Chapter 2

Simultaneous Determination of Multiple CYP Inhibition Constants using a Cocktail-Probe Approach

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Abstract

To identify cytochrome P450 (CYP) drug–drug interaction (DDI) potential of a new chemical entity, the use of a specific clinically relevant probe substrate in the presence of a test compound is common place. In early discovery of new chemical entities, a balance of rigor, the ability to predict clinical DDI, and throughput is desired in an in vitro assay. This chapter describes a high-throughput CYP-mediated DDI assay method that balances these characteristics. The method utilizes a cassette approach using a cocktail of five selective probe substrates for the major clinically relevant CYPs involved in drug interactions. CYP1A2, 2C9, 2C19, 2D6, and 3A activities are assessed with liquid chromatography/tandem mass spectrometry (LC-MS/MS) quantification of metabolite formation. The method also outlines specific inhibitors to evaluate dynamic range and as a positive control. The benefits and needs for caution of this method are noted and discussed.

Key words Drug–drug interaction, Cytochrome P450, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A, Cocktail of substrates, Cassette of substrates, LC-MS/MS, Liquid chromatography/tandem mass spectrometry

1 Introduction

Drug–drug interactions (DDI) are a major liability for any new drug entering the marketplace. Adverse drug reactions, of which DDI are a significant component, are a leading cause of hospital administrations (1) and drug withdrawals (2). Coadministration of multiple drugs to patients for one disease (e.g., HIV infection or cancer) or treatment for several diseases concurrently (geriatrics) is common place. Therefore, the potential is great for DDI in a polypharmacy environment. The FDA guidance related to CYP DDIs recommends both the accepted probe substrates and measured metabolites for clinical DDI assessment (3–5). In practice, in the early stages, five major enzymes are investigated, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, accounting for greater than 90% of total hepatic CYPs (6, 7) and 70% of metabolism of all marketed drugs (8). While this guidance sets the effect

Ian R. Phillips et al. (eds.), *Cytochrome P450 Protocols*, Methods in Molecular Biology, vol. 987,
DOI 10.1007/978-1-62703-321-3_2, © Springer Science+Business Media New York 2013

thresholds that trigger clinical studies, the agency will also accept appropriate in vitro data indicating no significant CYP inhibition as justification that subsequent in vivo studies are not necessary (4). Patient safety concerns and regulatory requirements have led the pharmaceutical industry to adopt the general practice of only progressing development candidates with acceptably low predicted CYP DDI liabilities which have been accessed in vitro (e.g., $IC_{50} \geq 1{,}000$-fold efficacious concentration). When this goal is not met, such early knowledge enables the planning of clinical studies at the appropriate time in the development program.

A competitive- and/or reversible-inhibition screen is often the first step in understanding the DDI potential of a new chemical entity. The definitive assessment of inhibition is the inhibition constant (K_i), which provides not only the inhibition potency but also information on the mechanism of inhibition (competitive, noncompetitive). However, in the lead optimization profiling environment this approach is overcomplex for the question being asked, and generates far too many samples to enable rapid screening of compound series. The substrate-cocktail approach has been developed to provide a balance of sufficient throughput without compromising data relevance. This method has been made possible due to advances in chromatographic methods and mass-spectrometry sensitivity, in addition to further understanding of specific probes for each clinically relevant CYP isoform (9–19). Immediate benefits seen from a cocktail or cassette method is a complete evaluation of the major metabolizing enzyme in a single reaction under the same conditions and the number of compounds that can be assessed by such a reaction. Chemical series modifications can be assessed, providing information on minor changes in structure-activity relationship (SAR) revealing shifts in potency toward or away from one or many isoforms. The assay consists of human liver microsomes and a cocktail of probe substrates metabolized by the five major CYP isoforms (tacrine for CYP1A2, diclofenac for CYP2C9, (S)-mephenytoin for CYP2C19, dextromethorphan for CYP2D6, and midazolam for CYP3A). The assay has been fully automated in both a 96- and a 384-well format (19, 20).

