KINETIC CONSTANTS AND SAMPLE-TO-SAMPLE VARIATION IN THE RATE OF METABOLISM OF TWO OR MORE SUBSTRATES FOR HUMAN LIVER MICROSOMAL CYP1A2, CYP2B6, CYP2C8, CYP2D6 AND CYP3A4/5

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INTRODUCTION

XenoTech has processed and characterized over 300 human liver samples during the past decade. During this time, marker substrates for certain CYPs have changed for several reasons, including the need for improved selectivity and sensitivity, a need for reliable substrates for assays that employ LC/MS/MS analysis, and substrates that help provide greater assay repeatability.

CYP1A2: Historically, 7-ethoxyresorufin *O*-dealkylation (EROD) has been employed as an industrial standard *in vitro* reaction for measuring the activity of CYP1A2. Although 7-ethoxyresorufin is acceptable to the FDA, it is not a drug, for which reason phenacetin has emerged as the preferred substrate to quantify CYP1A2 activity.[1] Phenacetin *O*-dealkylation is readily measurable with LC/MS/MS, which affords highly sensitive and selective assays with low limits of quantitation allowing for low concentrations of microsomal protein (*i.e.*, ≤0.1 mg/mL) in the assay incubation.[2]

CYP2B6: *S*-Mephenytoin *N*-demethylation is often used as a marker reaction to measure CYP2B6 activity and this reaction is acceptable to the FDA[1]. However, CYP2B6 catalyzes *S*-mephenytoin *N*-demethylation with low affinity (K_m in the millimolar range) at concentrations that are >500-fold higher than its plasma C_{max}, and the metabolite, nirvanol, contaminates the substrate. The FDA-preferred reaction for measuring CYP2B6 activity is bupropion hydroxylation.

CYP2C8: Paclitaxel 6α-hydroxylation is the FDA-preferred reaction for measuring CYP2C8 activity *in vitro*;[1] however, this reaction shows variances in repeatability and high costs for authentic standards. Amodiaquine *N*-dealkylation is an FDA-acceptable marker reaction for CYP2C8 activity[1] and maintains high specificity, low K_m, and high turnover.[2]

CYP2D6: Dextromethorphan *O*-dealkylation and bufuralol 1'-hydroxylation are both FDA-preferred marker reactions for CYP2D6.[1] Both assays are readily analyzed by LC/MS/MS, and both are utilized by the pharmaceutical industry to measure CYP2D6 activity; however, bufuralol seems to be the preferred substrate by industry investigators.[3]

CYP3A4/5: The FDA recommends that more than one structurally unrelated CYP3A4/5 substrate be used to evaluate CYP3A inhibition *in vitro*.[1] Taking this recommendation into account, three marker reactions for CYP3A were compared in this study: testosterone 6β-hydroxylation, midazolam 1'-hydroxylation and alfentanil *N*-dealkylation, of which the first two are preferred by the FDA.

The objectives of this study were to compare sample-to-sample variation in cytochrome P450 enzymatic rates when two or more CYP-specific substrates were used to measure the activity of the same enzyme, and to determine Michaelis-Menten kinetic constants (K_m and V_{max}) for these same reactions using a pool of human liver microsomes.

MATERIALS & METHODS

Human liver microsomes:
Human liver microsomes from donated tissue were prepared by XenoTech, LLC as previously described.[4] Sixteen individual samples of human liver microsomes were used to evaluate the correlation between two or more marker substrates for a single CYP. A pool of microsomes was used to determine kinetic constants (K_m and V_{max}) for the metabolism of the marker substrates used in this study.

Enzymatic assays for the comparison of multiple marker substrates:
Comparison of multiple marker substrates to determine CYP metabolic activity was carried out with two or more substrates (with [substrate] > K_m in all cases) for each CYP enzyme. Metabolite analysis was by fluorescence spectroscopy, HPLC with UV detection or LC/MS/MS according to the conditions summarized in **Table 1**. For a recent summary of general assay conditions see Ogilvie *et al.*, 2006.[5]

Correlation analysis:
The correlation between the rates of metabolism of two substrates by 16 individual samples of human liver microsomes was determined from the square of the Pearson correlation coefficient (r^2).

