

High-Throughput Cytochrome P450 Cocktail Inhibition Assay for Assessing Drug-Drug and Drug-Botanical Interactions

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ABSTRACT

Detection of drug-drug interactions is essential during the early stages of drug discovery and development, and the understanding of drug-botanical interactions is important for the safe use of botanical dietary supplements. Among the different forms of drug interactions that are known, inhibition of cytochrome P450 (P450) enzymes is the most common cause of drug-drug or drug-botanical interactions. Therefore, a rapid and comprehensive mass spectrometry-based in vitro high-throughput P450 cocktail inhibition assay was developed that uses 10 substrates simultaneously against nine CYP isoforms. Including probe substrates for CYP1A2, CYP2A6, CYP2B6, CYP2C8,

CYP2C9, CYP2C19, CYP2D6, CYP2E1, and two probes targeting different binding sites of CYP3A4/5, this cocktail simultaneously assesses at least as many P450 enzymes as previous assays while remaining among the fastest due to short incubation times and rapid analysis using ultrahigh pressure liquid chromatography–tandem mass spectrometry. The method was validated using known inhibitors of each P450 enzyme and then shown to be useful not only for single-compound testing but also for the evaluation of potential drug-botanical interactions using the botanical dietary supplement licorice (*Glycyrrhiza glabra*) as an example.

Introduction

Drugs and botanical dietary supplements can interact with some therapeutic agents by inhibiting or inducing drug-metabolizing enzymes or drug transporters. Inhibition of these enzymes and transporters can result in longer half-lives and higher and possibly toxic concentrations of therapeutic agents, whereas induction can have the opposite effect. The most common form of drug-drug or drug-botanical interaction is inhibition of cytochrome P450 (P450) enzymes. Examples include inhibition of CYP2D6 by paroxetine (Bertelsen et al., 2003) and inhibition of CYP3A4 by goldenseal (*Hydrastis canadensis*) (Chatterjee and Franklin, 2003; Gurley et al., 2005).

In vitro P450 inhibition assays are widely used to study potential drug-drug and drug-botanical interactions. Although these assays typically evaluate inhibition of one P450 isoform at a time, the U.S. Food and Drug Administration (FDA) recommends that at least seven P450 isoforms should be investigated for possible inhibition by each new drug entity under development (Food and Drug Administration, 2012). To expedite these assays, several cocktail approaches, also known as n-in-one assays, have been developed to test for inhibition of several P450 isoforms simultaneously. Most of these assays test for inhibition of five to eight P450 isoforms and use a wide variety of experimental conditions and probe substrates (Dierks et al., 2001; Testino and Patonay, 2003; He et al., 2007; Li et al., 2007; Smith et al., 2007; Workman and Raynaud, 2007; Zientek et al., 2008; Alden et al., 2010; Otten et al., 2011; Yao et al., 2012; Kozakai et al., 2012; Lee and

Kim, 2013; Qiao et al., 2014; Qin et al., 2014; Liu et al., 2015). Although a few approaches have claimed using 9 or 10 substrates to evaluate nine isoforms, they actually carry out separate incubations of subsets of probe substrates to avoid P450 interactions before pooling the mixtures immediately prior to a quantitative analysis step, use P450 substrates which are not recommended by the FDA, and/or use long incubation times of up to 60 minutes (Kim et al., 2005; Turpeinen et al., 2005; Tolonen et al., 2007; Dinger et al., 2014).

To address these assay limitations for the investigation of drug-drug interactions while including drug-botanical interactions which are important to our laboratory, we developed and validated a high-throughput P450 cocktail inhibition assay using 10 substrates against nine P450 enzymes. Simultaneously assessing the inhibition of nine P450 isoforms can significantly reduce the cost and time needed for the evaluation of drug-drug and drug-botanical interactions. Our in vitro high-throughput cocktail approach optimized enzyme protein concentration, minimized probe substrate interactions, minimized solvent effects, and used a fast and sensitive ultrahigh pressure liquid chromatography (UHPLC)–tandem mass spectrometry (MS/MS) quantitative assay (Chauret et al., 1998; Busby et al., 1999; Yuan et al., 2002; Turpeinen et al., 2005; Jia and Liu, 2007; Smith et al., 2007; Otten et al., 2011; Kozakai et al., 2012; Lee and Kim, 2013; Spaggiari et al., 2014). After validating the new assay using nine known P450 inhibitors, an extract of the botanical dietary supplement licorice (*Glycyrrhiza glabra*) was evaluated for P450 inhibition.

