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Identification of the Human Cytochromes P450 Responsible for in Vitro Formation of *R*- and *S*-Norfluoxetine

BARBARA J. RING, JAMES A. ECKSTEIN, JENNIFER S. GILLESPIE, SHELLY N. BINKLEY, MARK VANDENBRANDEN, and STEVEN A. WRIGHTON

Department of Drug Disposition, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Indiana

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ABSTRACT

The formation of *R*- and *S*-norfluoxetine was analyzed in vitro in human liver microsomes. Low apparent K_m values for *R*-norfluoxetine formation of ≤8 μM and *S*-norfluoxetine of <0.2 μM were determined. *R*-Norfluoxetine formation rates in a characterized microsomal bank correlated with the catalytic activities for cytochrome P450 (CYP) 2D6, CYP2C9, and CYP2C8. Expressed CYP2C9, CYP2C19, and CYP2D6 formed *R*-norfluoxetine following incubation with 1 μM *R*-fluoxetine and exhibited apparent K_m values of 9.7, 8.5, and 1.8 μM, respectively. Multivariate correlation analysis identified CYP2C9 and CYP2D6 as significant regressors with *R*-norfluoxetine formation. Antibodies to the CYP2C subfamily and CYP2D6 each exhibited moderate inhibition of *R*-norfluoxetine formation. Therefore, CYP2D6 and CYP2C9 contribute to this biotransformation. At pharmacological concentrations of *S*-fluoxetine, *S*-norfluoxetine formation rates in the bank of microsomes were found to correlate only with CYP2D6 catalytic activity and only expressed CYP2D6 was found to be capable of forming *S*-norfluoxetine. Thus, it would appear that both CYP2D6 and CYP2C9 contribute to the formation of *R*-norfluoxetine, whereas only CYP2D6 is responsible for the conversion to *S*-norfluoxetine. Since the enantiomers of fluoxetine and norfluoxetine are inhibitors of CYP2D6, upon chronic dosing, the CYP2D6-mediated metabolism of the fluoxetine enantiomers would likely be inhibited, resulting in *R*-norfluoxetine formation being mediated by CYP2C9 and *S*-norfluoxetine formation being mediated by multiple high K_m enzymes.

Fluoxetine, a racemic mixture of *R*- and *S*-fluoxetine, is a selective serotonin reuptake inhibitor currently marketed for the treatment of depression and other disorders. *R*-fluoxetine was a drug candidate in development for use in psychiatric illness. The major route of metabolism of the enantiomers of fluoxetine is *N*-demethylation (Wong et al., 1995). Identification of the enzymes involved in the formation of norfluoxetine would help explain interindividual differences observed in the metabolic clearances of these compounds. To date, however, the definitive identification of the cytochrome P450s (CYPs) responsible for the metabolism of *R*- and *S*-fluoxetine to *R*- and *S*-norfluoxetine has proven to be elusive.

It has long been recognized that the enantiomers of fluoxetine and norfluoxetine are inhibitors of CYP2D6-mediated reactions. *S*-Fluoxetine and *S*-norfluoxetine are approximately 5-fold more potent in their ability to inhibit CYP2D6-mediated reactions than *R*-fluoxetine and *R*-norfluoxetine (K_i values of 0.22, 0.31, 1.38, and 1.48 μM, respectively) (Stevens and Wrighton, 1993). In spite of this CYP2D6 inhibitory potential, previous studies performed in vitro suggested that CYP2D6 plays only a partial role the biotransformation of *R*- and *S*-fluoxetine to their respective *N*-desmethyl metabolites (Stevens and Wrighton, 1993). A similar conclusion was reached by von Moltke et al. (1997) who determined that although CYP2D6, CYP2C19, and CYP3A partially contributed to the formation of racemic norfluoxetine from racemic fluoxetine, CYP2C9 was the primary CYP responsible for norfluoxetine formation. Recently a third study concluded that CYP2D6, CYP2C9, and CYP3A were the greatest contributors to fluoxetine *N*-demethylation (Margolis et al., 2000).

There have been a few studies in humans that have examined the clearance of the enantiomers of fluoxetine following both single and multiple doses of racemic fluoxetine. In a study examining the pharmacokinetics of a single dose of racemic fluoxetine a major role for CYP2D6 in *S*-fluoxetine metabolism was proposed, for *S*-fluoxetine clearance was 12-fold slower in poor metabolizers (PMs) of CYP2D6-mediated reactions than that observed in extensive metabolizers (EMs) (Fjordside et al., 1999). However, the clearance of *R*-fluoxetine was only 2-fold slower in PMs compared with EMs. These differences in the clearances of *S*- and *R*-fluoxetine in PMs after a single dose indicated that the formation of *S*-norfluoxetine is highly dependent on CYP2D6, whereas other CYPs in addition to CYP2D6 participate in the formation of *R*-norfluoxetine. This differential dependence on

ABBREVIATIONS: CYP, cytochromes P450; PM, poor metabolizer of CYP2D6 substrates; EM, extensive metabolizer of CYP2D6 substrates; HL, human liver; FMO, flavin-containing monooxygenase; HPLC, high-performance liquid chromatography; bql, below quantifiable limit.

CYP2D6 for *S*- and *R*-norfluoxetine formation was also demonstrated by the pharmacokinetic profiles of *S*- and *R*-fluoxetine after multiple dosing of racemic fluoxetine (Bergstrom et al., 1991). These researchers found that when compared with a single dose of fluoxetine, after multiple dosing of fluoxetine the clearance of *S*-fluoxetine was substantially decreased, but that of *R*-fluoxetine was only slightly decreased. Thus, it appears that self-inhibition of the CYP2D6-mediated metabolism by the enantiomers of *S*- and *R*-fluoxetine occurred and had a greater effect on the clearance of *S*-fluoxetine compared with the clearance of *R*-fluoxetine. Therefore, although fluoxetine disposition is different in PMs and EMs following single dosing, these differences are significantly diminished upon multiple dosing. With this information as background, and noting the conflicting conclusions in the previous studies performed in vitro, the aim of this study was to definitively identify the enzymes involved in the *N*-demethylation of the enantiomers of fluoxetine to help explain population variability in the pharmacokinetics of both racemic and *R*-fluoxetine.