2 Materials

2.1 Assay

1. Substrates: Dextromethorphan hydrobromide monohydrate (mol wt 370.33), diclofenac sodium salt (mol wt 304.11), midazolam (mol wt 325.77) (see Note 1), (S)-mephenytoin (mol wt 218.26), tacrine hydrochloride (mol wt 234.72) (all from Sigma-Aldrich (St. Louis, MO)) (see Note 2).
2. Metabolite standards: Dextrorphan (mol wt 257.2), 4′-hydroxydiclofenac (mol wt 311.0), 1′-hydroxymidazolam (mol wt 341.1), 4′-hydroxymephenytoin (mol wt 234.1) (all from BD Biosciences,

Discovery Labware, Woburn, MA), 1′-hydroxytacrine (mol wt 214.3) (Pfizer internal compound library, but may be purchased from TLC PharmaChem) (see Note 3).

3. Assay buffer: 100 mM potassium phosphate buffer pH 7.4 with 1 mM magnesium chloride (see Note 4).
4. Cofactor: NADPH-regeneration system (β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADP+), isocitric dehydrogenase from porcine heart, dl-isocitric acid trisodium salt (all from Sigma-Aldrich (St. Louis, MO))). For convenience, 1 mM NADPH can be substituted for the regeneration system (see Note 5).
5. Enzyme source: Human liver microsomes (HLM), pooled from ≥50 male and female donors (BD Gentest, Bedford, MA) (see Note 6).
6. Control inhibitor: Miconazole (mol wt 479.15), a pan-CYP inhibitor, or individual-specific inhibitors for each CYP isoform (4) (see Note 7).
7. Labware: Dilutions and reactions were prepared in deep-well, polypropylene, 1 mL/well-capacity plates (Beckman Coulter, Fullerton, CA).
8. Incubations should be conducted at 37°C in a water-heated block or heated 96/384-well shaker or for higher throughput can be adapted to a robotic system with the capability to control temperature (i.e., Peltier system, recirculating water bath, or incubator).
9. A deproteinizing agent combined with an internal standard, such as chilled acetonitrile with triazolam (mol wt = 342) Sigma RBI (Natick, MA), acts as a reaction quenching reagent and a LC-MS/MS standard.

2.2 Preexperiment Preparations

1. Initial stock solutions of CYP isoform-specific substrates:
   (a) Diclofenac (7.5 mM) in water.
   (b) Dextromethorphan (10 mM) in 90% acetonitrile/10% methanol.
   (c) (S)-mephenytoin (50 mM) in acetonitrile.
   (d) Midazolam (10 mM) in 66.7% methanol/33.3% acetonitrile.
   (e) Tacrine (10 mM) in DMSO.
2. Working stock of the cocktail of substrates: For a 10-reaction experiment, combine stocks of 5 μL of 7.5 mM diclofenac, 3.75 μL of 10 mM dextromethorphan, 6 μL of 50 mM S-mephenytoin, 1.5 μL of 10 mM midazolam, and 1.5 μL of 10 mM tacrine with 5,982 μL of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM $MgCl_2$. This may be scaled up appropriately to the number of reactions needed.

3. Test compound or control inhibitor: Can be prepared as either a single concentration assessment or as an IC_{50} determination. If a single assessment is being conducted a stock of 300 μM in 10% DMSO, 90% acetonitrile is prepared to achieve a final concentration of 3 μM in the assay. If a full IC_{50} determination is desired, a starting conc. of 3 mM in 10% DMSO, 90% acetonitrile is appropriate to achieve a 30 μM starting concentration. Half to one-third log dilutions in 10% DMSO, 90% acetonitrile of the 3 mM starting concentration will provide a wide concentration range at ≥6 concentration points. In the case of a potent control pan-inhibitor, such as miconazole, a 1 μM final concentration will inhibit at least 80% of CYP activity for all isoforms (see Note 8).
4. Individual stock solutions of 500 mM isocitric acid, 100 mM NADP+, and 100 U/mL isocitrate dehydrogenase in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM $MgCl_2$ should be prepared, and are stable in the refrigerator for 1 week. At the time of the experiment, the three reagents should be combined with 100 mM potassium phosphate buffer, pH 7.4, with 1 mM magnesium chloride in a ratio of 1:1:1:2 and pre-incubated for 15 min at 37°C.
5. Thaw stock human liver microsomes (HLM) on ice and then prepare a stock of HLM to a concentration of 0.71 mg/mL in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM $MgCl_2$ and keep on ice until use.