Determination of kinetic constants:
To determine Michaelis-Menten kinetic constants, human liver microsomes were incubated with marker substrates at 13 different concentrations that ranged from approximately 0.14 to 10 times the anticipated K_m. These kinetic constants were determined under initial rate conditions which required, in some cases, the use of lower concentrations of microsomal protein and shorter incubation times than listed in **Table 1**.

TABLE 1

Assay conditions used to compare CYP metabolic activity between marker substrates for the same CYP enzyme

CYP	Marker Substrate	Incubation Volume	Incubation Time	Metabolite of Interest	Protein Concentration	Substrate Concentration	Method of Analysis
CYP1A2	7-Ethoxyresorufin	1.0 mL	2 min	Resorufin	0.4 mg/mL	10 µM	Fluorescence spectroscopy
	Phenacetin	400 µL	5 min	Acetaminophen	0.1 mg/mL	80 µM	LC/MS/MS
CYP2B6	*S*-Mephenytoin	200 µL	30 min	Nirvanol	0.4 mg/mL	400 µM	HPLC
	Bupropion	500 µL	30 min	Hydroxybupropion	0.25 mg/mL	500 µM	HPLC
CYP2C8	Amodiaquine	200 µL	10 min	*N*-Desethylamodiaquine	0.1 mg/mL	50 µM	LC/MS/MS
	Paclitaxel	1.02 mL	10 min	6α-Hydroxypaclitaxel	0.1 mg/mL	20 µM	HPLC
CYP2D6	Bufuralol	200 µL	10 min	1'-Hydroxybufuralol	0.1 mg/mL	12 µM	LC/MS/MS
	Dextromethorphan	1 mL	10 min	Dextrorphan	0.1 mg/mL	80 µM	HPLC
CYP3A4/5	Testosterone	1 mL	8 min	6β-Hydroxytestosterone	0.2 mg/mL	250 µM	HPLC
	Midazolam	1 mL	5 min	1'-Hydroxymidazolam	0.05 mg/mL	30 µM	HPLC
	Alfentanil	200 µL	10 min	*N*-Phenylpropionamide (AMX)	0.1 mg/mL	100 µM	LC/MS/MS

TABLE 2

K_m and V_{max} values for selected CYP marker substrates in human liver microsomes

Cytochrome P450 Reaction	Enzyme	V_{max} (pmol/mg protein/min)	K_m (µM)	Cytochrome P450 Reaction	Enzyme	V_{max} (pmol/mg protein/min)	K_m (µM)
7-Ethoxyresorufin *O*-dealkylation	CYP1A2	120 ± 0	0.26 ± 0.01	Dextromethorphan *O*-demethylation	CYP2D6	210 ± 0	7.2 ± 0.6
Phenacetin *O*-dealkylation		1200 ± 0	63 ± 3	Bufuralol 1'hydroxylation		90 ± 10	12 ± 2
S-Mephenytoin *N*-demethylation	CYP2B6	1800 ± 30	1700 ± 40	Midazolam 1'-hydroxylation	CYP3A4/5	2100 ± 0	5.4 ± 0.3
Bupropion hydroxylation		2000 ± 0	50 ± 2	Alfentanil *N*-dealkylation		850 ± 10	45 ± 3
				Testosterone 6β-hydroxylation		9500 ± 200	130 ± 10
Amodiaquine *N*-dealkylation	CYP2C8	3500 ± 120	2.2 ± 0.3				
Paclitaxel 6α-hydroxylation		570 ± 40	11 ± 2				

Kinetic constants are shown ± standard error (where constants are rounded to two significant figures, and standard errors are rounded to the same degree of accuracy).
The constants were calculated with GraFit software based on rates of metabolite formation (duplicate) at 13 substrate concentrations. A standard error value of "0" indicates the standard error is less than 1% of the mean.