Materials and Methods

Materials and Chemicals

Phenacetin was purchased from Tokyo Chemical Industry (Tokyo, Japan). Acetaminophen, coumarin, bupropion hydrochloride, tolbutamide, dextrophan tartrate, chlorzoxazone, 6 β -hydroxytestosterone, furafylline, ticlopidine hydrochloride,

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ABBREVIATIONS: FDA, Food and Drug Administration; MS/MS, tandem mass spectrometry; P450, cytochrome P450; UHPLC, ultrahigh pressure liquid chromatography.

quercetin, sulfaphenazole, quinidine, ketoconazole, ammonium diethyldithiocarbamate, NADPH, formic acid, potassium phosphate monobasic, and potassium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO). 1'-Hydroxymidazolam and 6-hydroxychlorzoxazone were purchased from Cayman Chemical (Ann Arbor, MI). Dextromethorphan hydrobromide was obtained from MP Biomedicals (Santa Ana, CA). 7-hydroxycoumarin was purchased from Indofine Chemical (Hillsborough, NJ). Hydroxybupropion and hydroxybupropion-*d*₆ were purchased from Santa Cruz Biotechnology (Dallas, TX). Midazolam and testosterone were obtained from Cerilliant Corporation (Round Rock, TX). [d₅]-7-Hydroxycoumarin, [d₇]-6β-hydroxytestosterone, and [¹³C₃]-1'-hydroxymidazolam were purchased from BD Gentest (Woburn, MA). Amodiaquine, *N*-desethylamodiaquine hydrochloride, [d₅]-*N*-desethylamodiaquine, (*S*)-mephenytoin, [d₄]-acetaminophen, [d₂]-6-hydroxychlorzoxazone, hydroxytolbutamide, [d₆]-4-hydroxytolbutamide, (±)-4'-hydroxymephenytoin, [d₃](±)-4'-hydroxymephenytoin, and [d₃]-dextrophan tartrate were obtained from Toronto Research Chemicals (Toronto, Canada).

Pooled human liver microsomes from 200 donors were purchased from XenoTech (Lenexa, KS). Liquid chromatography/mass spectrometry-grade acetonitrile and methanol were purchased from Thermo Fisher (Fair Lawn, NJ). Water was prepared using an Elga Purelab Ultra water purification system (Siemens Water Technologies, Woodridge, IL). An extract of licorice roots (*G. glabra*) was prepared from botanically authenticated plant material provided by the University of Illinois at Chicago/National Institutes of Health Center for Botanical Dietary Supplements Research (Chicago, IL). The licorice root was extracted with 90% ethanol, 5% isopropanol, 5% water (v/v/v) [weight root powder (grams)/volume solvent (milliliters)]: 1:15].

Microsomal Incubations

Potassium phosphate buffer (100 μl, 0.1 M, pH 7.4) containing 1.3 mM NADPH, 0.2 mg/ml human liver microsomes, and a cocktail of 10 probe substrates [phenacetin for CYP1A2, coumarin for CYP2A6, bupropion for CYP2B6, amodiaquine for CYP2C8, tolbutamide for CYP2C9, (*S*)-mephenytoin for CYP2C19, dextromethorphan for CYP2D6, chlorzoxazone for CYP2E1, and midazolam and testosterone for CYP3A4/5] (Table 1) or a single substrate (around *K_m*) was incubated at 37°C for 10 minutes. Methanol was used to dissolve the substrate cocktail or individual substrate and comprised <0.3% (v/v) of the total incubation mixture. The reactions were terminated by adding 20 μl of a stop solution consisting of water/acetonitrile/formic acid (92:5:3, v/v/v) containing stable isotope-labeled surrogate standards (Table 1). The samples were then centrifuged at 13,000 × *g* at 4°C for 15 minutes prior to analysis using UHPLC-MS/MS.

Michaelis Constant Determination. Human liver microsomes (0.05–0.20 mg/ml) were incubated at 37°C for 5–20 minutes with each P450 substrate at 8–10 different concentrations. After quantitative analysis using UHPLC-MS/MS, *K_m* values were calculated.