2.3 Bioanalysis

1. Column: Phenomenex Onyx Monolithic C18, 50×4.6 mm (product number = CH0-7644).
2. Mobile phases: Aqueous solvent (A & C) with 0.1% formic acid and acetonitrile (B) with 0.1% formic acid were used as mobile phases.

3 Methods

3.1 Experimental Methodology

1. Perform reactions in a final volume of 500 μL/well (see Note 9). To each well add:
   (a) 400 μL of the working stock of the cocktail of substrates warmed to 37°C. The final concentration of the substrates in a 500 μL reaction is 40 μM S-mephenytoin, 5 μM dextromethorphan, 5 μM diclofenac, 2 μM midazolam, and 2 μM tacrine (see Note 10).
   (b) 70 μL of 0.71 mg/mL human liver microsomes stock (0.1 mg/mL final concentration) (see Note 11).
   (c) 5 μL of test or control compound in 10% DMSO and 90% acetonitrile (see Note 12).

2. Samples (compound, microsomes, and substrate cocktail) are pre-incubated for 5 min at 37°C.
   (d) After the 5-min preincubation, add 25 μL of pre-warmed NADPH regeneration system (~1 mM NADPH generated) to initiate the reaction.
3. Following an 8-min incubation (see Note 13) at 37°C, terminate reactions by the addition of 500 μL/well chilled internal standard (acetonitrile containing 0.2 μg/mL triazolam) (see Subheading 2.1, item 9).
4. CYP inhibition is quantitated by simultaneously analyzing 4-hydroxymephenytoin, dextrorphan, 4-hydroxydiclofenac, 1-hydroxymidazolam, and 1-hydroxytacrine using LC-MS (Fig. 1).

3.2 Bioanalysis

1. Standard curve preparation
   (a) Prepare small volumes (500 μL) of 200 μg/mL (free base or free acid equivalent concentration) metabolite stock solutions of individual metabolites in 10% DMSO/90% acetonitrile. These can be stored in a refrigerator for up to 1 month.
   (b) Add 100 μL of each solution to 500 μL acetonitrile to make a total volume of 1 mL of 20,000 ng/mL (20 μg/mL) metabolite cocktail stock solution.
   (c) Prepare ½ serial dilutions from the metabolite cocktail stock solution by adding 0.5 mL to 0.5 mL acetonitrile to give standard concentration ranging from 10,000 to 2.44 ng/mL (Table 1).
   (d) Prepare denatured human liver microsome solution at 0.1 mg/mL in buffer. This solution can be stored in a refrigerator for 3 months (see Note 14).
   (e) Add 5 μL of each standard into 95 μL of denatured microsomes to give final concentration range for the standard curve of 500 to 0.12 ng/mL (Table 1).
   (f) Centrifuge for 15 min at 2400×*g*, 4°C prior to injecting into mass spectrometer (MS).
2. Analytical conditions (see Note 15)
   (a) Multiple-reaction monitoring (MRM) LC-MS/MS analysis should be conducted on the most sensitive quadrupole mass spectrometer available, and measured in positive ion mode (see Note 16).
   (b) For metabolites of interest, their MRM transitions, collision energies (CE), declustering potentials (DP), and collision cell exit potential (CXP) are shown in Table 2 (Fig. 1) (based upon Sciex API4000). Optimization of values will be dependent on specific MS. Values found in Table 2 serve as a guide.

