

## Differential Time- and NADPH-dependent Inhibition of CYP2C19 by Enantiomers of Fluoxetine

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# Application Note

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### Abstract.

Fluoxetine [ $\pm$ -N-methyl-3-phenyl-3-[( $\alpha,\alpha,\alpha$ -trifluoro-p-tolyl)oxy]propylamine)] is a widely used selective serotonin reuptake inhibitor useful in treating depression and other serotonin-dependent disease conditions. Racemic fluoxetine and its (R)- and (S)- enantiomers are potent reversible inhibitors of CYP2D6 and the racemate has been shown to be a mechanism-based inhibitor of CYP3A4. Rac-fluoxetine also demonstrates time- and concentration-dependent inhibition of CYP2C19 catalytic activity *in vitro*. In the course of developing our laboratory's time-dependent inhibition assay for CYP2C19, we tested model compounds that might serve as a reference (positive control) to measure time-dependent inhibition of CYP2C19. In this study, we compared fluoxetine, its (R)- and (S)-enantiomers, ticlopidine, and the reversible CYP2C19 inhibitor S-benzyl nirvanol. In a reversible inhibition protocol (30 minute preincubation with liver microsomes without NADPH), we found (R)-, (S)-, and racemic fluoxetine to be moderate inhibitors with  $IC_{50}$  values of 17, 67, and 27  $\mu$ M, respectively. However, when the preincubation was supplemented with NADPH,  $IC_{50}$ -values shifted to 4.1, 3.4, and 1.8  $\mu$ M. Thus, (S)-fluoxetine demonstrated a 20-fold shift in the  $IC_{50}$  value. Follow-up  $K_I$  and  $k_{inact}$  determinations confirmed time-dependent inhibition [e.g.  $K_I = 46 \mu$ M and  $k_{inact} = 0.064 \text{ min}^{-1}$  for (S)-fluoxetine];  $K_I = 5.3 \mu$ M,  $0.018 \text{ min}^{-1}$  for (R)-fluoxetine]. By contrast, ticlopidine showed an approximate 2-fold shift in  $IC_{50}$  value and (S)-benzyl nirvanol exhibited no shift, as expected. Although the (S)-isomer exhibits a much lower affinity for CYP2C19 inactivation relative to the (R)-enantiomer, it exhibits a more rapid rate of inactivation. From a practical viewpoint, (S)-fluoxetine appears to be a highly suitable reference inhibitor for time-dependent inhibition of liver microsomal CYP2C19. These data may have implications for explaining inhibition of S mephenytoin metabolism in healthy volunteers following administration of fluoxetine.



## Introduction.

Racemic fluoxetine and/or its enantiomers can be reversible inhibitors of CYP2D6 (Brosen, K. and Skjelbo, 1991; Stevens and Wrighton, 1993), CYP2C19 (Kobayashi, et al., 1995; Foti and Wahlstrom, 2008), CYP3A4 (von Moltke, et al., 1994; Ring, et al., 1995), and CYP2C9 (Schmider, et al., 1997; Hemeryck, et al., 1999). Fewer studies have examined the potential for fluoxetine to be a mechanism-based inhibitor (MBI) of cytochrome P450. Mayhew, et al. (2000) showed fluoxetine to be a MBI of CYP3A4 and McGinnity, et al. (2006) recently demonstrated time- and concentration-dependent inhibition of CYP3A4 and CYP2C19 in multiple *in vitro* systems. With heightened awareness of links between MBIs, covalent binding and idiosyncratic toxicity as well as the appearance of regulatory guidance for drug-drug interaction testing (USFDA, 2006), many laboratories are establishing procedures for conducting time-dependent CYP inhibition testing. Ticlopidine is often used as a positive control time-dependent inhibitor of CYP2C19, but we and others have found it be only weakly inhibitory, and therefore unsatisfactory as a benchmark. Here we show that the enantiomers of fluoxetine are kinetically different, time-dependent inhibitors of CYP2C19. Although the (R)-isomer appears to be a more efficient inactivator, as determined by  $k_{\text{inact}}/K_I$  ratios, from a practical viewpoint, (S)-fluoxetine appears to be a suitable reference inhibitor for time-dependent inhibition of liver microsomal CYP2C19.