Linearity of Metabolite Formation in the Cocktail Assay. Each cocktail reaction mixture was incubated at 37°C for 2.5, 5, 10, 15, or 20 minutes. After quenching and quantitative analysis, the linearity of metabolite formation was evaluated.

Comparison of Cocktail and Single-Substrate Assays Using Known P450 Inhibitors and Licorice Root Extract. Known P450 inhibitors recommended by the FDA were used at 10 different concentrations as follows: 0.01–200 μM furafylline for CYP1A2, 0.005–100 μM methoxsalen for CYP2A6, 0.05–500 μM ticlopidine for CYP2B6 and CYP2C19, 0.01–200 μM quercetin for CYP2C8, 0.05–500 μM sulfaphenazole for CYP2C9, 0.005–100 μM quinidine for CYP2D6, 0.1–2000 μM diethyldithiocarbamate for CYP2E1, and 0.005–100 μM ketoconazole for CYP3A4/5. Licorice root extract at 11 concentrations from 0.005 to 250 μg/ml was also evaluated for inhibition. After incubation with probe substrates as described earlier and quantitative analysis of metabolites as described later, *IC*₅₀ values were calculated.

UHPLC-MS/MS

All metabolites and surrogate standards were analyzed in a single run using UHPLC-MS/MS on a Shimadzu Nexera UHPLC and LCMS-8050 triple quadrupole mass spectrometer (Kyoto, Japan). The 10 metabolites of the probe substrates and their corresponding isotope-labeled internal standards were separated on a Waters ACQUITY UPLC BEH C₁₈ column (2.1 × 100 mm, 1.7 μm) (Milford, MA) using a 3-minute gradient from 20 to 75% acetonitrile in water containing 0.1%

TABLE 1
K_m values and concentrations of P450-specific probe substrates, and the MS/MS selected reaction monitoring (SRM) transitions and collision energies (CEs) for substrate metabolites and stable isotope-labeled internal standards used in the cocktail assays

Enzyme	Substrate	<i>K_m</i> μM	Concentration μM	Metabolite	SRM Transition (Polarity)	CE	Internal Standard	SRM Transition (Polarity)	CE
CYP1A2	Phenacetin	112.7 ± 10.9	100	Acetaminophen	152.2 > 110.0 (+)	-17	[d ₄]-Acetaminophen	156.2 > 114.1 (+)	V
CYP2A6	Coumarin	1.5 ± 0.2	1.5	7-Hydroxycoumarin	161.1 > 133.1 (-)	23	[d ₅]-7-Hydroxycoumarin	166.1 > 138.1 (-)	22
CYP2B6	Bupropion	125.2 ± 14.0	12	Hydroxybupropion	256.2 > 139.1 (+)	-26	[d ₆]-Hydroxybupropion	262.2 > 139.0 (+)	-26
CYP2C8	Amodiaquine	1.0 ± 0.1	1	<i>N</i> -desethylamodiaquine	328.1 > 283.0 (+)	-21	[d ₅]- <i>N</i> -desethylamodiaquine	333.1 > 283.1 (+)	-29
CYP2C9	Tolbutamide	110.7 ± 11.6	100	Hydroxytolbutamide	285.1 > 186.0 (-)	18	[d ₄]-Hydroxytolbutamide	294.2 > 186.0 (-)	18
CYP2C19	(<i>S</i>)-mephenytoin	52.5 ± 10.6	50	(±)-4'-Hydroxymephenytoin	235.1 > 133.1 (+)	-18	[d ₃](±)-4'-Hydroxymephenytoin	238.1 > 133.1 (+)	-22
CYP2D6	Dextromethorphan	2.9 ± 0.5	2.5	Dextrophan	258.1 > 157.0 (+)	-37	[d ₃]-Dextrophan	261.2 > 157.1 (+)	-40
CYP2E1	Chlorzoxazone	149.8 ± 12.6	15	6-Hydroxychlorzoxazone	184.0 > 120.0 (-)	18	[d ₂]-6-Hydroxychlorzoxazone	186.2 > 122.0 (-)	18
CYP3A4/5	Midazolam	2.7 ± 0.1	2.5	1'-Hydroxymidazolam	342.1 > 324.0 (+)	-21	[¹³ C ₃]-1'-hydroxymidazolam	347.2 > 329.0 (+)	-22
	Testosterone	50.5 ± 5.6	50	6β-Hydroxytestosterone	305.2 > 269.2 (+)	-18	[d ₇]-6β-Hydroxytestosterone	312.2 > 276.2 (+)	-16

formic acid. The flow rate was 0.5 ml/min, and the column oven temperature was 40°C. Detection was carried out using electrospray with polarity switching, collision-induced dissociation, and selected reaction monitoring (Table 1).

