IN VITRO RESEARCH ADME SOLUTIONS

The most rapid path to more sound decision making

High-Throughput CYP Inhibition Screening with Drug Probe Substrates: The RapidFire® Advantage

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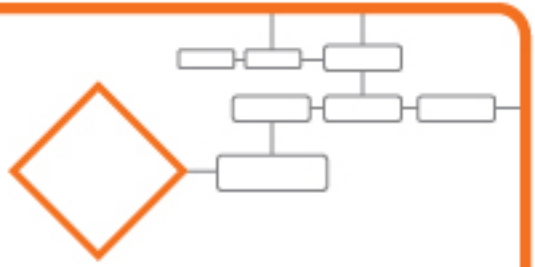
November 11, 2009

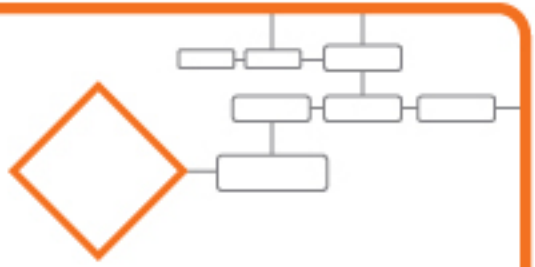
BD

Presentation Overview



- What makes a robust CYP inhibition assay?
- What is RapidFire®?
- CYP inhibition assay comparison
  - LC/MS vs RapidFire-MS
- Drug Discovery ADME Services from BD Gentest℠ Contract Services

Recent Example of CYP DDI- Tamoxifen and SSRI Interaction



- Retrospective analysis of 1300 breast cancer patients ca. 2003-2005

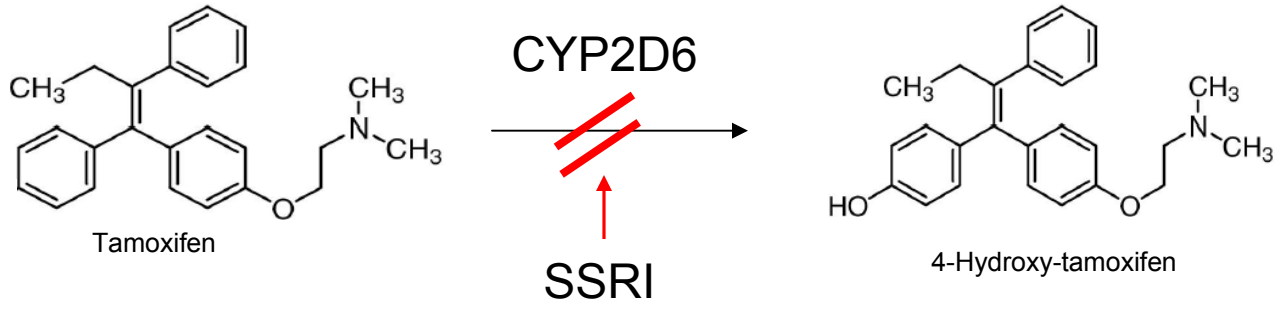
Outcome	Tamoxifen with CYP2D6 inhibitor (n=353), %	Tamoxifen w/o CYP2D6 inhibitor (n=945), %	Adjusted odds ratio
Breast cancer recurrence	13.9	7.5	1.9

- Analysis of subset of patients taking SSRI

Outcome	Tamoxifen with potent/moderate CYP2D6 inhibitors Fluoxetine, Paroxetine, Sertraline (n=213), %	Tamoxifen with weak CYP2D6 inhibitors citalopram, escitalopram, fluvoxamine (n=137), %	Adjusted odds ratio
Breast cancer recurrence	16	Not statistically different than patients not taking inhibitor	~1.9

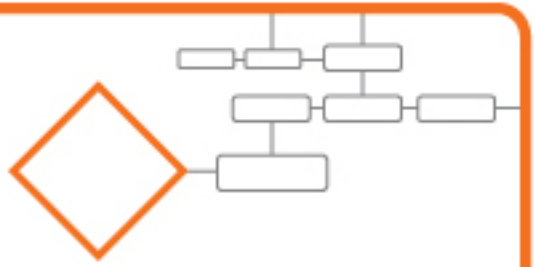
Reference: Aubert et al (2009) Presented at the Amer. Soc. Clin. Oncol., May 30, 2009, Orlando, FL (www.medcoresearch.com/community/oncology/tamoxifen)

Potential Mechanism of Tamoxifen-SSRI Interaction



- Tamoxifen itself is a prodrug, converted by CYP2D6 into 4-hydroxytamoxifen (Dehal & Kupfer, 1997)
- 4-hydroxtamoxifen has 100X more affinity for ER than parent tamoxifen.
- Fluoxetine, Paroxetine, Sertraline
  - Well established, potent inhibitors of CYP2D6
  - IC_{50} values often < 1 μM

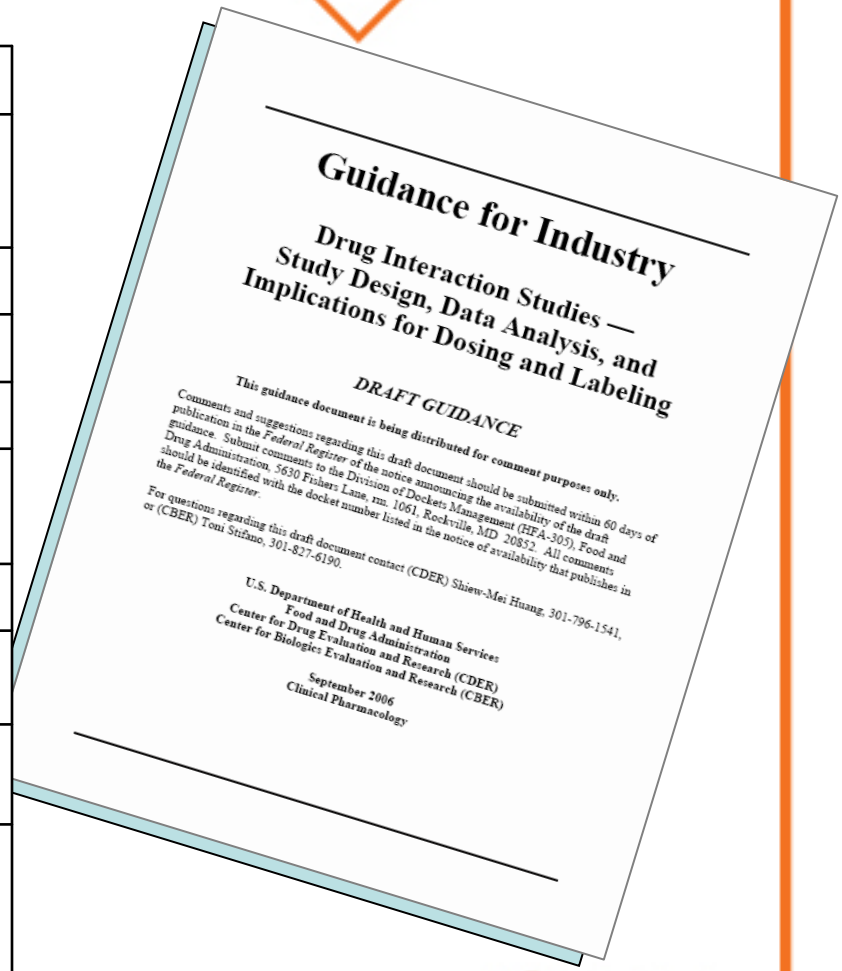
Characteristics of Robust P450 Inhibition Assays