Experimental Procedures

Materials. *R*-fluoxetine, *R*-norfluoxetine, *S*-fluoxetine, *S*-norfluoxetine, and LY110086 (internal standard) were synthesized by Eli Lilly and Co. (Indianapolis, IN). Diclofenac, phenacetin, chlorzoxazone, zoxazolamine, 7-hydroxy coumarin, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Midazolam was obtained from Hoffmann La Roche (Nutley, NJ) and 4′-hydroxy diclofenac was obtained from GENTEST (Woburn, MA). *S*-Mephenytoin, 4′-hydroxy mephenytoin, and 1′-hydroxy midazolam were purchased from Ultrafine (Manchester, UK). 6-Hydroxy chlorzoxazone was obtained from Sigma/RBI (Natick, MA) and acetaminophen was obtained from Kodak (Rochester, NY). Coumarin and trolox were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bufuralol and 1′-hydroxy bufuralol were obtained from GENTEST.

Monoclonal antibodies to CYP2D6 and CYP2C in ascites fluid were obtained from Panvera (Madison, WI) and control ascites fluid was obtained from ICN Biochemicals (Aurora, OH). The specificity of these antibodies was shown by the observation that the CYP2C monoclonal antibody inhibited by 90% the form-selective CYP2C19 biotransformation of *S*-mephenytoin 4′-hydroxylase and CYP2C9-specific diclofenac 4′-hydroxylation. The CYP2D6 antibody inhibited >90% of the CYP2D6 form-selective biotransformation of bufuralol 1′-hydroxylation (Panvera).

Microsomes. Human liver samples designated HLA through HLT were obtained from the Medical College of Wisconsin (Milwaukee, WI), Medical College of Virginia (Richmond, VA), or Indiana University School of Medicine (Indianapolis, IN), under protocols approved by the appropriate committee for the conduct of human research. Hepatic microsomes were prepared by differential centrifugation (van der Hoeven and Coon, 1974) and characterized for their relative levels of CYPs and flavin-containing monooxygenase (FMO) via immunoquantification or through the use of form-selective catalytic activities (see below). Microsomes prepared from a human β-lymphoblastoid cell line engineered to express CYPs (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4) were obtained from GENTEST.

Examination of *R*- or *S*-Norfluoxetine Formation. The conversions of *R*-fluoxetine to *R*-norfluoxetine and *S*-fluoxetine to *S*-norfluoxetine were accomplished under linear rate conditions. Incubations were performed for 10 min at 37°C, after a 3-min preincubation, with incubation mixtures containing the indicated concentrations of substrate with human hepatic microsomes (0.5 mg/ml) and 1 mM NADPH in 100 mM sodium phosphate buffer, pH 7.4. The reactions were stopped with an equal volume of acetonitrile followed by the addition of internal standard. The denatured protein was removed by centrifugation and the supernatant analyzed for metabolite formation.

Estimations of apparent enzyme kinetic parameters for the formation of *R*-norfluoxetine were determined by human liver microsomal samples HLM, HLG, HLK, and HLO. Samples HLM and HLG were chosen as representative of livers that contain an average complement of CYPs, and HLO was chosen for its high level of CYP2D6, CYP3A, and CYP2A6 (Table 1). Sample HLK was chosen because it is deficient in CYP2D6 as determined by immunoblot analysis (Wrighton et al., 1993b). In these studies concentrations of *R*-fluoxetine ranged from 1 to 300, 0.25 to 100, 2.5 to 300, and 0.2 to 100 μM, respectively. Similarly, to examine the enzyme kinetics for the formation of *S*-norfluoxetine concentrations of *S*-fluoxetine ranged from 0.5 to 150, 0.11 to 120, and 5 to 150 μM with HLG, HLM, and HLK, respectively.

TABLE 1
CYP form-selective catalytic activities and *R*- and *S*-norfluoxetine formation rates (pmol/min/mg) by a bank of human liver microsomal samples[a]

Sample	Phenacetin *O*-Deethylation (1A2)	Coumarin 7-OH (2A6)	Taxol 6-OH (2C8)	Diclofenac 4′-OH (2C9)	*S*-Mephenytoin 4′-OH (2C19)	Bufuralol 1′-OH (2D6)	Chlorzoxazone 6-OH (2E1)	Midazolam 1′-OH (3A4/5)	*R*-Norfluoxetine Formation	*S*-Norfluoxetine Formation
HLA	1490	470	157	597	58	17	2210	898	1.79	1.90
HLB	492	308	279	337	14	30	1210	833	2.42	3.74
HLC	357	116	185	254	66	19	2440	595	1.71	1.64
HLD	421	836	496	659	0	41	2790	439	5.94	5.51
HLE	747	484	456	517	158	97	1890	4257	5.38	6.01
HLF	862	546	141	305	0	28	1470	2382	1.32	1.77
HLG	595	413	114	373	44	44	920	1632	3.06	3.66
HLH	970	324	5	546	49	25	1630	1143	2.81	2.28
HLI	947	1338	184	501	47	38	3400	5836	3.28	3.62
HLJ	937	388	110	308	88	34	920	1365	2.40	2.47
HLK	769	1031	76	532	35	11[b]	880	1020	1.39	bql
HLL	254	80	52	150	0	15	1030	681	bql	1.01
HLM	197	406	94	591	21	70	1650	909	4.45	5.25
HLN	877	428	198	463	99	14[b]	1280	1244	1.93	1.01
HLO	862	1468		298	26	92	1300	7897	3.74	5.20
HLP	159	728		292	14	44	3800	3157	2.27	3.40
HLQ	293	510		677	13	50	1010	734	4.65	3.60
HLR	121	138		413	32	13	1080	722	2.10	1.39
HLS	1487	1594		441	126	37	1890	2209	3.01	2.08
HLT							1300		1.15	1.78

[a] Assays were performed as described under *Materials and Methods.*
[b] HLK and HLN are CYP2D6-deficient as determined by immunoblot analysis (Wrighton et al., 1993b).