Table 1
Analytical standard curve preparation via ½ dilutions from the metabolite cocktail stock solution

Metabolite cocktail stock solution concentration (ng/mL)	Volume of stock to be diluted (mL)	Volume of acetonitrile diluent (mL)	Final metabolite stock concentration (ng/mL)	Final metabolite standard concentration in assay matrix (ng/mL)
20,000	0.5	0.5	10,000	500
10,000	0.5	0.5	5,000	250
5,000	0.5	0.5	2,500	125
2,500	0.5	0.5	1,250	62.5
1,250	0.5	0.5	625	31.3
625	0.5	0.5	312.5	15.6
312.5	0.5	0.5	156.3	7.81
156.3	0.5	0.5	78.1	3.91
78.1	0.5	0.5	39	1.95
39	0.5	0.5	19.5	0.977
19.5	0.5	0.5	9.8	0.488
9.77	0.5	0.5	4.9	0.244
4.9	0.5	0.5	2.4	0.122
0	0	1.0	0	0

Table 2
Multiple-reaction monitoring (MRM) LC-MS/MS analysis transitions, collision energies (CE), declustering potentials (DP), and collision cell exit potential (CXP) for specific metabolites formed by each cytochrome P450 isoform

Metabolite	P450	MRM	CE	DP	CXP
1-OH-tacrine	CYP1A2	M/Z 152>110	30	50	15
4-OH-diclofenac	CYP2C9	M/Z 312.3>230.1	45	45	15
4-OH-S-mephenytoin	CYP2C19	M/Z 235.2>150.1	27	45	15
Dextrorphan	CYP2D6	M/Z 256.2>157.1	53	90	15
1-OH-midazolam	CYP3A4	M/Z 342.3>168.1	53	65	15
Internal standard/triazolam		M/Z 343.2>308.1	40	70	15

Gradient conditions

Initial conditions: Total flow 0.200 mL/min.

Pump B Pct 1.0%; Pump C flow 3.000 mL/min. (Teed into liquid path immediately before the column.)

Remaining conditions can be found in Table 3.