- Reaction must be single P450 isoform-specific
  - Use “probe” substrate with enzyme source (typically human liver microsomes or BD Supersomes™)
- Rapid metabolism of substrate
  - Get more metabolite, faster
- Short incubation time
  - Reduce substrate and inhibitor depletion (that can lead to artifacts)
  - Improves sensitivity in detecting time-dependent inhibitors
- Low protein concentration
  - ≤ 0.3 mg/mL
  - Reduce nonspecific binding to microsomes (that can lead to artifacts)
- Metabolite formation linear with:
  - Incubation time
  - Microsomal protein concentration
- Result is scalable across discovery and development

Guidance for Industry – Sept, 2006

CYP	Substrate Preferred	Substrate Acceptable
1A2	phenacetin-O-deethylation	7-ethoxyresorufin-O-deethylation theophylline-N-demethylation caffeine-3-N-demethylation tacrine 1-hydroxylation
2A6	coumarin-7-hydroxylation nicotine C-oxidation	
2B6	efavirenz hydroxylase bupropion-hydroxylation	propofol hydroxylation S-mephenytoin-N-demethylation
2C8	Taxol 6α-hydroxylation	amodiaquine N-deethylation rosiglitazone para-hydroxylation
2C9	tolbutamide methyl-hydroxylation S-warfarin 7-hydroxylation diclofenac 4'-hydroxylation	flurbiprofen 4'-hydroxylation phenytoin-4-hydroxylation
2C19	S-mephenytoin 4'-hydroxylation	omeprazole 5-hydroxylation fluoxetine O-dealkylation
2D6	(±)-bufuralol 1'-hydroxylation dextromethorphan O-demethylation	debrisoquine 4-hydroxylation
2E1	chlorzoxazone 6-hydroxylation	p-nitrophenol 3-hydroxylation lauric acid 11-hydroxylation aniline 4-hydroxylation
3A4/5*	midazolam 1-hydroxylation testosterone 6ß -hydroxylation	erythromycin N-demethylation dextromethorphan N-demethylation triazolam 4-hydroxylation terfenadine C-hydroxylation nifedipine oxidation



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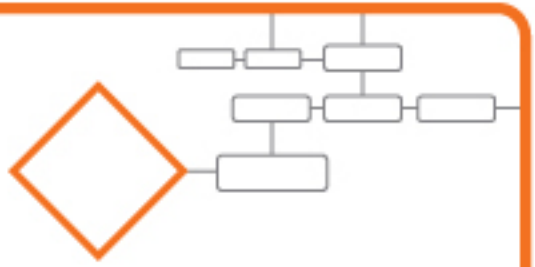
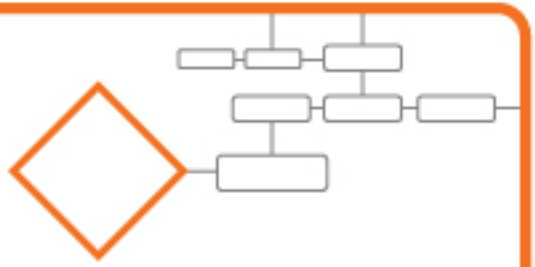
* Recommend use of 2 structurally unrelated CYP3A4/5 substrates for evaluation of in vitro CYP3A inhibition. If the drug inhibits at least one CYP3A substrate in vitro, then in vivo evaluation is warranted.

Ideal High Throughput Method…



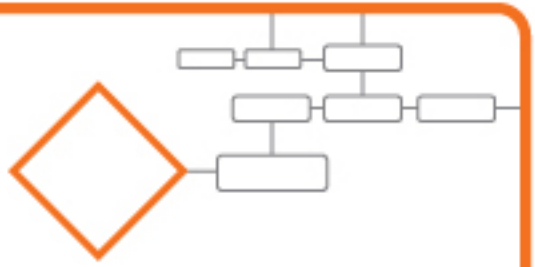
- Methodology is similar to that used in drug development
  - No need to re-validate the approach
  - Chemists are more likely to believe and use the data
  - Conforms to FDA guidance (drug probes)

Ideal High Throughput Method (cont.)



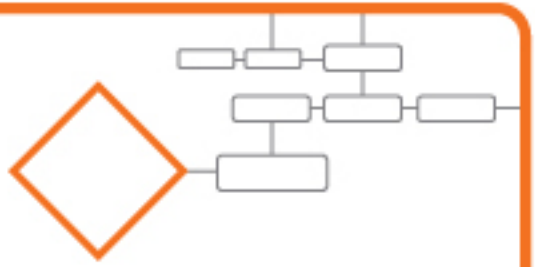
- No pooling (prior to or after incubation)
  - Avoids substrate-substrate interactions
  - No need to re-validate the approach
  - Flexibility to optimize incubation time and protein
  - Obviates need to deconvolute data
  - Maintains analytical robustness

In a CRO…



- The CRO should meet expectations for:
  - Data reliability
  - Value
  - Turnaround time

BD Biosciences – RapidFire Partnership



The in vitro ADME market leader in products and services, **BD Biosciences**, and a technology leader in high-throughput LC/MS, **BIOCIUS Life Sciences**, combine to provide a complete service package for cytochrome P450 inhibition.

- BD Biosciences' validated assay methods combined with RapidFire high throughput LC-MS/MS technology.

BIOCIUS
LIFESCIENCES

a wholly owned subsidiary of BioTrove, Inc.

RapidFire Business Unit forms BIOCIUS Life Sciences

November 10, 2009

www.BIOCIUS.com.