The rates of formation of *R*- and *S*-norfluoxetine were determined following incubations with 0.9 μM *R*-fluoxetine or 2.5 μM *S*-fluoxetine by a human liver microsomal bank characterized for nine CYP form-selective catalytic activities for correlation studies.

Microsomes prepared from human β-lymphoblastoid cells transfected with human CYP cDNA were examined for their ability to form *R*- or *S*-norfluoxetine following 60-min, 37°C incubations with 2 mM NADPH and 1 and 30 μM *R*-fluoxetine or 1 and 75 μM *S*-fluoxetine. For the estimation of apparent enzyme kinetic parameters for the formation of *R*-norfluoxetine by expressed CYP2C9, CYP2C19, or CYP2D6, incubations were performed under initial rate conditions with *R*-fluoxetine concentrations ranging from 0.5 to 75, 1 to 300, and 0.05 to 50 μM, respectively. The enzyme kinetic parameters by expressed CYP2D6 were determined for *S*-norfluoxetine formation under linear rate conditions following incubations with *S*-fluoxetine concentrations ranging from 0.05 to 25 μM.

Monoclonal antibodies to the CYP2C subfamily or CYP2D6 were used with human liver microsomal samples HLM, HLG, and HLS to assess their effect on *R*-norfluoxetine formation. In these studies, microsomes, 2 μM *R*-fluoxetine, and ascites fluid containing antibodies or control ascites fluid were preincubated for 5 min at 37°C prior to the initiation of the 10-min reaction with 1 mM NADPH. Preliminary experiments to determine maximum inhibition of *R*-norfluoxetine formation by each of these antibodies were performed in incubations with HLM containing 1, 2, 4, or 8 μl of each of the ascites preparation. Maximum inhibition for both anti-2C and anti-2D6 occurred with the addition of 4 μl of ascites fluid (data not shown). Therefore, 4 μl of these inhibitory antibodies was used to examine their ability to inhibit the formation of *R*-norfluoxetine.

Phosphate buffer (0.1 M) was added to supernatants from the centrifuged samples and they were loaded onto Isolute HCX solid phase extraction cartridges (130 mg, 3 ml) (Jones Chromatography, Lakewood, CO) preconditioned with sequential washes of methanol and 0.1 M potassium phosphate buffer, pH 6.0. Cartridges were washed sequentially with methanol, acetonitrile and 50:50 (v/v) hexane/ethyl acetate, and eluted with methylene chloride/methanol/concentrated ammonium hydroxide (78.4:19.6:0.2, v/v/v). The eluted samples were dried at 50°C under nitrogen, 1 ml of 2% heptafluorobutyric acid anhydride in hexane added, and samples heated at 80°C for 30 min. Derivatized samples were dried at 50°C under nitrogen, solubilized in hexane, and analyzed for *R*- or *S*-norfluoxetine formation by gas chromatography/mass spectral analysis using a 15-m Restek Rtx-5 MS column (Restek Corp., Bellefonte, PA) and negative chemical ionization with methane as the reagent gas. The lower and upper limits of detection of the analytical assay for both *R*- and *S*-norfluoxetine were 1 and 501 pmol per the 200-μl incubation volume.

CYP Form-Selective Catalytic Activities. The *O*-deethylation of phenacetin (acetaminophen formation) was used as a marker of CYP1A2-mediated metabolism. Acetaminophen was detected by HPLC with UV detection (254 nm) using an Alltima Phenyl column (Alltech, Deerfield, IL) (5 μm, 4.6 × 150 mm) and a mobile phase of 25 mM sodium phosphate buffer, pH 3.0/methanol (95:5, v/v) delivered at 1.0 ml/min with a retention time of 6 min. To characterize the human liver microsomal bank for acetaminophen formation, microsomes were incubated under initial rate conditions with 50 μM phenacetin (Table 1).

The 4′-hydroxylation of diclofenac was used as a marker of CYP2C9-mediated metabolism. 4′-Hydroxy diclofenac and internal standard (trolox) had retention times 1.8 and 1.3 min, respectively, following HPLC with electrochemical detection using a Zorbax SB-CN column (Mac-Mod Analytical, Inc., Chadds Ford, PA), 3.5 μm, 4.6 × 75 mm and a mobile phase of 100 mM potassium phosphate, pH 3.0/ acetonitrile (60:40, v:v) delivered at 1.5 ml/min. To characterize the human liver microsomal bank, incubations were performed under initial rate conditions with 5 μM diclofenac (Table 1).

Chlorzoxazone biotransformation to 6-hydroxy chlorzoxazone was used as a marker of CYP2E1-mediated metabolism. 6-Hydroxy chlorzoxazone and internal standard zoxazolamine were detected by HPLC with UV detection (287 um) with retention times of 11 and 13 min, respectively, using a Zorbax SB-CN column (Mac-Mod Analytical, Inc.), 3.5 μm, 4.6 × 75 mm and a mobile phase of 100 mM potassium phosphate, pH 3.0/acetonitrile (60:40, v/v) delivered at 1.5 ml/min. To characterize the human liver microsomal bank, incubations were performed under initial rate conditions with 400 μM chlorzoxazone (Table 1).