## Methods.

Inhibition parameters were determined using pooled HLMs (BD Biosciences – Discovery Labware, Woburn, MA, cat. no. 452161). Incubations were conducted in 100 mM KPi pH 7.4. For  $IC_{50}$  shift experiments, multiple concentrations of inhibitors were incubated with HLMs with and without a NADPH regenerating system (BD Biosciences – Discovery Labware, Woburn, MA, cat. nos. 451220, 451200) for 30 minutes prior to 5x dilution into a secondary incubation containing 40  $\mu$ M S-mephenytoin (at  $\sim K_m$ ). For  $K_I/k_{inact}$  experiments, increasing concentrations of racemic, S-, or R-fluoxetine were incubated with HLMs and a NADPH regenerating system for multiple time points prior to dilution into a secondary incubation containing 200  $\mu$ M S-mephenytoin.  $IC_{50}$  values were calculated by linear interpolation.  $IC_{50}$  “shift” was calculated as the ratio of  $IC_{50}$  values in the absence and presence of NADPH.  $K_I$  and  $k_{inact}$  values were determined by non-linear regression (SigmaPlot v8.0 with EK module v1.1). Quantitation of 4'-OH-S-mephenytoin was determined using LC/MS/MS with a stable-labeled isotope internal standard, 4-hydroxymephenytoin-d3 (BD Biosciences – Discovery Labware, Woburn, MA, cat. no. 451007).

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## Results

**Table 1.** Summary of  $IC_{50}$  shift experiments

Compound	$IC_{50}$ - NADPH in a 30 min preincubation					$IC_{50}$ + NADPH in a 30 min preincubation					$IC_{50}$ shift (mean)
	Run #1	Run #2	Run #3	Run #4	Mean	Run #1	Run #2	Run #3	Run #4	Mean	
(±)-Fluoxetine	27	22	22	37	27	6.0	1.8	3.3	3.9	3.8	8.2
(S)-Fluoxetine	67	109	91	79	87	3.4	4.0	2.7	3.3	3.3	26
(R)-Fluoxetine	17	21	20	22	20	4.1	4.7	3.5	3.9	4.1	4.9
(S)-Benzylrivanol	0.17	0.19	0.16	N.D.	0.2	0.44	0.41	0.43	N.D.	0.43	0.41
Ticlopidine	1.1	1.2	1.4	N.D.	1.3	0.81	0.76	0.59	N.D.	0.72	1.8

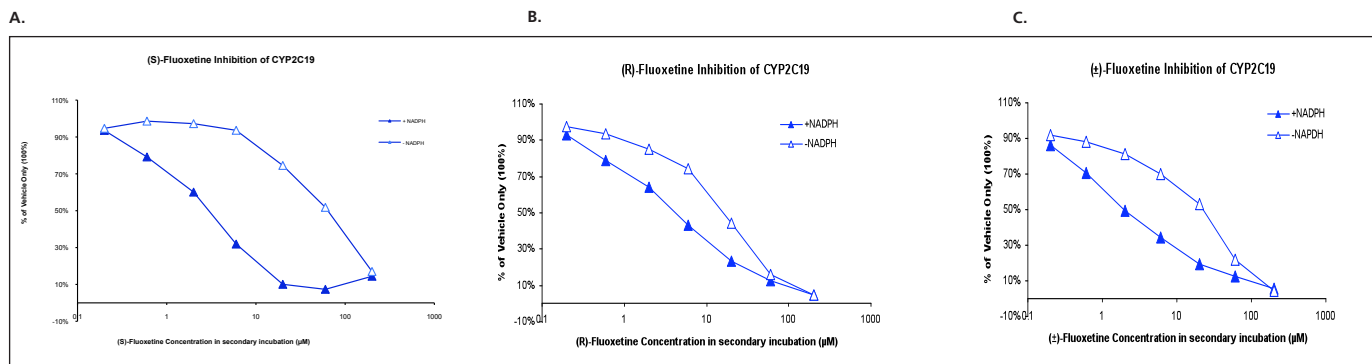
\*Each run was conducted on independent days. Global interday CV for all  $IC_{50}$  values was 0.17. Values were not corrected for free fraction.

Highlighted cell shows (S)-fluoxetine displays a mean 26-fold shift in  $IC_{50}$  values found after preincubation with and without a NADPH regenerating system, making it more robust than ticlopidine as a time-dependent inhibitor of CYP2C19.

