Validation of Time-Dependent Cytochrome P450 Inhibition assays: Use of Stable-Labeled Isotope Internal Standards for Optimal Quantitation

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Abstract. Time-dependent inhibition of cytochrome P450 (CYP) metabolism can lead to nonlinear pharmacokinetics and drug-drug interactions. Recent guidance from the USFDA and drug failures attributable to toxicity that may have a basis in time-dependent inhibition, have elevated interest in this long-known undesirable property of drug candidates. We have validated cytochrome P450 inhibition assays in pooled human liver microsomes that incorporate IC_{50}, IC_{50} shifts, K_I, K_i and k_{inact} endpoints. The assays incorporate CYP-isoform selective probe substrates, LC/MS/MS analysis using stable-labeled isotope internal standards, positive and negative control inhibitors, low total protein to minimize non-specific binding and a step-wise analysis designed to improve k_{inact} experimental design. Our data was found to compare well with literature values. Among the enzymes, probes and inhibitors validated, with enhanced consideration for CYP3A4 because of its major role in drug metabolism and its propensity to exhibit substrate-dependent inhibition response, were as follows: CYP1A2/phenacetin/alpha naphthoflavone/furafylline; CYP2C9/diclofenac/sulfaphenazole/tienilic acid; CYP2C19/S mephenytoin/S-benzylnirvanol; CYP2D6/dextromethorphan/quinidine/paroxetine; CYP3A4/testosterone/midazolam/ketoconazole/azamulin/verapamil/diltiazem.

Introduction.
Drug candidate failures resulting from metabolism-based drug interactions are now becoming infrequent, attributable to robust and routine screening procedures aimed at eliminating potent inhibitors of cytochrome P450 (CYP)[a]. Addressing drug toxicity as a cause of drug failures is now a top-priority. In turn, this has driven more focus on screening for mechanism-based CYP inhibitors. This is because mechanism-based inhibitors can be associated with toxicity, ostensibly as a result of reactive metabolite formation and subsequent covalent binding to cellular macromolecules. Moreover, recent guidance from the USFDA has advocated testing for this endpoint[1]. Although this testing in not required to be performed according to GLP as outlined in Title 21 CFR pt. 58, many pharmaceutical companies prefer a conservative approach to *in vitro* drug-drug interaction testing and therefore, this was incorporated as a success criteria for this project.

Analytical Methods.
Metabolites were quantitated by LC/MS using authentic reference standards obtained from BD Biosciences or Sigma-Aldrich. To control for ion suppression and provide optimal quantitation, stable-labeled isotopes, obtained from BD Biosciences, were used as internal standards. Metabolites were quantified using an ABI/MDS Sciex 4000 Q TRAP™ system, equipped with TurboIonSpray® source. Samples were injected onto a C18 column [Waters® Symmetry® (C18, 2.1x50 mm, 5 μm)] with a mobile phase consisting of 0.1% FA in H_2O, 0.1% FA in ACN at a flow rate of 0.4 mL/min. Run times were <3.5 min. Parameters for analytical method validation, including mass transitions, range, interday precision are shown in **Table 1**. All standard curves were prepared in a matrix of 0.1 mg/mL pooled HLM (BD Cat. No. 452161) in potassium phosphate buffer pH 7.4 containing an NADPH regeneration system. All methods were validated in accordance with the FDA Bioanalytical Method Validation Guidance, 2001.

Assay Methods.
Assays were conducted in 0.1 M potassium phosphate buffer pH 7.4 in a volume of 400 μL. For IC_{50} shift experiments, test articles were incubated with HLM with and without NADPH for 10 or 30 min prior to transfer of 40 μL into 360 μL into a secondary incubation containing probe substrate at a concentration at or near the K_m, and supplemental NADPH. Secondary incubations proceeded according parameters in **Table 2**. Reactions were terminated by addition of 0.1% FA in ACN containing IS to give final concentrations between 0.02 and 2 μM, which in general is in the lower range of each metabolite standard curve. The ratio of the IC_{50} value determined with incubations lacking NADPH in the preincubation compared to same with NADPH in preincubation are reported as a "shift". For K_I and k_{inact} experiments, at multiple time-points, 40 μL aliquots were transferred into secondary incubations as above, except the substrate concentration was approximately 5X the K_m. The K_I and k_{inact} values were determined using non-linear regression (SigmaPlot v. 8.0 equipped with Enzyme Kinetics module v 1.1).

Considerations for Time-dependent Inhibition Assays

Assays were developed with several considerations to maximize robustness[4] while maintaining efficiency that is vital for keeping costs low but rapid delivery of data to customers.