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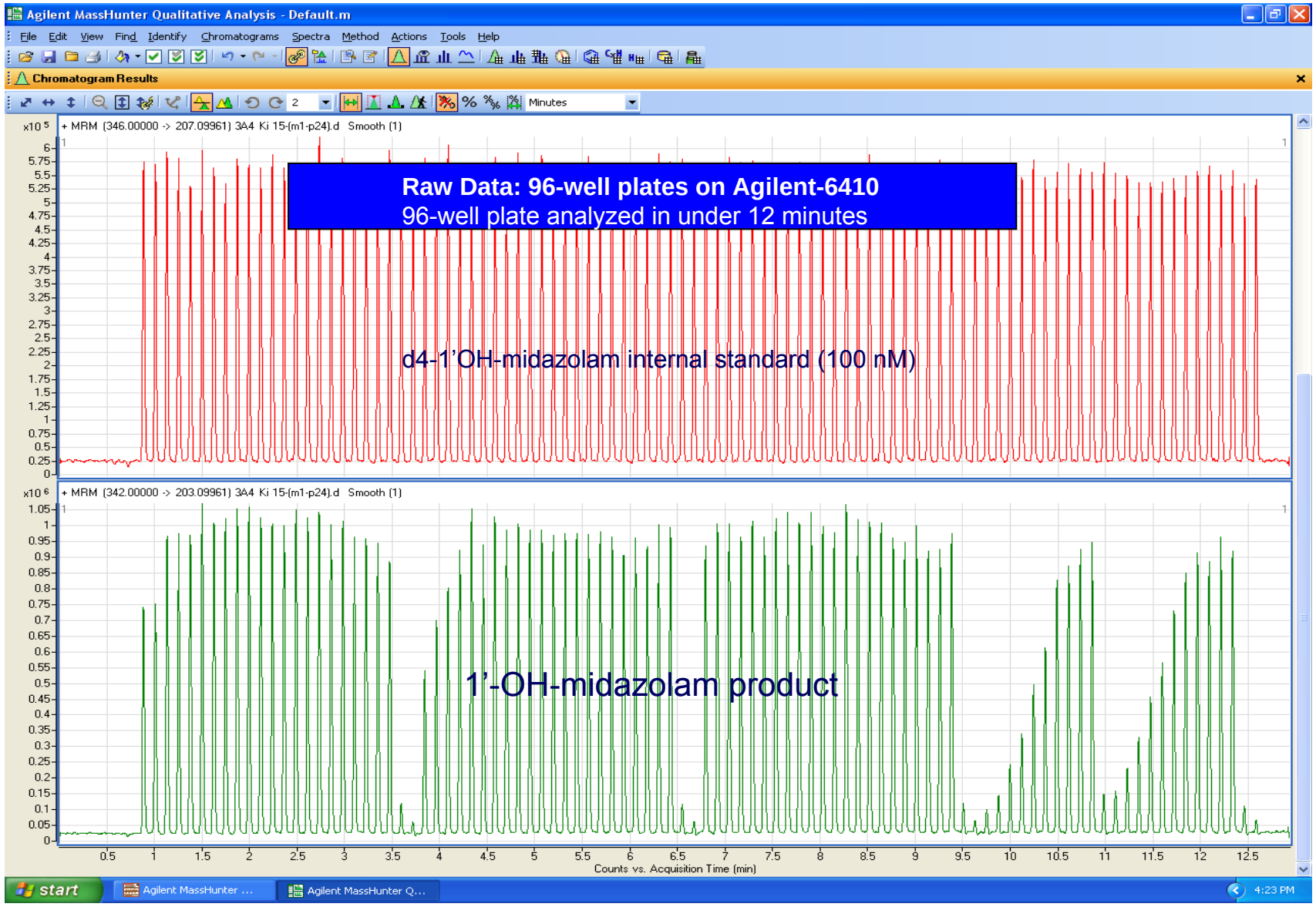
RapidFire Mass Spectrometry

- RapidFire from BIOCIUS
- Replaces the LC of LC/MS with a rapid sample purification system
- Micro scale solid-phase extraction (μSPE)
- Isocratic run
- 5-8 sec cycle times
- No sample prep
- Permits ultra-rapid data turnaround!

RapidFire 300 for in vitro ADME


Agilent MassHunter Qualitative Analysis - Default.m
File Edit View Find Identify Chromatograms Spectra Method Actions Tools Help
Chromatogram Results
Minutes
+ MRM (346.00000 -> 207.09961) 3A4 Ki 15-(m1-p24).d Smooth (1)
Raw Data: 96-well plates on Agilent-6410
96-well plate analyzed in under 12 minutes
d4-1'OH-midazolam internal standard (100 nM)
+ MRM (342.00000 -> 203.09961) 3A4 Ki 15-(m1-p24).d Smooth (1)
1'-OH-midazolam product
Counts vs. Acquisition Time (min)
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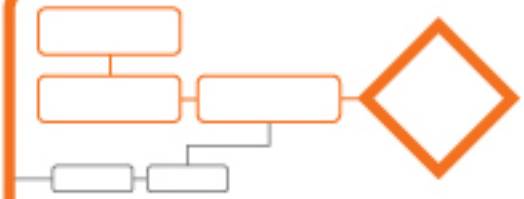


IN VITRO RESEARCH ADME SOLUTIONS

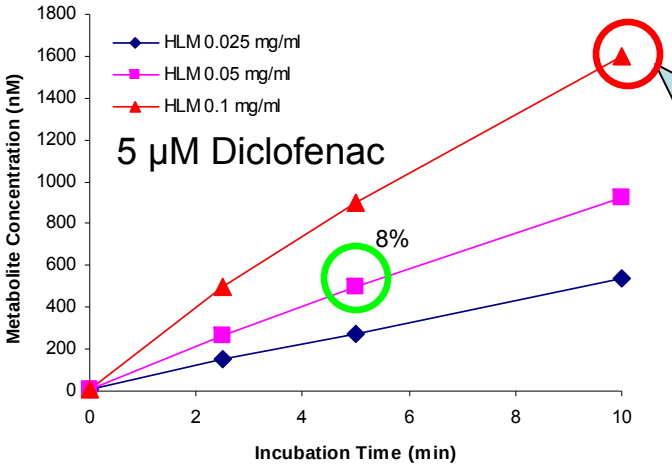
The most rapid path to more sound decision making

How We Validated Our Assays and How We Conduct Them for Clients - Using Conventional LC/MS

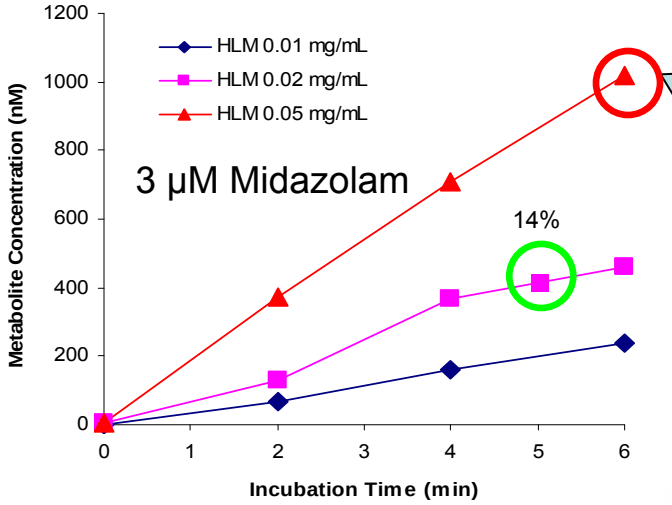
BD

Optimization of Metabolite Formation with Time & Protein

- Linearity of metabolite formation with incubation time and HLM protein concentration
- Remember, v ~ [S]
- *"Preferably no more than 10-30% substrate or inhibitor depletion should occur." – FDA guidance*