Coumarin 7-hydroxy formation, form-selective for CYP2A6 activity, was characterized in the human liver microsomal bank following incubation under initial rate conditions with 100 μM coumarin by a modification of the method of Greenlee and Poland (1978) (Table 1). Immunoquantification of CYP2B6 in the human liver bank was previously reported by Ekins et al. (1998). Taxol 6-hydroxylation, form-selective for CYP2C8 activity, was determined by the method of Harris et al. (1994) following incubation of the human liver microsomal bank under initial rate conditions with 10 μM Taxol (Table 1). The 4′-hydroxylation of *S*-mephenytoin, as determined by the method of Wrighton et al. (1993a), was used as a marker of CYP2C19-mediated metabolism. Incubations were performed under initial rate conditions with 50 μM *S*-mephenytoin for characterization of the human liver bank (Table 1). The microsomal bank was characterized for 1′-hydroxy bufuralol formation, form-selective for CYP2D6, following incubation under initial rate conditions with 25 μM bufuralol by the method of Ring et al. (1996) (Table 1). The 1′-hydroxylation of midazolam was used as a marker of CYP3A4/5-mediated metabolism and was analyzed by a modification of the method of Wrighton and Ring (1994) using a 4.6 × 150 mm YMC Basic column (YMC Inc., Wilmington, NC) following 1-min incubations. Incubations were performed with 25 μM midazolam for characterization of the human liver bank (Table 1).

Calculations. Enzyme kinetic parameters were determined following fit of the data to the appropriate kinetic equations using nonlinear regression analysis (WinNonlin, version 1.5; Statistical Consultants, Inc., Cary, NC). Formation rates of *R*- or *S*-norfluoxetine were fit to one of the following models (Segel, 1975; Copeland, 1996):

Equation 1 (Michaelis-Menten kinetics):

$$v = (V_{max} \times S)/(K_m + S)$$

Equation 2 (product inhibition observed at high substrate concentrations):

$$v = (V_{max})/(1 + (K_m/S) + (S/K_i))$$

where K_i is the product inhibition constant.

Equation 3 (substrate activation at low substrate concentrations):

$$v = (V_{max} \times S^N)/(K_m{}^N + S^N)$$

where N is the number of apparent substrate binding sites.

Equation 4 (two enzymes involved in the biotransformation):

$$v = ((V_{max1} \times S)/(K_{m1} + S)) + ((V_{max2} \times S)/(K_{m2} + S))$$

Choice of the correct model and weighting within the model were determined by a number of criteria, which included visual inspection of a plot of the data (Michaelis-Menten plot or a transformed Eadie-Hofstee data plot), the random distribution of residuals, size of the sum of squares of the residuals, and the standard error of the parameter estimate.

Univariate correlation analyses were performed (JMP, version 3.2.1; SAS Institute, Inc., Cary, NC) between the rates of metabolite formation and the enzymatic activities or immunoquantified levels of various oxidative enzymes (see above) in a human liver microsomal bank of up to 20 samples. When more than one regressor was found to be significant ($p \leq 0.05$) following univariate regression analysis, multivariate regression analysis was performed. In these analyses, a

stepwise analysis was performed in a forward mode. This procedure added regressors to the correlation that most improve the fit, given that the added term was significant at least at the $p = 0.15$ level. Once potential significant regressors were identified, leverage plots were examined to assess the significance of the added regressors to the correlation model.

Results

***R*-Fluoxetine *N*-Demethylation to *R*-Norfluoxetine.** Enzyme kinetic parameters for the formation of *R*-norfluoxetine were determined under initial rate conditions in four human liver samples: HLO, HLG, HLM, and HLK. The Eadie-Hofstee plot (data not shown) for the formation of *R*-norfluoxetine by microsomal sample HLO was biphasic, suggesting the involvement of at least two enzymes in this biotransformation. This microsomal sample demonstrated high activities of CYP2D6, CYP3A, and CYP2A6 relative to the other human liver microsomal samples examined (Table 1). The apparent low K_m (1.6 μM) and high K_m (34 μM) values for this reaction by HLO were determined using eq. 4 (Table 2). The formation of *R*-norfluoxetine in the three additional human liver microsomal samples exhibited Michaelis-Menten kinetics at low substrate concentrations and product inhibition at high substrate concentrations (Fig. 1). Therefore, the apparent kinetic parameters for this biotransformation by these samples were estimated using eq. 2. Two of these samples, HLG and HLM, contained a full complement of CYP enzymes and exhibited apparent K_m values of <10 μM (Table 2). Sample HLK was deficient in CYP2D6 and exhibited an apparent K_m value of 20 μM (Table 2). The apparent K_i values for these three microsomal samples ranged from 65 to 294 μM.

The rates of formation of *R*-norfluoxetine were determined in a bank of 20 characterized human liver microsomal samples following incubation with 0.9 μM *R*-fluoxetine (Table 1). The formation rates of this metabolite were correlated with the form-selective activities or immunoquantified levels of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A, or FMO as described under *Materials and Methods*. Since the *R*-norfluoxetine formation rate from the microsomal sample HLL was below the quantifiable limit (bql) the correlation analysis was performed including a rate for HLL as zero (Table 3). Significant univariate correlations were obtained between the formation rates of *R*-norfluoxetine and form-selective activities for CYP2C8, CYP2C9, and CYP2D6 (Table 3). Following multivariate correlation analyses, including the form-selective activities for CYP2C8, CYP2C9, and CYP2D6 as potential coregressors with *R*-norfluoxetine formation, a significant correlation was obtained with all three CYPs (Table 3).