1. There should be at least a 10-fold dilution of the test article into a secondary incubation for both IC_{50} shift and K_I/k_{inact} assays. This serves to minimize the reversible or irreversible inhibition occurring in the secondary incubation.
2. The substrate concentration should be ~5X the K_m. This further reduces reversible inhibition and increases the velocity of the probe substrate reaction that can improve the analytical sensitivity.
3. For accurate K_I/k_{inact} determinations, the number and spacing of preincubation times should be appropriate to accurately determine slope.

A two-time point IC_{50} shift strategy to optimize K_I/k_{inact} study design
A common element to sensitive CYP TDI testing is to preincubate test articles with a metabolic activation system prior to measuring enzyme activity with a probe substrate in a secondary incubation. Quantitative endpoints often include K_I and k_{inact}. A significant challenge in the study design is selecting preincubation time points that adequately define the slope of the inactivation rate curve while avoiding an excessive number of incubations – too few at longer time points (e.g., >15 mins.) fails to detect slopes of slow inactivators and too few at shorter time points (e.g., <10 mins.) fails to adequately define the slope for rapid inactivators. A two-time point IC_{50} shift provides a robust preliminary experiment to assist preincubation time point number and spacing in the K_I/k_{inact} assay.

1 Single IC_{50} Shift experiment used to design K_I/k_{inact}
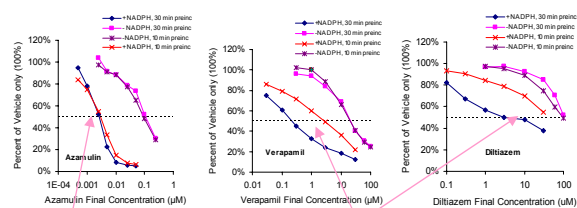
IC_{50} Shift plot showing essentially no change in IC_{50} shift after a 10- or 30-minute preincubation demonstrates rapid inactivation suggesting a compressed and early spacing of time points for follow-up K_I/k_{inact} study design.

Conversely, IC_{50} shift plots for verapamil and diltiazem show a significant shift between 10- and 30-minute preincubation time points. This demonstrates verapamil and diltiazem are slow inactivators by comparison to azamulin.
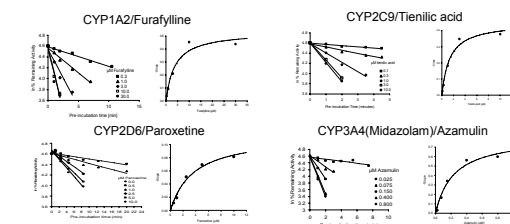
2 Representative KI/kinact Plots

Inactivation plots (left panels) showing negative slopes for the natural logarithm of the percentage of control enzyme activity versus time. Adjacent plots show nonlinear regression analysis used to obtain K_I and k_{inact}. Values for K_I and k_{inact} were generally in agreement (within 3-fold) with those reported by Obach, et al.[3] with the exception of the 4.4-fold higher K_I value for CYP2D6 and paroxetine.

Table 1 Analytical method parameters

Enzyme	Substrate	Metabolite	Mass Transition	Internal Standard	Mass Transition	Ionization	LLOQ (μM)	RE	CV%	Range	R^2	RE	CV
CYP1A2	Phenacetin	Acetaminophen	151→111	Acetaminophen-[$^{13}C_2$$^{15}N$]	155→110	ESI+	0.0760	106	11.9	0.076-5.0	0.9988	98-101	5.3
CYP2C9	Diclofenac	4'-OH Diclofenac	312→268	4'-OH Diclofenac-[$^{13}C_6$]	316→272	ESI+	0.0087	107	4.20	0.0087-2.0	0.9998	99-102	3.4
CYP2C19	S-mephenytoin	4'-OH S-Mephenytoin	235→150	4'-OH S-Mephenytoin-[D3]	238→150	ESI+	0.0040	101	10.4	0.004-10.0	0.9979	97-103	9.8
CYP2D6	Dextromethorphan	Dextrorphan	258→157	Dextrorphan-[D3]	261→157	ESI+	0.0025	94	7.90	0.0025-1.0	0.9975	94-103	7.9
CYP3A4	Midazolam	1'-OH Midazolam	342→203	1'-OH Midazolam-[$^{13}C_3$]	347→208	ESI+	0.0025	93	10.0	0.0025-1.0	0.9967	93-112	13.2
CYP3A4	Testosterone	6β-OH Testosterone	305→269	6β-OH Testosterone-[D7]	312→276	ESI+	0.0160	107	5.3	0.016-10.0	0.9994	98-102	7.2