Figure 1 **Comparison of phenacetin *O*-dealkylation and 7-ethoxyresorufin *O*-dealkylation as measures of CYP1A2 activity in 16 samples of human liver microsomes**

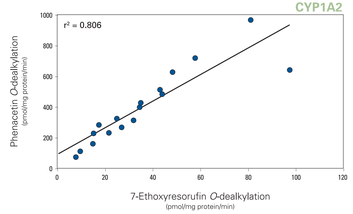


Figure 2 **Comparison of *S*-mephenytoin *N*-demethylation and bupropion hydroxylation as measures of CYP2B6 activity in 16 samples of human liver microsomes**

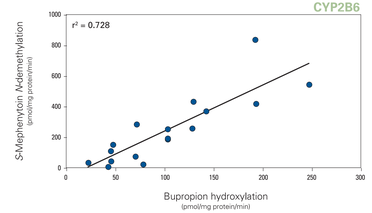


Figure 3 **Comparison of paclitaxel 6α-hydroxylation and amodiaquine *N*-dealkylation as measures of CYP2C8 activity in 16 samples of human liver microsomes**

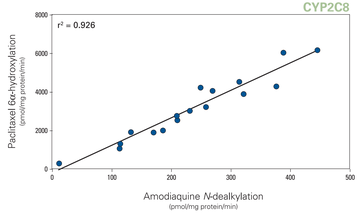


Figure 4 **Comparison of dextromethorphan *O*-demethylation and bufuralol 1'-hydroxylation as measures of CYP2D6 activity in 16 samples of human liver microsomes**

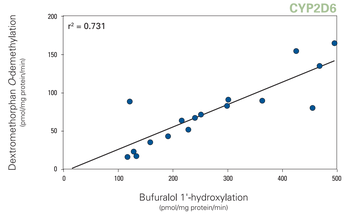


Figure 5 **Comparison of testosterone 6β-hydroxylation and alfentanil *N*-dealkylation as measures of CYP3A4/5 activity in 16 samples of human liver microsomes**

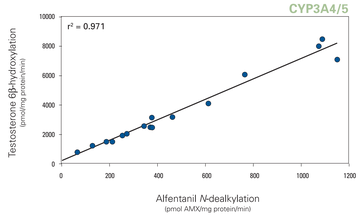


Figure 6 **Comparison of alfentanil *N*-dealkylation and midazolam 1'-hydroxylation as measures of CYP3A4/5 activity in 16 samples of human liver microsomes**

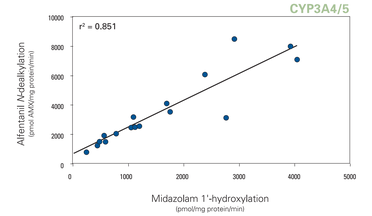
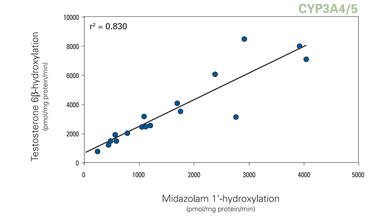


Figure 7 **Comparison of testosterone 6β-hydroxylation and midazolam 1'-hydroxylation as measures of CYP3A4/5 activity in 16 samples of human liver microsomes**



CONCLUSION

The results of this study demonstrate that there is a good correlation (r^2>0.70) between the rates obtained with two or more substrates for CYP1A2 (7-ethoxyresorufin *O*-dealkylation and phenacetin *O*-dealkylation), CYP2B6 (*S*-mephenytoin *N*-demethylation and bupropion hydroxylation), CYP2C8 (paclitaxel 6α-hydroxylation and amodiaquine *N*-dealkylation), CYP2D6 (bufuralol 1'-hydroxylation and dextromethorphan *O*-dealkylation) and CYP3A4/5 (testosterone 6β-hydroxylation, midazolam 1'-hydroxylation and alfentanil *N*-dealkylation).

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