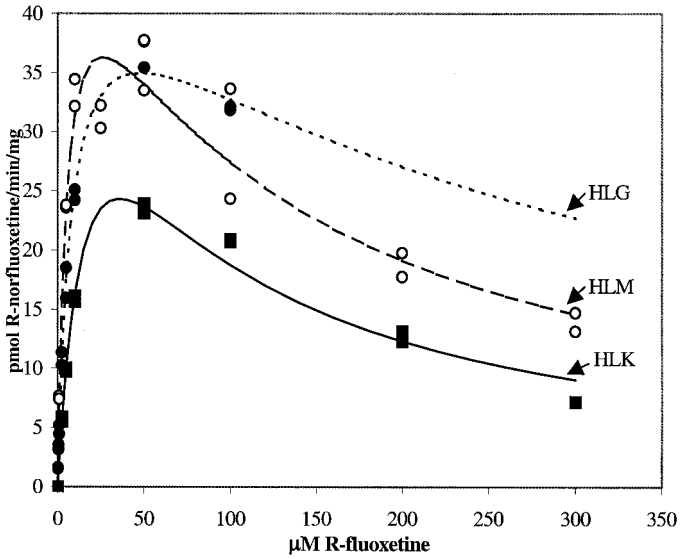

Fig. 1. Kinetics of formation of *R*-norfluoxetine in human liver microsomal samples HLG (●), HLM (○), and HLK (■).

TABLE 3
Significant univariate and multivariate correlations between the formation of *R*-norfluoxetine and CYP form-selective activities or immunoquantified levels in a bank of characterized human liver microsomes

Form-Selective Catalytic Activity	Univariate r^2	Multivariate r^2
1′-Hydroxy bufuralol (CYP2D6)	0.53	
4′-Hydroxy diclofenac (CYP2C9)	0.41	
6α-Hydroxy taxol (CYP2C8)	0.48	
CYP2D6, CYP2C9, and CYP2C8		0.88

Microsomal samples containing expressed cDNA enzymes CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were examined for their ability to form *R*-norfluoxetine following incubation with 1 or 30 μM *R*-fluoxetine. The only enzymes capable of forming *R*-norfluoxetine following incubations of 1 μM *R*-fluoxetine were CYP2C9, CYP2C19, and CYP2D6. At the higher concentration (30 μM), all CYPs examined, except CYP2C8, formed *R*-norfluoxetine; however, the formation rates by CYP2C9, CYP2C19, and CYP2D6 ranged from 7- to 573-fold greater than that observed by the other CYPs. Multivariate regression analyses were again performed taking into consideration the information obtained through the use of expressed CYPs and their ability to form *R*-norfluoxetine following incubation with 1 μM *R*-fluoxetine. Specifically, these analyses examined CYP2C9, CYP2D6, and CYP2C19 as possible coregressors with *R*-norfluoxetine formation rates. Al-

TABLE 2
Enzyme kinetic analyses of the formation of *R*-norfluoxetine by human liver microsomes

Microsomal Sample	K_{m1}	V_{max1}	K_i	K_{m2}	V_{max2}
	μM	*pmol/min/mg*	*μM*	*μM*	*pmol/min/mg*
HLO	1.6 ± 0.4[a]	11 ± 3	n/a	34 ± 8	74 ± 4
HLG	8.0 ± 1.0	47 ± 4	294 ± 112	n/a	n/a
HLM	5.9 ± 0.9	53 ± 4	116 ± 19	n/a	n/a
HLK[b]	20 ± 4	51 ± 9	65 ± 17	n/a	n/a

n/a, not applicable.
[a] Parameter estimate ± standard error of the parameter estimate.
[b] Human liver deficient in CYP2D6.

though CYP2C8 was identified as a possible coregressor in the correlation studies described previously, expressed CYP2C8 was not able to form *R*-norfluoxetine, therefore CYP2C8 was not included in these analyses. The only significant correlation observed with the formation of *R*-norfluoxetine was with both CYP2C9 and CYP2D6.

Apparent enzyme kinetic parameters for the formation of *R*-norfluoxetine were determined under initial rate conditions with expressed CYP2C9, CYP2C19, and CYP2D6 (Table 4). The formation of *R*-norfluoxetine by CYP2C19 exhibited traditional Michaelis-Menten kinetics (eq. 1), exhibiting an apparent K_m value of 8.5 μM. Formation of *R*-norfluoxetine by CYP2C9 exhibited substrate activation at low *R*-fluoxetine concentrations and substrate inhibition at a high *R*-fluoxetine concentration of 75 μM. Since there is not an established enzyme kinetic model for this formation rate profile (substrate activation followed by substrate inhibition) and the substrate inhibition occurred at a concentration of *R*-fluoxetine (75 μM) that was well above that expected to be observed in patients (<1 μM), the 75 μM point was removed from the analysis. As a result, the apparent kinetic parameters for this biotransformation were determined using the Hill equation (eq. 3), resulting in an apparent K_m value of 9.7 μM and a calculated N of 1.2. The formation of *R*-norfluoxetine by CYP2D6 exhibited substrate inhibition at high substrate concentrations, therefore the enzyme kinetic parameters were estimated by fit of the data to eq. 2, resulting in an apparent K_m value of 1.8 μM.

Monoclonal antibodies to the CYP2C subfamily or CYP2D6 were examined for their ability to inhibit *R*-norfluoxetine formation by three human liver microsomal samples containing a full complement of the CYPs (HLG, HLM, and HLS) following incubation with 2 μM *R*-fluoxetine. The CYP2C antibody inhibited *R*-norfluoxetine formation 40, 41, and 80% (average 54%) in incubations with HLG, HLM, and HLS, respectively. The antibody to CYP2D6 inhibited this biotransformation 40, 45, and 33% (average 39%) when incubated with HLG, HLM, and HLS, respectively.

S-Fluoxetine N-Demethylation to S-Norfluoxetine. Enzyme kinetic parameters for the formation of *S*-norfluoxetine were determined under initial rate conditions in three human liver samples: HLM, HLG, and HLK. Eadie-Hofstee plots of the formation of *S*-norfluoxetine by HLM and HLG were biphasic in nature, consistent with two enzymes being responsible for this biotransformation. Therefore, the kinetic parameters for the formation of *S*-norfluoxetine by HLG and HLM were estimated using eq. 4. The low K_m values for this biotransformation in HLG and HLM were 0.17 and 0.18 μM, respectively (Table 5). The high K_m values for the formation of *S*-norfluoxetine were 88 and 67 μM in HLG and HLM, respectively (Table 5). The Eadie-Hofstee plot of the data generated for HLK, a liver deficient in CYP2D6, was monophasic in nature, which suggests that one enzyme was responsible for this biotransformation in this sample. The kinetic parameters obtained for the formation of *S*-norfluoxetine in HLK were determined using eq. 1, resulting in an apparent K_m value of 109 μM for this biotransformation (Table 5).