0.1 mg/mL
10 min
27% [S] depletion

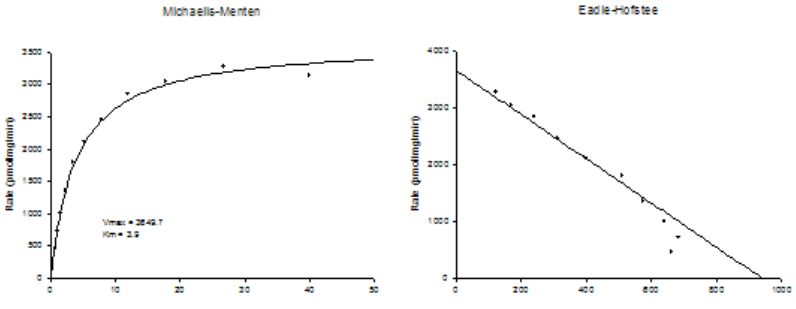


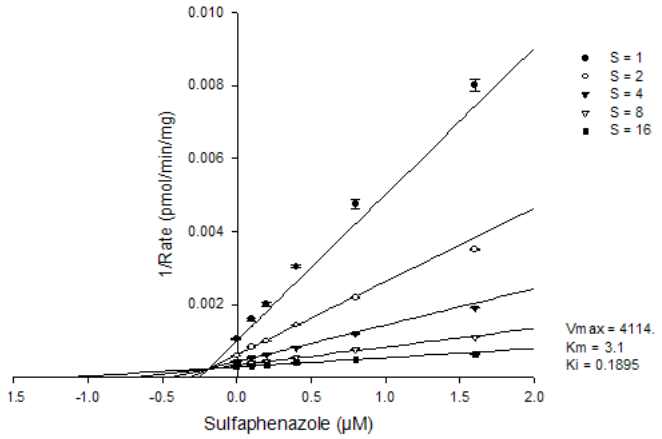
0.05 mg/mL
6 min
34% [S] depletion

BD

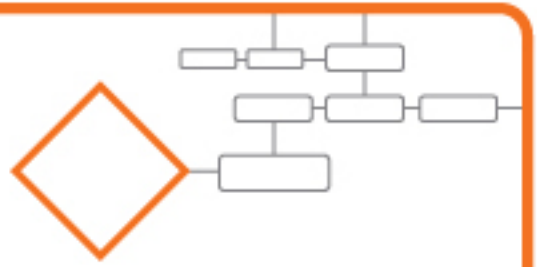
Example Assay Validation Data Set: Diclofenac 4'-hydroxylase

- Resulting Data Set
  - K_M determination
    - 3.5 µM, 3.9 µM
  - IC_{50} and K_i determination wi sulfaphenazole
    - IC_{50}: 0.41 µM, 0.63 µM
    - K_i: 0.20 µM, 0.19 µM





Cytochrome P450 Inhibition Assay Parameters

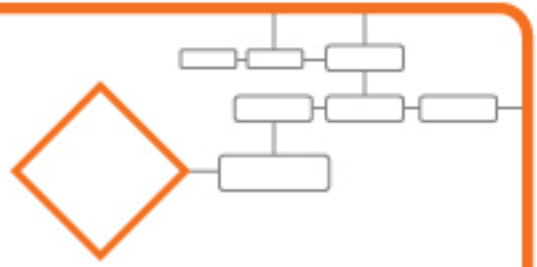


Enzyme	Substrate	K_M	Model	[S]	Inc time (min)	HLM (mg/mL)	Analytical method	Competitive inhibitor	Time Dependent inhibitor
CYP1A2	Phenacetin	37	MM	40	10	0.2	LC/MS	α-Naphthoflavone	Furafylline
CYP2A6	Coumarin	1.3	MM	1.5	5	0.05	LC/MS	Tranylcypromine	8-Methoxypsoralen
CYP2B6	Bupropion	79	MM	80	10	0.1	LC/MS	Ketoconazole	Ticlopidine
CYP2C8	Amodiaquine	1.1	MM	2	5	0.02	LC/MS	Montelukast	Gemfibrozil-gluc
CYP2C9	Diclofenac	3.7	MM	5	5	0.05	LC/MS	Sulfaphenazole	Tienilic acid
CYP2C19	S-mephenytoin	43	MM	40	10	0.3	LC/MS	S-Benzylnirvanol	S-fluoxetine
CYP2D6	Dextromethorphan	4.9	MM	5	5	0.1	LC/MS	Quinidine	Paroxetine
CYP2E1	Chlorzoxazone	60	MM	60	5	0.1	LC/MS	Chlormethiazole	Diethyldithiocarbamate
CYP3A4	Midazolam	2.2	MM	3	5	0.02	LC/MS	Ketoconazole	Azamulin
CYP3A4	Testosterone	65[1]	Hill	50	10	0.05	LC/MS	Ketoconazole	Azamulin

[1] – K_s, Hill coefficient n = 1.3

- Parameters validated: Linearity of metabolite formation with time & protein, K_M, IC_{50}, TDI
- Aligned with FDA guidance: Drug-drug interaction studies (Sept, 2006)
- Perloff et al (2009) Validation of cytochrome P450 time-dependent inhibition assays: a two-time point IC_{50} shift approach facilitates kinact assay design. Xenobiotica 39:99-112

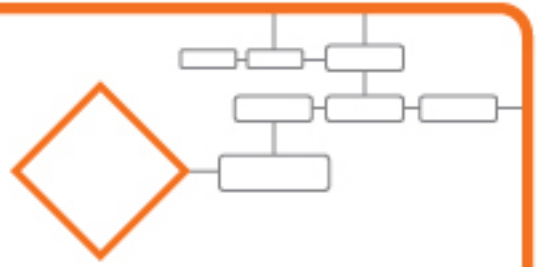
Validated Analytical Methods



Enzyme	Substrate	Metabolite	Mass Transition	Internal Standard	Mass Transition	Ionization	LLOQ (μM)	Std. Curve Range
CYP1A2	Phenacetin	Acetaminophen	151→111	Acetaminophen-[$^{13}C_2{}^{15}N$]	155→110	ESI+	0.0760	0.076-5.0
CYP2A6	Coumarin	7-hydroxycoumarin	161→133	7-OH-Coumarin-[D_5]	166→138	ESI -	0.0020	0.002-1.0
CYP2B6	Bupropion	Hydroxybupropion	256→139	Hydroxybupropion-[D_6]	262→244	ESI+	0.0005	0.0005-0.8
CYP2C8	Amodiaquine	Des-ethyl amodiaquine	328→283	Des-ethyl amodiaquine-[D_3]	331→283	ESI+	0.0047	0.0047-1.5
CYP2C9	Diclofenac	4´-OH Diclofenac	312→268	4´-OH Diclofenac-[$^{13}C_6$]	316→272	ESI -	0.0087	0.0087-2.0
CYP2C19	S-mephenytoin	4´-OH S-Mephenytoin	235→150	4´-OH S-Mephenytoin-[D_3]	238→150	ESI+	0.0040	0.004-10.0
CYP2D6	Dextromethorphan	Dextrorphan	258→157	Dextrorphan-[D_3]	261→157	ESI+	0.0025	0.0025-1.0
CYP2E1	Chlorzoxazone	6-OH Chlorzoxazone	184→120	6-OH Chlorzoxazone-[D_2-^{15}N]	187→67	ESI -	0.0022	0.0022-20.0
CYP3A4	Midazolam	1´-OH Midazolam	342→203	1´-OH Midazolam-[$^{13}C_3$]	347→208	ESI+	0.0025	0.0025-1.0
CYP3A4	Testosterone	6β-OH Testosterone	305→269	6β-OH Testosterone-[D_7]	312→276	ESI+	0.0160	0.016-10.0