Table 2 Reversible Inhibition Assay Parameters, IC_{50} and K_i values

Enzyme	Substrate	K_m	Model	[S]	Inc time (min)	HLM (mg/mL)	Competitive inhibitor	IC_{50}[a] (nM)	K_i[a] (nM)	Model for K_i
CYP1A2	Phenacetin	37	MM	40	15	0.1	α-Naphthoflavone	13, 12	18, 20	Mixed
CYP2C9	Diclofenac	3.7	MM	5	5	0.05	Sulfaphenazole	410, 630	200, 190	Competitive
CYP2C19	S-mephenytoin	43	MM	40	20	0.3	S-Benzylnirvanol	440, 310	130	Competitive
CYP2D6	Dextromethorphan	4.9	MM	5	5	0.1	Quinidine	58, 65	58, 41	Competitive
CYP3A4	Midazolam	2.2	MM	3	5	0.02	Ketoconazole	13, 19	8.6, 9.2	Mixed
CYP3A4	Testosterone	65[b]	Hill	50	10	0.05	Ketoconazole	18, 19	24, 18	Competitive

[a] - values determined in duplicate on independent days unless a single value is shown
[b] – K_s, Hill cooefficient n = 1.3

Table 3 Time-Dependent Inhibition Assay Parameters, IC_{50} Shift, K_I and k_{inact} values[a]

Enzyme	Substrate	Time-depend. inhibitor	IC_{50} (μM) 10 min Preincubation – NADPH	+ NADPH	Shift	IC_{50} (μM) 30 min Preincubation - NADPH	+ NADPH	Shift	Max preinc. time for K_I/k_{inact} (min)	K_I (μM) BD	Lit[b]	k_{inact} (min⁻¹) BD	Lit[b]
CYP1A2	Phenacetin	Furafylline	8.1	0.062	134	8.1	0.021	400	15	4.0	1.6	0.60	0.19
CYP2C9	Diclofenac	Tienilic acid	1.6	0.047	34	1.7	0.049	34	8	1.1	1.0	0.46	0.28
CYP2D6	Dextromethorphan	Paroxetine	1.4[c]	0.150	9	1.1	0.065	17	20	3.6	0.81	0.13	0.17
CYP3A4	Midazolam	Diltiazem	91	>28	4	>100	3.4	29	30	8.7	4.5	0.0050	0.012
CYP3A4	Midazolam	Verapamil	23	3.8	6	25	0.34	73	30	2.0	1.8	0.023	0.043
CYP3A4	Midazolam	Azamulin	0.10	0.0030	34	0.15	0.0025	60	7.5	0.17	NA	0.68	NA
CYP3A4	Testosterone	Diltiazem	79[c]	30	3	108	2.8	38	30	2.0	2.4	0.021	0.015
CYP3A4	Testosterone	Verapamil	21[c]	6.3	3	32	0.33	97	30	3.0	1.7	0.030	0.043
CYP3A4	Testosterone	Azamulin	0.081[c]	0.0088	9	0.094	0.0078	12	5	0.49	NA	0.82	NA

[a] – Unless otherwise indicated, values represent the mean of two experiments conducted in duplicate on independent days. Mean CV (range/mean) was 0.26
[b] – Literature values are from Obach, et al. (2007)
[c] – N = 1

Abbreviations:
CYP = cytochrome P450
FA = Formic acid
ACN = Acetonitrile
HLM = Human liver microsomes
MM = Michaelis-Menten
TDI = time-dependent inhibition
K_I = inactivator concentration yield half-maximal inactivation rate
k_i = inactivation rate constant
NA = Not available
ND = Not done

References
1. Draft Guidance for Industry: Drug Interaction Studies — Study Design, Data Analysis, and Implications for Dosing and Labeling (www.fda.gov/cder/guidance/6695dft.pdf)
2. Obach, R.S. and Walsky, R.L. *Drug Metab Dispos* **32**:647 (2004).
3. Obach, R.S., Walsky, R.L., and Venkatakrishnan, K. *Drug Metab Dispos* **35**:246 (2007).
4. Ghanbari, F., Rowland-Yeo, K., Bloomer, J.C., Clarke, S.E., Lennard, M.S., Tucker, G.T., and Rostami Hodjegan, A. *Curr Drug Metab* **7**:315 (2006).

Summary and Conclusions
1. We have validated time-dependent inhibition assay conditions for the major cytochrome P450 enzymes in pooled human liver microsomes
2. Values obtained for K_I and k_{inact} were generally in good agreement with representative literature values[3].
3. To mitigate risk for "trial and error" in obtaining accurate K_I/k_{inact} values, a preliminary, dual time point IC_{50} shift experiment may be employed to optimize experimental design.
4. Although not a requirement for testing, assay were developed with adherence to applicable FDA guidance documents and according to GLP.