Data Analysis

Quantitative UHPLC-MS/MS data were analyzed using Shimadzu LabSolutions software. The K_m and IC_{50} values were determined using the Enzyme Kinetics module of SigmaPlot (Systat Software, San Jose, CA). The percentage of control activity, linearity of metabolite formation, and other calculations were carried out using Excel (Microsoft, Seattle, WA).

Results

The 10 probe substrates specific to nine P450 isoforms (Table 1) were selected based on U.S. FDA recommendation (<http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm>), specificity of the enzymatic reaction, sensitivity of analytical detection, and availability of the stable isotope-labeled surrogate standard of the corresponding metabolites.

The initial concentration of each substrate was determined based on its K_m value (Table 1) and systematic evaluation of probe interactions. The selected reaction monitoring transitions for all 10 metabolites and their corresponding isotope-labeled internal standards were selected based on the most abundant fragment ions of each protonated or deprotonated molecule and are summarized in Table 1. The elution profiles of all metabolites and internal standards detected during UHPLC-MS/MS are shown in Fig. 1. Note that all compounds eluted within 3 minutes.

Although the metabolic transformation of most probe substrates in the cocktail was linear for at least 20 minutes, the rates of metabolism of

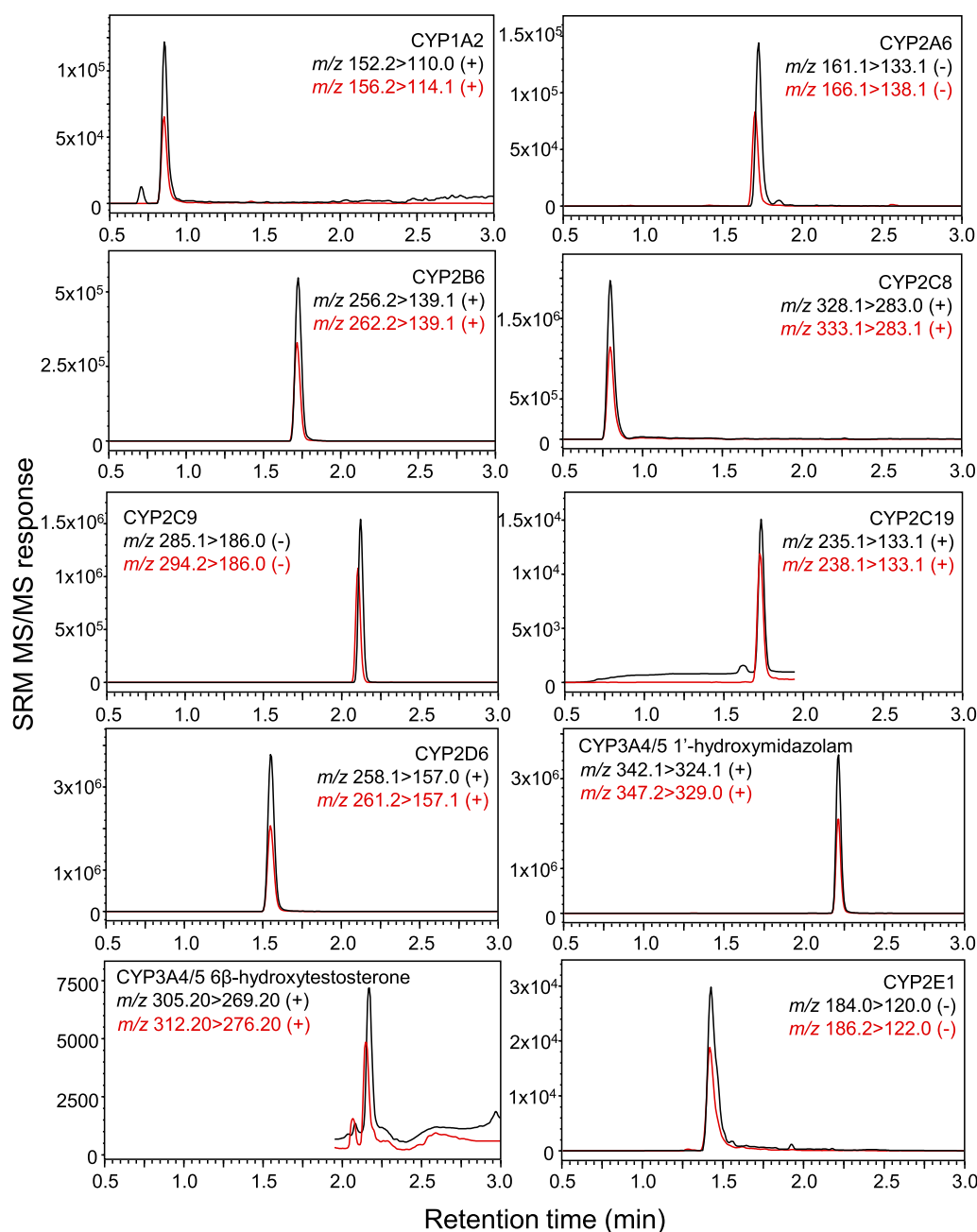


Fig. 1. UHPLC-MS/MS chromatograms of probe substrate metabolites (100 nM) and their corresponding stable isotope-labeled internal standards. SRM, selected reaction monitoring.

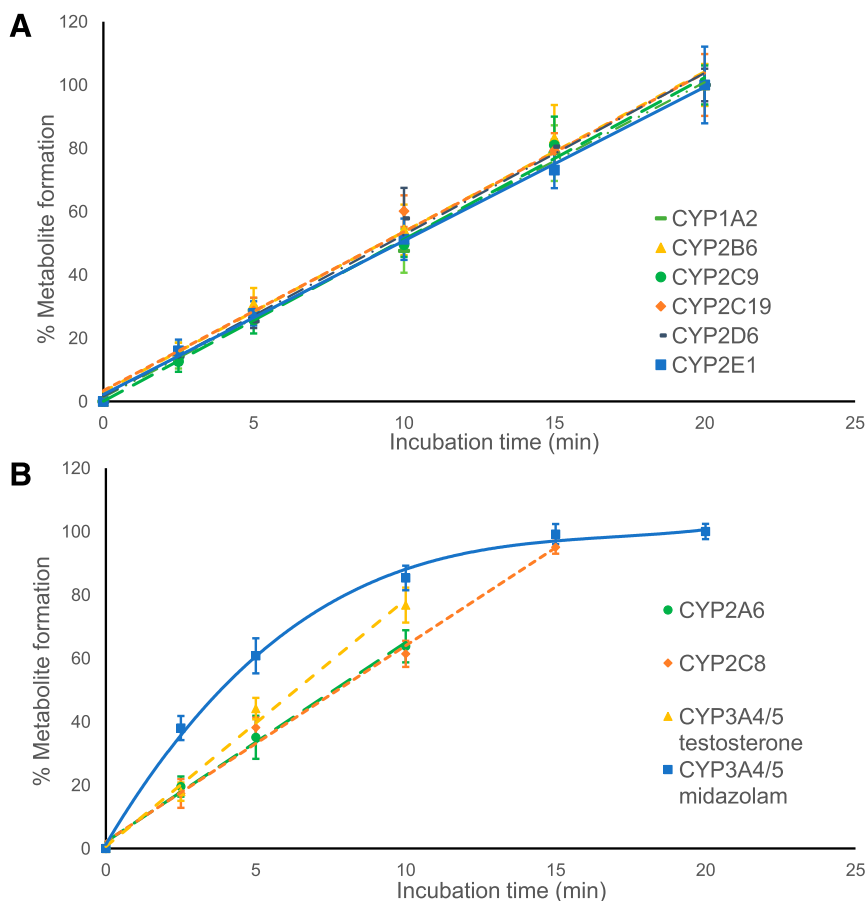


Fig. 2. Linearity of substrate metabolite formation during the cocktail assay. Metabolite formation is expressed as a percentage of the amount of metabolite at 20 minutes. A) P450 enzymes CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1. B) P450 enzymes CYP2A6, CYP2C8, CYP3A4/5 (testosterone substrate), and CYP3A4/5 (midazolam substrate).

coumarin by CYP2A6, of amodiaquine by CYP2C8, and of testosterone by CYP3A4/5 showed linearity for up to only 10–15 minutes. The formation of 1'-hydroxymidazolam was not linear under any incubation conditions but was most linear during the first 10 minutes (Fig. 2). Therefore, an incubation time of 10 minutes was selected for the entire cocktail assay based on the linearity of formation of most metabolites during this period. Using known inhibitors of each enzymatic reaction, validation of the cocktail assay was carried out by comparing the IC_{50} values obtained using the cocktail approach with those obtained using only single substrates. Inhibition curves and IC_{50} values (Fig. 3; Table 2) showed good accordance between the cocktail assay and the single-substrate method.