- Parameters validated: Selectivity, Standard curve and QC sample Accuracy and Precision, Carryover, Stability, Autosampler stability, LLOQ
- Full accordance with FDA guidance document for analytical method validation (2001)
- Matrix: 0.1 mg/mL HLM, NADPH regenerating system

IC_{50} and K_i Values Obtained With Conventional LC/MS



Enzyme	Substrate	Inhibitor	mean IC_{50} (nM)	mean K_i (nM)	Best fit model	ratio IC_{50}/K_i
CYP1A2	Phenacetin	7,8-Benzoflavone	9	3	Mixed	2.7
CYP2B6	Bupropion	Ketoconazole	2250	1400	Competitive	1.6
CYP2C8	Amodiaquine	Montelukast	22	13	Competitive	1.7
CYP2C9	Diclofenac	Sulfaphenazole	520	195	Competitive	2.7
CYP2C19	(S)-Mephenytoin	(S)-Benzylnirvanol	410	340	Competitive	1.2
CYP2D6	Dextromethorphan	Quinidine	62	50	Competitive	1.2
CYP3A4	Midazolam	Ketoconazole	16	9	Mixed	1.8
CYP3A4	Testosterone	Ketoconazole	19	21	Competitive	0.9

Values represent means of two independent determinations; Global CV = 0.13

Mean = 1.7

Extensive experimental detail available in the following publication:

Perloff et al (2009) Validation of cytochrome P450 time-dependent inhibition assays: a two-time point IC_{50} shift approach facilitates kinact assay design. *Xenobiotica* 39:99-112

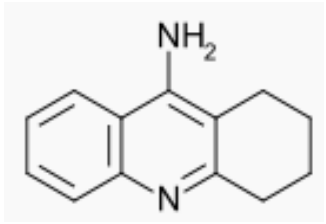
How We Validated Our Assays and How We Conduct Them for Clients – Using RapidFire MS

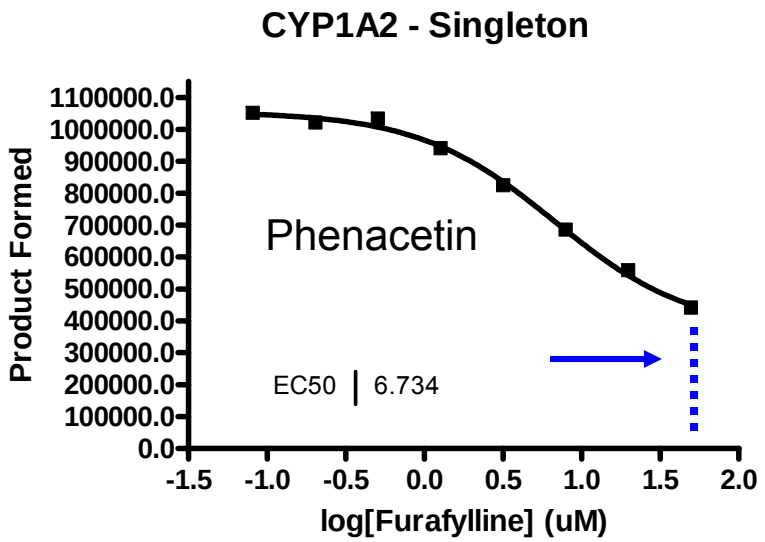
Assay Methods for RapidFire Analysis

- Assay are conducted in an identical manner
- No add'l validation, except CYP1A2 and CYP2B6 (next slides)

Tacrine Used as the Probe for CYP1A2

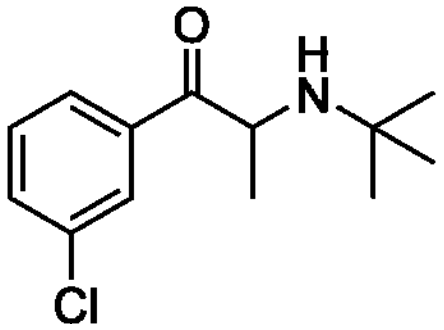
- Tacrine used as an alternate to phenacetin
- In-source fragmentation
- Tacrine is also FDA-recommended



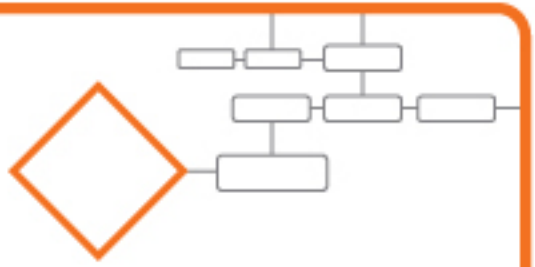


CYP2B6 - Bupropion

- Criteria – 5 fold s/n at the IC_{50}
- Boosted the protein concentration (0.2 mg/mL)
- Extended the incubation time (20 min)



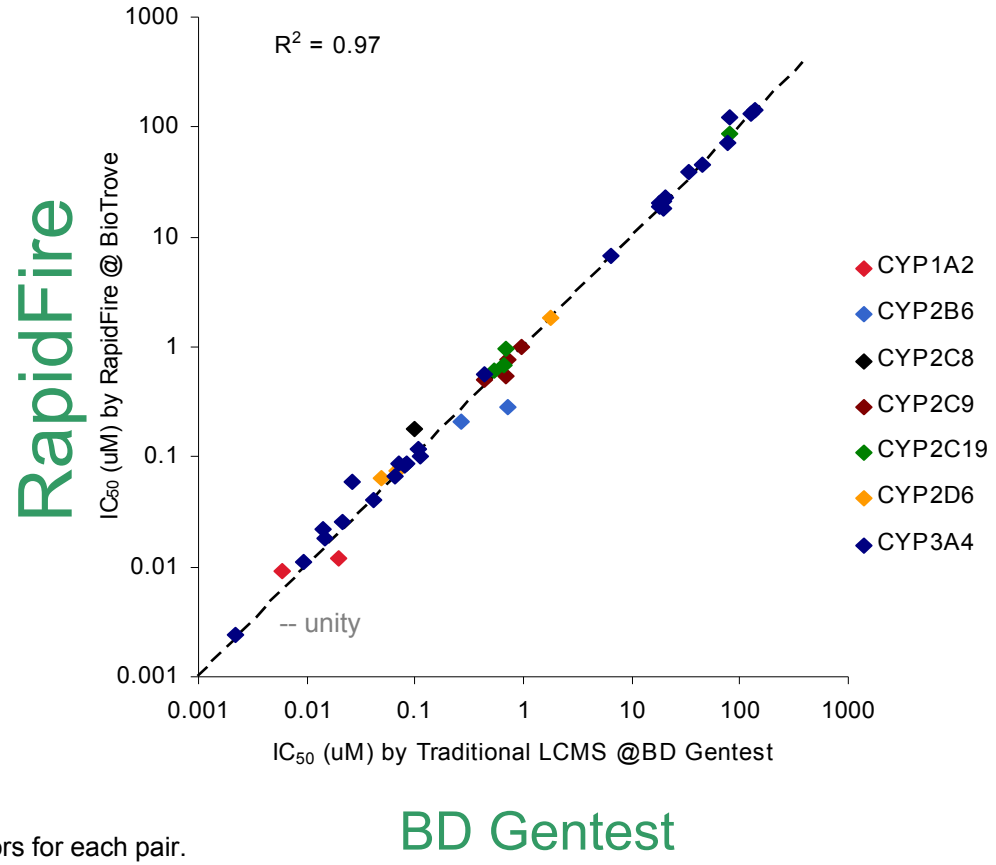
Comparison of RapidFire with Conventional