The rates of formation of *S*-norfluoxetine were determined by a bank of 20 characterized human liver microsomal samples following incubation with 2.5 μM *S*-fluoxetine (Table 1). The formation rates of this metabolite were then correlated to the CYP-selective activities indicated in Table 1, including a formation rate from HLK as zero since it was bql (Table 6). Significant univariate correlations were obtained between the formation rates of *S*-norfluoxetine and the catalytic activities for CYP2C8 and CYP2D6. Following multivariate correlation analyses, which included as possible coregressors CYP2D6 and CYP2C8, the only significant correlation obtained was with CYP2D6 and the formation of *S*-norfluoxetine.

The expressed cDNA enzymes, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, were examined for their ability to form *S*-norfluoxetine following incubation with 1 or 75 μM *S*-fluoxetine. The only enzyme capable of forming *S*-norfluoxetine following incubations with 1 μM *S*-fluoxetine was CYP2D6. After incubation with 75 μM *S*-fluoxetine, all CYPs except CYP2A6 and CYP2B6 formed *S*-norfluoxetine. Of the enzymes that formed *S*-norfluoxetine during the incubation with 75 μM *S*-fluoxetine, the formation rate by CYP2D6 was the greatest observed. Multivariate regression analysis was again performed adding as possible coregressors with *S*-nor-

TABLE 4
Enzyme kinetic analyses of the metabolism of *R*-fluoxetine to *R*-norfluoxetine by expressed CYP2C9, CYP2C19, and CYP2D6

Microsomal Sample	K_m	V_{max}	K_i	*N*
	μM	*pmol/min/pmol CYP*	μM	
CYP2C9	9.7 ± 0.6[a]	0.396 ± 0.011	n/a	1.2 ± 0.0
CYP2C19	8.5 ± 0.7	0.373 ± 0.014	n/a	n/a
CYP2D6	1.8 ± 0.1	0.183 ± 0.008	120 ± 46	n/a

n/a, not applicable.
[a] Parameter estimate ± standard error of the parameter estimate.

TABLE 5
Enzyme kinetic analyses of the metabolism of *S*-fluoxetine to *S*-norfluoxetine by human liver microsomes and expressed CYP2D6

Microsomal Sample	K_{m1}	V_{max1}	K_{m2}	V_{max2}
	μM	*pmol/min/mg*	μM	*pmol/min/mg*
HLG	0.17 ± 0.05[a]	2.4 ± 0.2	88 ± 9	49 ± 3
HLM	0.18 ± 0.02	4.4 ± 0.2	67 ± 9	38 ± 3
HLK[b]	109 ± 26	49 ± 7	n/a	n/a

	K_m	V_{max}
	μM	*pmol/min/pmol CYP*
Expressed CYP2D6	0.58 ± 0.02	0.173 ± 0.003

n/a, not applicable.
[a] Parameter estimate ± standard error of the parameter estimate.
[b] Human liver deficient in CYP2D6.

TABLE 6
Significant univariate and multivariate correlations between the formation of *S*-norfluoxetine and CYP form-selective activities or immunoquantified levels in a bank of characterized human liver microsomes

Form-Selective Catalytic Activity	Univariate r^2	Multivariate r^2
HLK included as zero		
1′-Hydroxy bufuralol (CYP2D6)[a]	0.76	
6α-Hydroxy taxol (CYP2C8)	0.43	
CYP2D6[a]		0.76

[a] Activity found to be a significant coregressor following multivariate analysis.

fluoxetine formation all the CYPs able to form this metabolite following incubations with 75 μM *S*-fluoxetine. Once again, the only significant correlation with the formation of *S*-norfluoxetine was with the catalytic activity for CYP2D6.

Apparent enzyme kinetic parameters for the formation of *S*-norfluoxetine were determined with expressed CYP2D6 under initial rate conditions. The formation of *S*-norfluoxetine by CYP2D6 exhibited traditional Michaelis-Menten kinetics (eq. 1), resulting in an apparent K_m value of 0.58 $\pm$ 0.02 μM (Table 5).

Discussion

The identification of the enzyme(s) responsible for the oxidative metabolism of a drug allows one to predict and/or explain interindividual differences in the effects of the drug that are due to differences in its metabolic clearance. Previous in vitro (Stevens and Wrighton, 1993; von Moltke et al., 1997, Margolis et al., 2000) and in vivo studies (Bergstrom et al., 1991; Fjordside et al., 1999) suggested CYP2D6 contributes to the biotransformation of the enantiomers of fluoxetine to their *N*-demethylated metabolites. However, these studies also suggested that additional enzymes were involved in these biotransformations. Therefore, the current studies were designed to definitively identify the enzymes involved in the *N*-demethylation of *R*- and *S*-fluoxetine.

An examination of the enzyme kinetics of *R*-norfluoxetine formation suggested that at least two enzymes were involved in this biotransformation, which exhibited apparent K_m values ranging from about 1 to 35 μM. It is interesting to note that inhibition of *R*-norfluoxetine formation was observed at high substrate concentrations in three of four microsomal samples examined. This observation is similar to that reported by Stevens and Wrighton (1993) and von Moltke et al. (1997). This phenomenon is most likely due to product inhibition (Copeland, 1996). In these studies the apparent affinity of the enzyme-product complex (K_i values) ranged from 65 to 294 μM. This greatly exceeds the expected in vivo steady-state concentration of <1 μM *R*-fluoxetine; therefore, product inhibition would not be expected to be a factor in the in vivo clearance of *R*-fluoxetine.