The new cocktail assay was then used to assess the potential for drug-botanical interactions of a licorice root extract from *G. glabra* (Fig. 4). The licorice root extract inhibited CYP2B6 and the CYP2C family of enzymes (CYP2C8, CYP2C9, and CYP2C19), with IC_{50} values $<20 \mu\text{g/ml}$. Note that good agreement was observed between the cocktail and single-substrate approaches as indicated by the data in Table 3.

Discussion

Probe Substrate Selection and Mass Spectrometry. Phenacetin *O*-deethylation by CYP1A2 was selected for the cocktail assay and is often used in other cocktails (Yuan et al., 2002; Spaggiari et al., 2014) because of its superior specificity compared with other FDA-recommended substrates. Among several possible probes for CYP2A6, CYP2B6, and CYP2E1, coumarin-7-hydroxylation, bupropion-hydroxylation, and chlorzoxazone 6-hydroxylation were selected based on the commercial availability of the corresponding stable isotope-labeled metabolites for use as surrogate standards during UHPLC-MS/MS

(Fig. 1). For CYP2C8, taxol and amodiaquine are frequently used as probe substrates; however, the higher solubility of amodiaquine makes it superior for cocktail applications (Spaggiari et al., 2014). Therefore, amodiaquine *N*-deethylation by CYP2C8 was measured instead of taxol hydroxylation.

Tolbutamide and diclofenac are frequently used as probe substrates for CYP2C9, and bufuralol and dextromethorphan are often used as substrates of CYP2D6. Although either probe substrate of each pair could have been used in our assay, tolbutamide methyl-hydroxylation and dextromethorphan *O*-demethylation were used to measure interactions with CYP2C9 and CYP2D6, respectively. As a probe for CYP2C19, (*S*)-mephenytoin 4'-hydroxylation is highly specific; however, cocktail assays typically use the less-specific omeprazole due to the sensitivity limitations of most detection methods (Yuan et al., 2002; Testino and Patonay, 2003; Spaggiari et al., 2014). Here, we were able to use the preferred probe substrate, (*S*)-mephenytoin, due to the high sensitivity of UHPLC-MS/MS (Fig. 1). For evaluation of CYP3A4/5 inhibition, the use of two structurally unrelated substrates is recommended (Yuan et al., 2002; <http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm>). Therefore, midazolam 1-hydroxylation and testosterone 6 β -hydroxylation were used to probe inhibition of CYP3A4/5 as recommended by the FDA. For [$^{13}\text{C}_3$]-1'-hydroxymidazolam, we monitored the second most abundant protonated ion m/z 247 instead of the most abundant protonated ion m/z 245 due to the isotopic interference from 1'-hydroxymidazolam.

7-Hydroxycoumarin, hydroxytolbutamide, and 6-hydroxychlorzoxazone were measured using negative electrospray, whereas the other seven metabolites were measured in positive ion mode (Fig. 1). Poor ionization efficiency of 6-hydroxychlorzoxazone during positive ion