- To compare performance:
  - We generated full, 7 point IC_{50} curves
  - Multiple compounds
  - 7 enzymes, 8 substrates
- Samples were split
- Analyzed at BD and at BIOCIUS
- Results to follow:

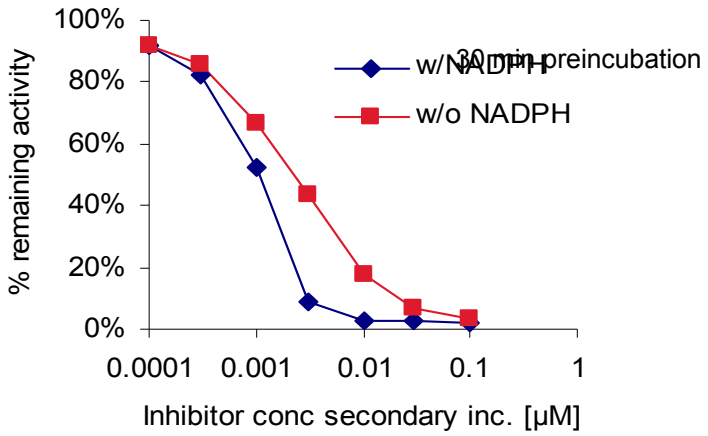
Results

- N = 43 IC_{50} curves
- R-squared = 0.97
- No systematic bias
  - Ratio of RapidFire to Conventional = 1.13

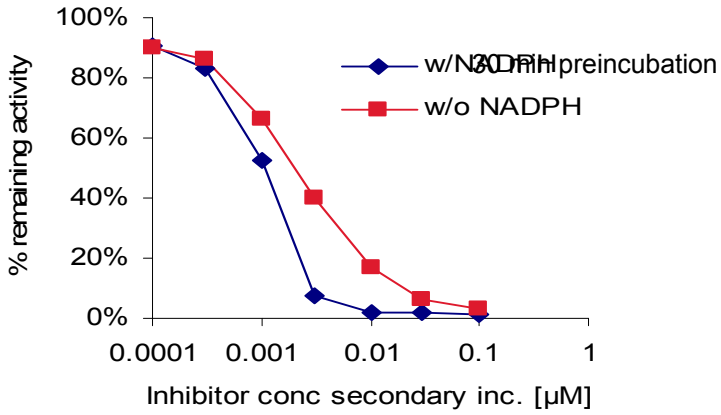


Data from 8 different enzyme/substrate pairs and 1 to 3 inhibitors for each pair. Inhibitors include ketoconazole, α-naphthoflavone, montelukast. S-benzylnirvanol, sulfaphenazole, azamulin, paroxetine, ticlopidine, S-fluoxetine, tienilic acid, verapamil and diltiazem, tamoxifen, ritonavir, erythromycin, mibefradil

Analysis of Ritonavir by IC_{50} Shift – Midazolam as Substrate for CYP3A4



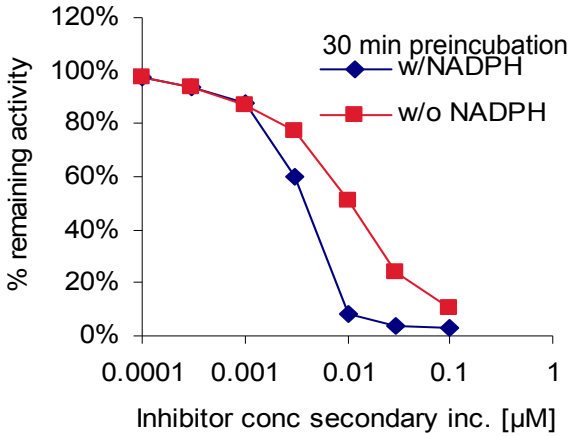
- IC_{50} - RapidFire
  - w/ NADPH = 11 nM
  - w/o NADPH = 24 nM
- Shift = 2.17

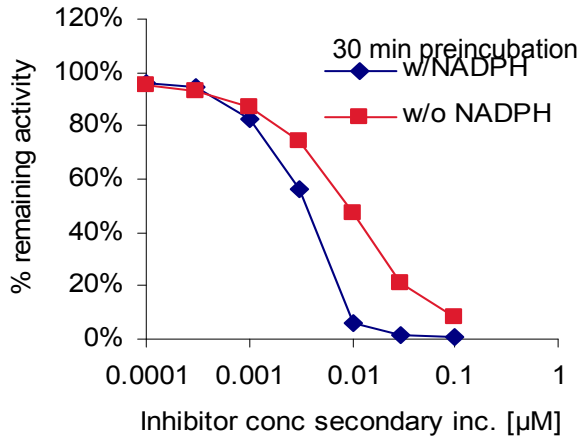


- IC_{50} - Conventional
  - w/ NADPH = 11 nM
  - w/o NADPH = 22 nM
- Shift = 2.04

IC_{50} shift with dilution to mimic recommended K_I/k_{inact} study design (Grimm et al, 2009)

Analysis of Ritonavir by IC_{50} Shift – Testosterone as Bubstrate for CYP3A4



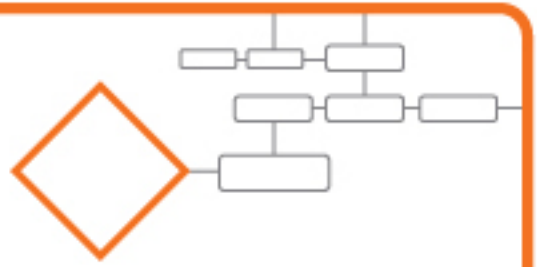


- IC_{50} - RapidFire
  - w/ NADPH = 4.4 nM
  - w/o NADPH = 11 nM
- Shift = 2.47

- IC_{50} - Conventional
  - w/ NADPH = 3.9 nM
  - w/o NADPH = 9.4 nM
- Shift = 2.40

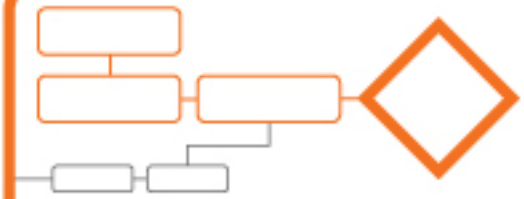
IC_{50} shift with dilution to mimic recommended K_I/k_{inact} study design (Grimm et al, 2009)