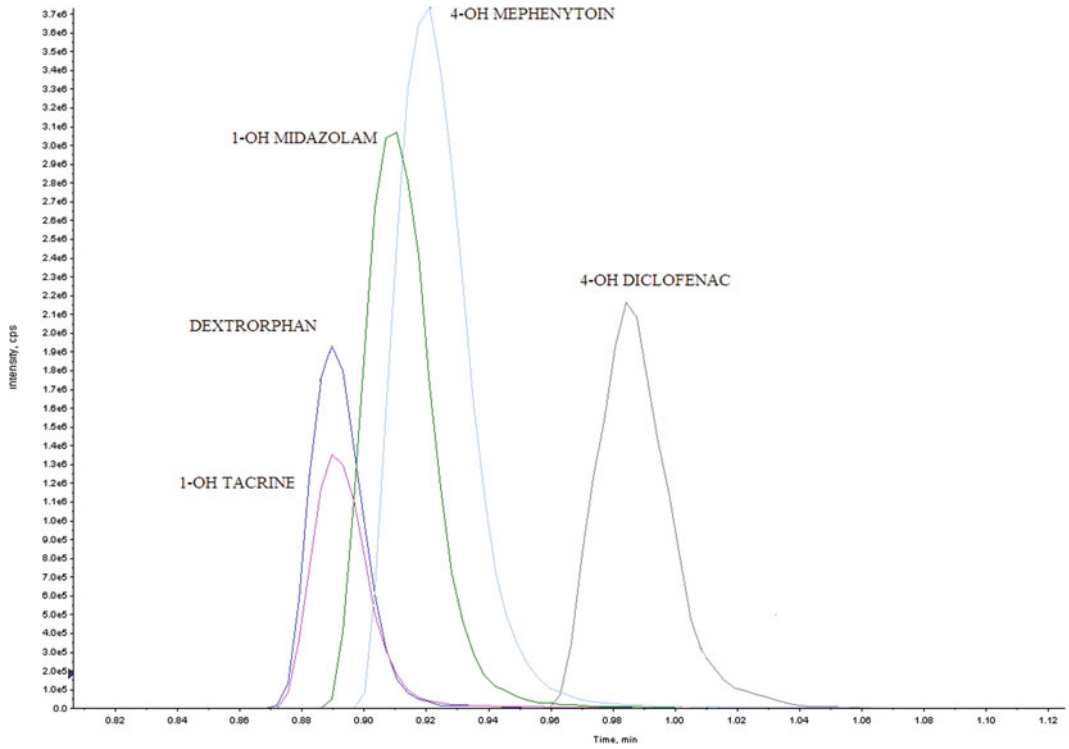

Fig. 1 Chromatogram detecting the simultaneous formation of the metabolites formed in the cocktail of substrates drug-drug interaction reaction (peak area intensity versus time eluted off the chromatography column)

Table 3
Liquid chromatography gradient conditions to separate the specific metabolites formed by each of the cytochrome P450 isoforms

Time	Events	Parameter
0.01	Pump B conc.	10
0.20	Total flow	0.20
0.21	Pump C flow	3.5
0.22	Total flow	3
0.23	Pump C flow	0.01
0.42	Pump B conc.	10
0.60	Pump B conc.	25
1.45	Pump B conc.	65
1.57	Pump B conc.	65
1.58	Pump B conc.	10
1.90	System controller	Stop

3.3 Data Analysis

3.3.1 Analytical

1. Acceptance criteria

Calibration line

Calibration standards should be within 20% of nominal conc. with at least six remaining on the line.

Linearity and model used (R2>0.95).

Same regression model throughout runs.

Calibration line the used to calculated.

Metabolite concentrations

Metabolite concentrations (nM) should be calculated using appropriate software on the MS from the standard curve using linear regression with $1/x2$ weighting. The metabolite concentrations can be converted to pmol/min/mg and should be used to ensure acceptable substrate turnover as well as assess experiment to experiment variability (see Note 17).

3.3.2 Percent Inhibition or IC_{50} Determination

1. If a single concentration percent inhibition is desired, the metabolic rate of formation of the metabolite for each substrate can then be expressed as a percentage of the control (uninhibited activity) and a percent inhibition value determined by subtraction from 100%.
2. If IC_{50} are being determined, estimations can be made from the data by fitting a standard four-parameter logistic using nonlinear regression Eq. 1. In this equation *Range* is the fitted uninhibited value (y_{max}) minus the *Background*, and "*s*" is a slope factor or Hill coefficient. The equation assumes that "*y*" (*enzyme velocity*) decreases with increasing "*x*" (*concentration*) (see Note 18).

$$y = \text{Range} / 1 + (x / \text{IC}_{50})^{s} + \text{Background} \quad (1)$$

4 Notes

1. In the USA and the UK, midazolam and the internal standard triazolam are controlled substances requiring the facility to carry certification in order to obtain these compounds.
2. The user may want to add other specific CYP isoform substrates to the cocktail of substrates, but a similar validation should occur to that performed by Zientek et al. before use (20). Also, potential competition of individual isoforms for cytochrome b_5 and NADPH cytochrome P450 reductase may limit the electron transport and thus metabolic rate (21, 22). It has been shown that such issues can be overcome and in an acceptable range to provide an appropriate level of quality data (20, 23).

