver (HL1) were used.

Acknowledgments

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