To identify the low K_m enzyme(s) involved in the formation of *R*-norfluoxetine further studies were performed using *R*-fluoxetine concentrations near the low K_m value observed for this biotransformation. These studies included correlating the rates of *R*-norfluoxetine formation to known activities mediated by the CYPs and FMO in a bank of liver microsomal samples, examination of the ability of cDNA expressed CYPs to form this metabolite, and the use of monoclonal antibodies in an attempt to inhibit this biotransformation. The formation of *R*-norfluoxetine following incubation with a pharmacological concentration of *R*-fluoxetine correlated to CYP2C9, CYP2D6, and CYP2C8 form-selective catalytic activities. Expressed CYP2C9, CYP2C19, and CYP2D6 were the only enzymes able to form *R*-norfluoxetine. Performing multivariate regression analysis using CYPs identified in these studies as possible coregressors with *R*-norfluoxetine formation, only CYP2C9 and CYP2D6 were found to be significant regressors with the formation of *R*-norfluoxetine. Monoclonal antibodies were used to quantify the role of particular CYPs in the formation of *R*-norfluoxetine (Gelboin et al., 1999). Through the use of these antibodies the role of CYP2C9 and CYP2D6 in this biotransformation by human liver microsomes was further confirmed. Finally, kinetic assessments of the formation of *R*-norfluoxetine by expressed CYP2D6, CYP2C9, and CYP2C19 determined apparent K_m values of 1.8, 9.7, and 8.5 μM, respectively. Interestingly, the kinetic analyses with expressed CYP2D6 and CYP2C9 exhibited product inhibition at high *R*-fluoxetine concentrations, which was similar to that observed in three microsomal liver samples. Expressed CYP2C9 also exhibited substrate activation at low concentrations.

Taken together, the data presented indicate that in microsomal samples containing a full complement of CYPs, the contribution of CYP2C9 and CYP2D6 to the formation of *R*-norfluoxetine at low *R*-fluoxetine concentrations is similar. Although present in relatively small amounts (~2%, Shimada et al., 1994) in the human liver, CYP2D6 has a low K_m value, which indicates a high affinity for *R*-fluoxetine. This coupled with the antibody inhibition data suggests that CYP2D6 plays an important role (~40%) in the formation of *R*-norfluoxetine. Although CYP2C9 appears to have a 5-fold higher K_m value for *R*-fluoxetine, results with inhibitory antibodies suggest it also plays a primary role in *R*-fluoxetine metabolism (~55%), which is likely due to CYP2C9 levels in the liver that are about 10-fold greater than those of CYP2D6 (Shimada et al., 1994). Expressed CYP2C19 and CYP2C9 have a similar affinity for *R*-fluoxetine; however, CYP2C19 represents only ~1% of the CYPs in the liver (Inoue et al., 1997), therefore it would not be expected to play a major role in this biotransformation. Furthermore, the correlation studies indicated that CYP2C19 levels were not related to the formation of *R*-norfluoxetine, suggesting that CYP2C19 does not play a significant role in this biotransformation.

Similar studies were performed examining the conversion of *S*-fluoxetine to *S*-norfluoxetine. Enzyme kinetic studies in two liver samples containing a full complement of enzymes were consistent with two enzymes being involved in this biotransformation, with the low K_m enzyme exhibiting an apparent K_m value of about 0.2 μM. Interestingly, a microsomal sample deficient in CYP2D6 (HLK) apparently contained only the high K_m enzyme (K_m = 109 μM) able to form *S*-norfluoxetine. In the correlation studies, the only activity that correlated with *S*-norfluoxetine formation following incubation with 2.5 μM *S*-fluoxetine was that for CYP2D6. Only expressed CYP2D6 was able to form this metabolite (apparent K_m value of 0.58 μM) at a low *S*-fluoxetine concentration. These results confirm the apparently exclusive role of CYP2D6 in this biotransformation at low, pharmacological *S*-fluoxetine concentrations.

In the current study, multiple CYPs were found to be capable of forming both *R*- and *S*-norfluoxetine following incubation with high concentrations of *R*- and *S*-fluoxetine. As reported herein, at high concentrations of substrate, CYP2D6 is only one of many CYPs that may participate in this biotransformation. This may explain the conclusions of von Moltke et al. (1997) and Margolis et al. (2000) who suggested that in addition to CYP2D6 and CYP2C9 playing a role in norfluoxetine formation, that CYP2C19 and CYP3A may also be involved. These conclusions were confirmed in the current studies where CYP2C19 and CYP3A (along with other CYPs) were able to form *R*- and *S*-norfluoxetine at high substrate concentrations.