Inter- and Intraplate Reproducibility in IC_{50} Values



Condition	Intraplate CV	Interplate CV
CYP3A4/ Midazolam/ Ketoconazole	0.29	0.28

N = 36 IC_{50} values; mean = 17 nM



IN VITRO RESEARCH ADME SOLUTIONS

The most rapid path to more sound decision making

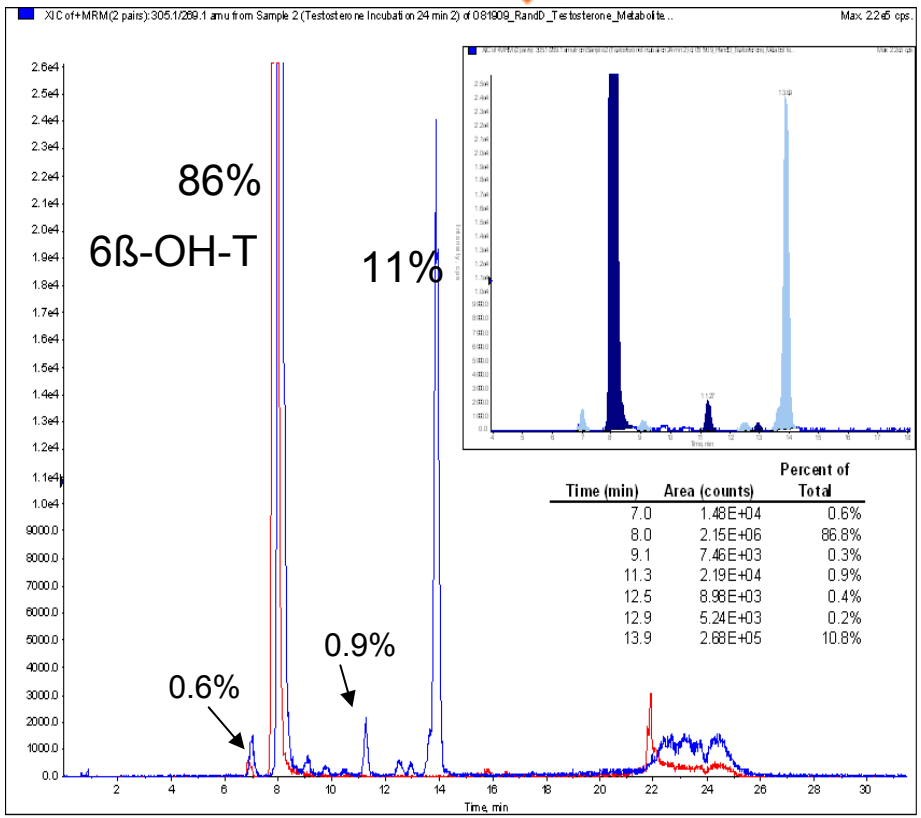
Examination of Analytical Selectivity in the Absence of Chromatography

Testosterone Metabolism as a Case Study

BD

Selectivity of Testosterone

- Testosterone is a major CYP3A4 probe
  - Hydroxytestosterone metabolites yield essentially identical fragmentation by MS
  - With conventional LC/MS, chromatography solves this issue
- RapidFire-MS uses a µSPE cartridge for sample clean up.
  - There is ~ no chromatographic separation of analytes
- Does MRM alone provide adequate selectivity?
  - Non-6ß-OH metabolites may be confounders

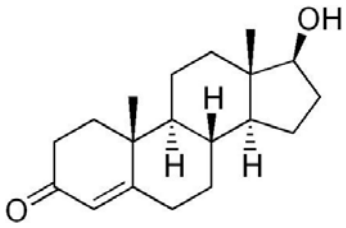


Time (min)	Area (counts)	Percent of Total
7.0	1.48E+04	0.6%
8.0	2.15E+06	86.8%
9.1	7.46E+03	0.3%
11.3	2.19E+04	0.9%
12.5	8.98E+03	0.4%
12.9	5.24E+03	0.2%
13.9	2.68E+05	10.8%

Conditions: 250 µM testosterone, 0.1 mg/mL 10 min. Red trace is 6β-OH Testosterone-[D7]

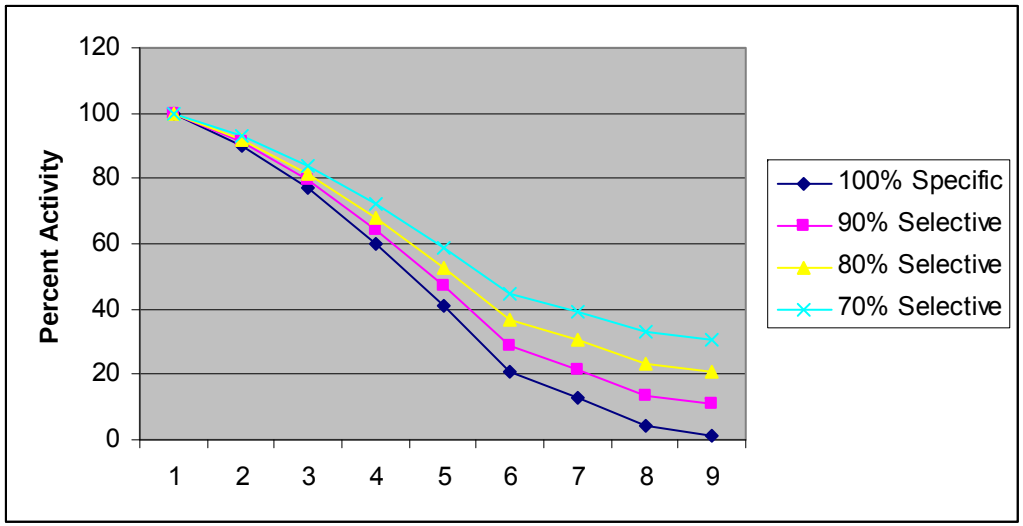
Review of Testosterone Metabolism In Vitro

- Multiple hydroxylated metabolites in HLM
  - 6ß, 2ß, 1ß, 15ß, 16ß, 11ß, 2α(?) [(Waxman et al (1988); Krauser et al (2004); Choi et al (2005)]
- 85-90% of total is 6ß-OH
- ~10% is 2ß-OH
- CYP3A4 > to >> 2C9, 2C19 for all
- There is a very minor contribution of non-CYP3A4 to total response



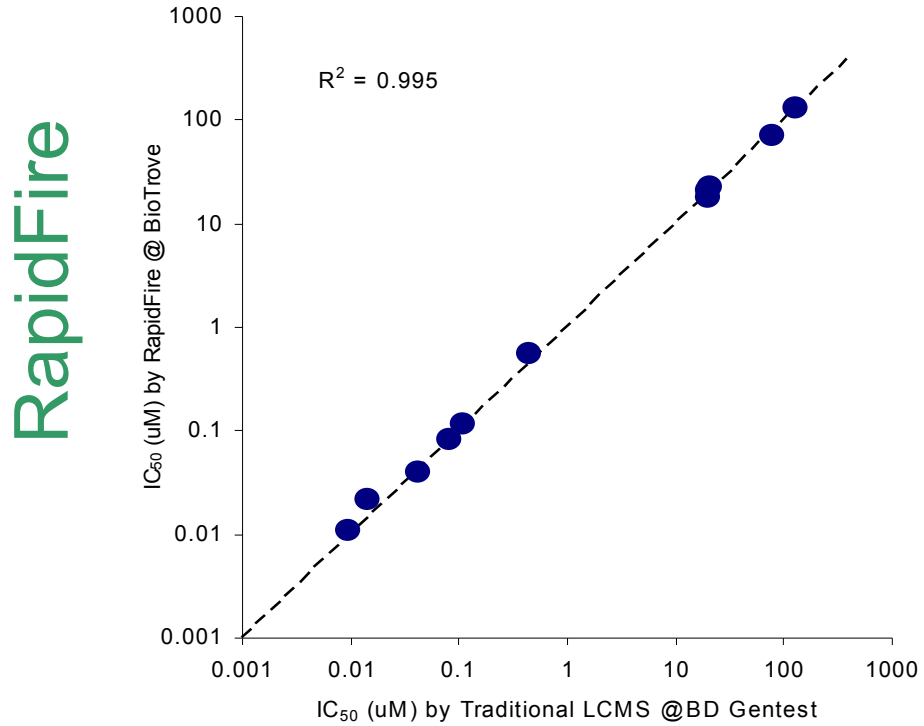
Potential Impact of Selectivity of CYP Probe Substrate