3. The addition of the magnesium ion from magnesium chloride is needed to stabilize the negative charge formed on the hydroxyl oxygen during dehydrogenation and then a consecutive transfer of a hydride to produce NADPH from $NADP^+$ (24).
4. Temperature may need to be adjusted to control the temperature loss between the heat source and the labware; reaction rate will suffer if not optimized.
5. If NADPH is used instead of the NADPH regeneration system, $MgCl_2$ can be removed from the buffer.
6. Human liver microsomes are from human-derived materials and require special handling and blood-borne-pathogen training.
7. In early discovery, a pan-CYP inhibitor is appropriate as a positive control inhibitor but should be left to the discretion of the scientist. There are many specific inhibitors for the five major CYP isoforms to choose from, and the FDA has made recommendations as to which ones are most appropriate (4). Some of these potent but specific inhibitors are furafylline (1A2), sulfaphenazole (2C9), ticlopidine (2C19), quinidine (2D6), and ketoconazole (CYP3A4/5). The concentration or concentration range should be appropriate to observe significant inhibition of the specified isoform while not affecting the activity of the other CYP isoforms.
8. Final concentrations of DMSO and non-DMSO organic solvents of 0.1% (v/v) and 1.0% (v/v), respectively. CYPs are quite sensitive to DMSO; therefore caution should be used when utilizing this solvent as an experimental reagent (20, 25–27).
9. This method can be miniaturized to accommodate 50 μL reactions, appropriate for a 384-well plate (19, 20).
10. All substrates utilized in the inhibition experiment are prepared at stock concentration to have final concentrations at the K_M, to assure appropriate sensitivity to the inhibitor (18, 20, 28–35). This also allows estimation of K_i values, using the Cheng-Prusoff equation, from the IC_{50} concentration (36).
11. Low microsomal protein concentrations of 0.1 mg/mL or below reduces the effect the inhibitor binding to microsomal matrix (5, 37, 38).
12. Typically, concentrations in the 1–5 μM range are used for single concentration DDI assessment (15, 39).
13. Strict adherence to initial rate and compliance with Michaelis–Menten kinetics (<10% substrate depletion) is not possible with very different reaction rates, although with an 8-min reaction time no more than 25% substrate depletion is observed for the most extensively metabolized substrate while maintaining sensitivity of the least metabolized substrate (20).

14. Denatured microsomes can be prepared using a number of approaches: (a) boiling for 3 min, (b) leaving at room temperature for 48 h, or (c) direct denaturing into acetonitrile. In each case the microsomal mix then needs to be prepared such that the organic:aqueous ratio matches that in the samples. This ensures all samples are matrix matched, thus mitigating the effects of any ion suppression on metabolite intensities (40).
15. Given the difference in analytical setup in different laboratories, users have flexibility to adapt analytical conditions to ensure maximum sensitivity achieved with their particular instrument. The conditions described below serve only as a guide (17, 19, 41).
16. Single-stage quadrupole mass spectrometers (SSQMS) as well as triple-stage quadrupole mass spectrometers (TSQMS) are commonly used for this type of analysis (41). Although quadrupole mass analyzers have the ability to operate in both negative and positive ion modes, specific advantages of SSQMS instruments include low cost and their relatively small size, whereas TSQMS instruments have greater discrimination against chemical background, resulting in real gains in selectivity and sensitivity. In TSQMS, the Q1 mass analyzer filters the desired ions such that they are fragmented by argon or nitrogen within Q2, and their fragment ions are subsequently scanned by Q3 before reaching the mass detector. Consequently, given that TSQMS acquires much richer, higher-value datasets than SSQMS in selected reaction monitoring (SRM) or multiple-reaction monitoring (MRM) modes would suggest it to be the instrument of choice in routine and high-throughput quantitative bioanalysis. However, detection sensitivity decreases dramatically when wide mass range is analyzed in a scanning mode, which can be a limitation in its application for screening of "*unknown*" drug metabolites.
17. It is suggested that a control activity chart be kept to monitor inter-experiment rate variability, and those experiments that lie outside 50% CV should be repeated.
18. When using Eq. 1, the maximal percent control (y_{max}) incorporated into the *Range* term refers to the lack of inhibition. Therefore, y_{max} or 100% control activity must be observed in the span of concentrations tested or the equation should be modified. Specifically, the y_{max} should be fixed at 100% of control to assure a proper IC_{50} determination. Another point to consider is when only a few points define the dose–response curve, the assumption is that the inhibitor binds to the enzyme via the law of mass action; therefore the Hill coefficient may be set to a standard slope of −1.0, although in most cases the data should dictate the appropriate fit.

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Cytochrome P450 Protocols
Phillips, I.R.; Shephard, E.A.; Ortiz de Montellano, P.R. (Eds.)
2013, XII, 319 p., Hardcover
ISBN: 978-1-62703-320-6
A product of Humana Press