The involvement of CYP2D6 and CYP2C9 in the metabo-

lism of the enantiomers of fluoxetine at pharmacological concentrations helps to explain the pharmacokinetic parameters observed with the administration of racemic fluoxetine. The identification of CYP2D6 as the principle enzyme responsible for the formation of *S*-norfluoxetine is important since CYP2D6 is polymorphically expressed where 5 to 10% of the Caucasian population and <1% of the Asian population lack functional enzyme. Therefore, the involvement of CYP2D6 in *S*-norfluoxetine formation explains the observation made in vivo where following a single dose of racemic fluoxetine the clearance of *S*-fluoxetine in PMs of substrates of CYP2D6 was 12-fold slower than that observed in an EM population (Fjordside et al., 1999). However, because both CYP2C9 and CYP2D6 contribute to *R*-norfluoxetine formation, the clearance of *R*-fluoxetine should be less affected in a CYP2D6 PM population than *S*-fluoxetine, since *R*-norfluoxetine would also be substantially formed by CYP2C9. This was also confirmed in the Fjordside et al. (1999) in which the change in *R*-fluoxetine clearance in PMs was reported to be only 2-fold slower than that observed in EMs. Interestingly, it has been observed that upon multiple dosing of racemic fluoxetine, the metabolism of coadministered dextromethorphan, a CYP2D6 substrate, in EMs was inhibited to a point where a majority of the subjects became phenotypically PMs of CYP2D6-mediated dextromethorphan *O*-deethylation (Alfaro et al., 1999). This is apparently due to the potent inhibition by *S*-fluoxetine and *S*-norfluoxetine of CYP2D6-mediated reactions [K_i values of 0.22 and 0.31, respectively (Stevens and Wrighton, 1993)]. Furthermore, since CYP2D6 has been identified as the primary enzyme involved in *S*-norfluoxetine formation, inhibition of its own metabolism would be predicted to occur upon multiple dosing. This is exactly what was observed after chronic dosing of racemic fluoxetine (Bergstrom et al., 1991). Specifically, upon chronic administration of 60 mg/day racemic fluoxetine, EM subjects were found to clear *S*-fluoxetine similarly to that of PMs. Therefore, with the chronic use of racemic fluoxetine, patients, no matter what their CYP2D6 genotype, would be phenotypically PMs of CYP2D6 substrates. Therefore, CYP2C9 and the other enzymes that exhibit a high K_m value for the conversion of the enantiomers of fluoxetine to norfluoxetine most likely mediate the metabolic clearance of racemic fluoxetine upon chronic dosing.

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References

Alfaro CL, Lam YWF, Simpson J and Ereshefsky L (1999) CYP2D6 status of extensive metabolizers after multiple-dose fluoxetine, fluvoxamine, paroxetine, or sertraline. *J Clin Psychopharmacol* **19:**155–163.

Bergstrom RF, Wolen RL, Peyton AL, Goodwin MR, Kasper SC, Barton R, Schwertschlag U and Lemberger L (1991) Pharmacokinetics of R- and S-fluoxetine enantiomers and the effect of P450IID6 activity on their disposition in man. *Pharm Res* **9:**S206.

Copeland RA (1996) *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*. John Wiley & Sons, New York.

Ekins S, VandenBranden M, Ring BJ, Gillespie JS, Yang TJ, Gelboin HV and Wrighton SA (1998) Further characterization of the expression in liver and catalytic activity of CYP2B6. *J Pharmacol Exp Ther* **286:**1253–1259.

Fjordside L, Jeppesen U, Eap CB, Powell K, Baumann P and Brosen K (1999) The stereoselective metabolism of fluoxetine in poor and extensive metabolizers of sparteine. *Pharmacogenetics* **9:**55–60.

Greenlee WF and Poland A (1978) An improved assay of 7-ethoxycoumarin O-deethylase activity: induction of hepatic enzyme activity in C57B:6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. *J Pharmacol Exp Ther* **205:**596–605.

Gelboin HV, Krausz KW, Gonzalez FJ and Yang TJ (1999) Inhibitory monoclonal antibodies to human cytochrome P450 enzymes: a new avenue for drug discovery. *Trends Pharmacol Sci* **20:**432–438.

Harris JW, Rahman A, Kim B-R, Guengerich FP and Collins JM (1994) Metabolism of Taxol by human hepatic microsomes and liver slices: participation of cytochrome P450 3A4 and an unknown P450 enzyme. *Cancer Res* **54:**4026–4035.

Inoue K, Yamazaki H, Imiya K, Akasaka S, Guengerich FP and Shimada T (1997) Relationship between CYP2C9 and 2C19 genotypes and tolbutamide methyl hydroxylation and S-mephenytoin 4′-hydroxylation activities in livers of Japanese and Caucasian populations. *Pharmacogenetics* **7:**103–113.

Margolis JM, O'Donnell JP, Mankowski DC, Ekins S and Obach RS (2000) (R)-, (S)-, and racemic fluoxetine N-demethylation by human cytochrome P450 enzymes. *Drug Metab Dispos* **28:**1187–1191.

Ring BJ, Binkley SN, VandenBranden M and Wrighton SA (1996) In vitro interaction of the antipsychotic agent olanzapine with human cytochromes P450 CYP2C9, CYP2C19, CYP2D6 and CYP3A. *Br J Clin Pharmacol* **41:**181–186.

Segel IH (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*, John Wiley & Sons, New York.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* **270:**414–423.

Stevens JC and Wrighton SA (1993) Interaction of the enantiomers of fluoxetine and norfluoxetine with human liver cytochromes P450. *J Pharmacol Exp Ther* **266:** 964–971.

van der Hoeven TA and Coon MJ (1974) Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. *J Biol Chem* **249:**6302–6310.

von Moltke LL, Greenblatt DJ, Duan SX, Schmider J, Wright CE, Harmatz JS and Shader RI (1997) Human cytochrome mediated N-demethylation of fluoxetine in vitro. *Psychopharmacology* **132:**402–407.

Wong DT, Bymaster FP and Engleman EA (1995) Prozac (fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: twenty years since its first publication. *Life Sci* **57:**411–441.

Wrighton SA and Ring BJ (1994) Inhibition of human CYP3A catalyzed 1′-hydroxy midazolam formation by ketoconazole, nifedipine, erythromycin, cimetidine, and nizatidine. *Pharm Res* **11:**921–924.

Wrighton SA, Stevens JC, Becker GW and VandenBranden M (1993a) Isolation and characterization of human liver cytochrome P450 2C19: correlation between 2C19 and S-mephenytoin 4′-hydroxylation. *Arch Biochem Biophys* **306:**240–245.

Wrighton SA, VandenBranden M, Stevens JC, Shipley LA and Ring BR (1993b) In vitro methods for assessing human hepatic drug metabolism: their use in drug development. *Drug Metab Rev* **25:**453–484.

Send reprint requests to: Barbara J. Ring, Lilly Corporate Center, Mail Drop 0730, Eli Lilly and Co., Indianapolis, IN 46285. E-mail: ring_barbara_j@lilly.com