**Table 2.** Summary of  $IC_{50}$  shift experiments

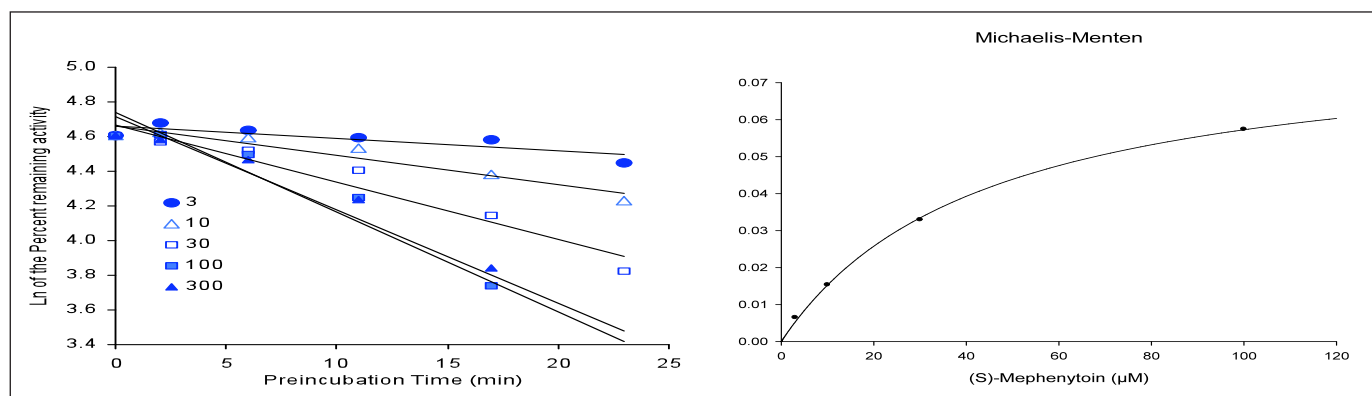
Compound	$k_{inact}$ ( $min^{-1}$ )			$K_i$ ( $\mu M$ )			$k_{inact}/K_i$ (mL/min/ $\mu mol$ )
	Run #1	Run #2	Literature <sup>1</sup>	Run #1	Run #2	Literature <sup>1</sup>	
(±)-Fluoxetine	0.031	N.D.	0.03	13	N.D.	8	2.38
(S)-Fluoxetine	0.064	0.082	Unavailable	46	44	Unavailable	1.64
(R)-Fluoxetine	0.018	N.D.	Unavailable	5.3	N.D.	Unavailable	3.47

1 – Literature values were from McGinness, et al. (2006) and all values represent apparent parameters not corrected for free fraction.



**Figure 1.** Representative  $IC_{50}$  Shift Plots.

Representative  $IC_{50}$  plot of (S)-fluoxetine (1A), (R)-fluoxetine (1B) or (±)fluoxetine (1C) inhibition of human liver microsomal 4'-hydroxylation of (S)-mephenytoin after preincubation with or without a NADPH regenerating system for 30 minutes.



**Figure 2.**  $K_i / k_{inact}$  Examples.

Representative plots showing time- and concentration- dependent loss of human liver microsomal S-mephenytoin 4'-hydroxylase activity by (S)-fluoxetine (2A). Figure 2B shows the michaelis-menten plot of (S)-fluoxetine vs. negative slope the natural log used to determine  $K_i$  and  $k_{inact}$  values. The 300  $\mu M$  point was omitted from the  $K_i/k_{inact}$  estimate as in both cases it displayed apparent substrate inhibition.

### Summary and Conclusions:

- The R and S enantiomers of fluoxetine were found to be kinetically different, time- and NADPH-dependent inhibitors of CYP2C19.
- (S)-fluoxetine displayed an average  $IC_{50}$  “shift” of 26-fold, making it more robust than ticlopidine (which gave a shift of only 1.8-fold) as a positive control for CYP2C19 time-dependent inhibition.
- Although large differences were found in  $IC_{50}$  shifts, mean “shifted”  $IC_{50}$  values were similar, ranging from 3.5 to 4.1  $\mu M$ , among fluoxetine and its enantiomers. Values were not corrected for non-specific binding.
- Efficiency of inactivation, as determined by  $k_{inact}/K_I$ , was found to be (R) > (±) > (S).
- The mechanism of time-dependent inhibition was not investigated. Future studies are planned to assess the inhibitory properties of the major dealkylated metabolites of each isomer, whether CYP2C19 itself or another enzyme present in HLM is catalyzing the formation of the inhibitory species and whether the time-dependent inhibition is reversible.
- These data provide a better understanding of the drug-interaction potential of fluoxetine.

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