- CYP probe substrates are generally selective but not specific in HLM.
- Model decreasing selectivity
- Any decrease in selectivity tends to increase IC_{50} values.



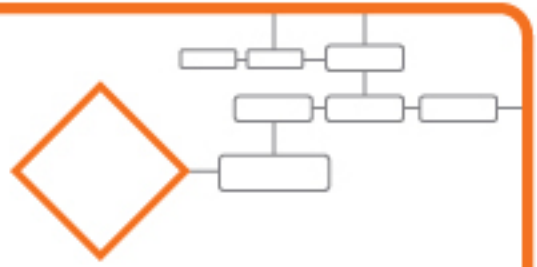
Substrate Selectivity	Relative IC50
100	1.00
90	1.28
80	2.06
70	3.58

Correlation Analysis – CYP3A4/testosterone



Inhibitors tested: Ketoconazole, Fluoxetine, Ritonavir, Azamulin, Mibefradil, Verapamil, Diltiazem

The BD-BIOCIUS Advantage



- Features
  - Full 7-point IC_{50} curves
    - Single point, percent inhibition is risky – especially for CYP3A4
  - MS with stable-labeled isotopes
  - Individually incubated and analyzed
  - Uses BD UltraPool HLM 150™ human liver microsomes or your company's pool
  - *GLP- Validated Assay Methodology - in Drug Discovery!*
- Complete sample preparation and data analysis
  - Customers provide compounds to BD, we conduct incubations, BIOCIUS performs analysis - BD provides completed data package to customer
- Rapid Turnaround
  - Data available in 1 week
- Cost-effective
  - $250 per 7 point curve
- Time-dependent inhibition assays available

BD Gentest Contract Services

- We offer a full spectrum of affordable, high throughput ADME Drug Discovery services
  - CYP inhibition by RapidFire
  - Metabolic stability in microsomes
  - Metabolic stability in hepatocytes
  - Plasma protein binding (Rapid Equilibrium Dialysis)
  - HT-reaction phenotyping with BD Supersomes™
  - Caco-2 or MDR1-LLC-PK1 monolayers
  - Solubility

RapidFire BioTrove

IN VITRO ADME RESEARCH SOLUTIONS

- BD UltraPool™ HLM 150 application

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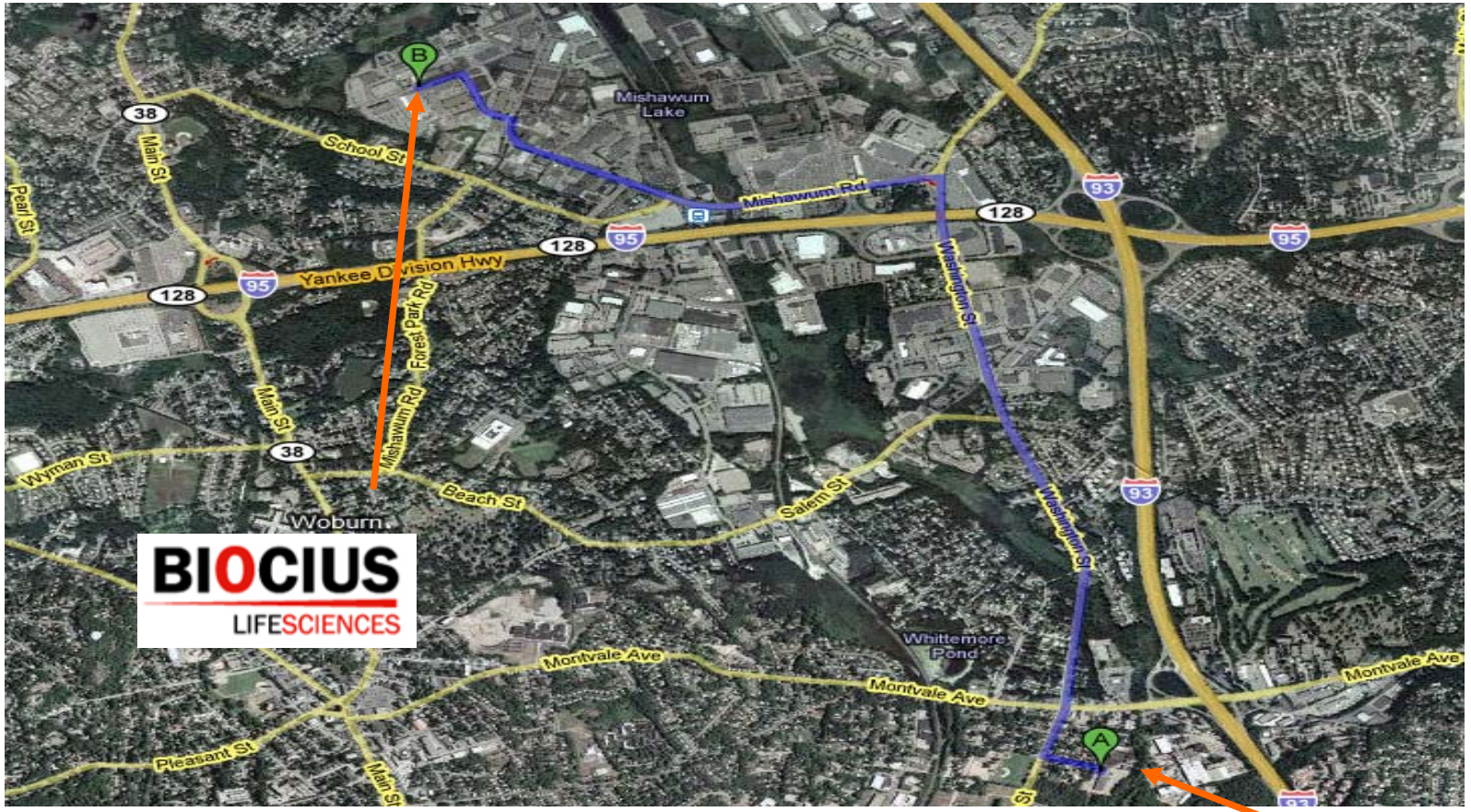
Come visit us in Woburn, MA USA



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BIOCIUS LIFESCIENCES

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Questions?

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