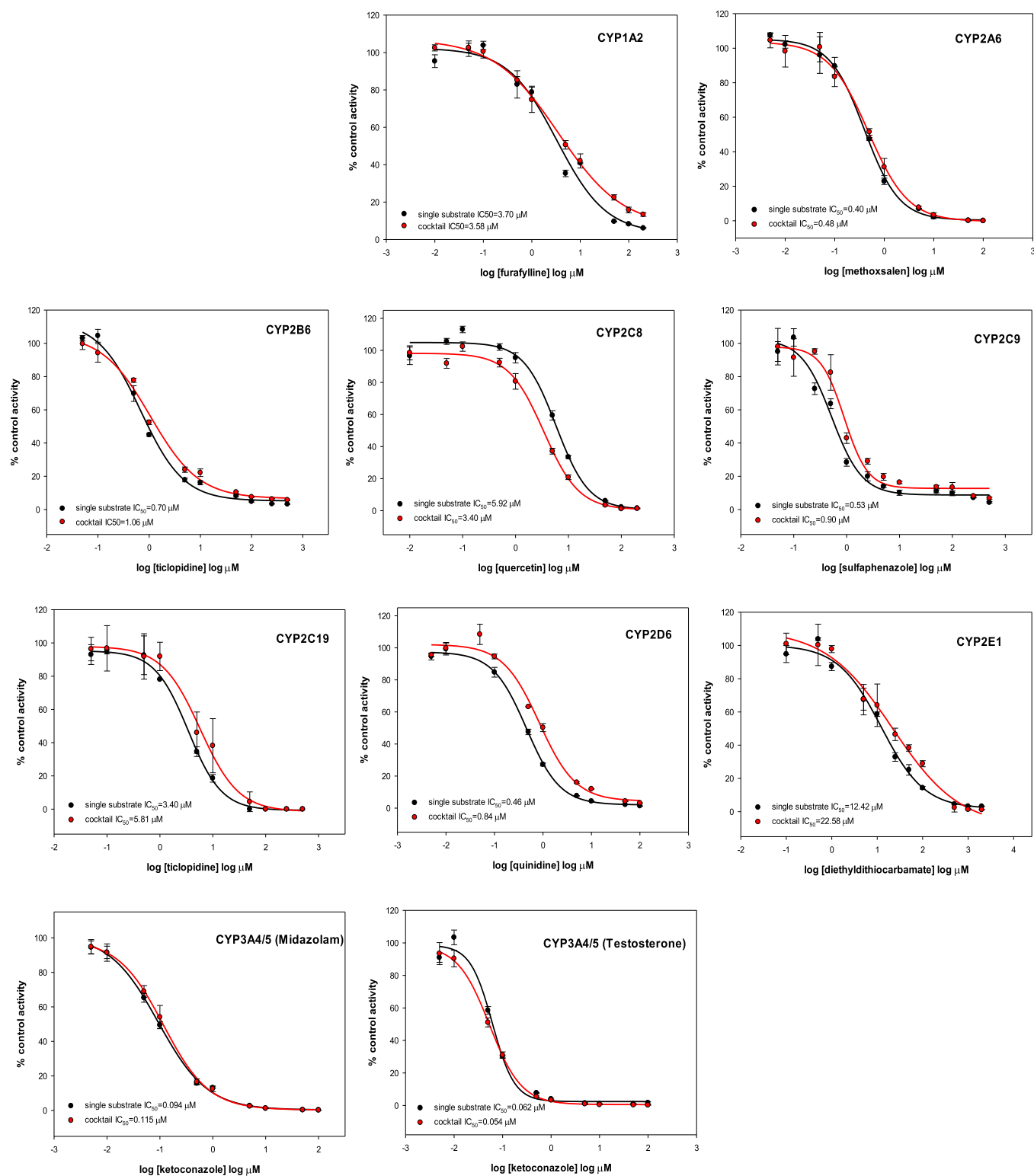


Fig. 3. Inhibition curves of known inhibitors obtained using single substrates and substrate cocktails. Each inhibitor was incubated in separate experiments with single substrates or the substrate cocktail. The activity is expressed as a percentage of remaining activity compared with the control containing no inhibitor. Experiments were carried out three times.

electrospray and the inability of some mass spectrometers to carry out rapid polarity switching have been cited as reasons for excluding CYP2E1 from some previous cocktail assays (He et al., 2007). Chlorzoxazone was included in our cocktail assay as a probe for CYP2E1 (Fig. 1), because the fast polarity switching and high sensitivity of this generation triple quadrupole mass spectrometer enabled the measurement of all 10

probes including 6-hydroxychlorzoxazone and their surrogate standards in a single analysis.

Optimization of Probe Substrate Concentrations and Incubation Conditions. The probe substrate concentrations in cocktail assays should be less than or equal to the K_m of the corresponding cytochrome P450 enzymes. Although K_m values from the literature have been used

TABLE 2
Comparison of IC₅₀ values of known inhibitors obtained using the single-substrate approach, the new cocktail approach, and literature values

Substrate	Inhibitor	Single Substrate IC ₅₀ ± S.D.	Cocktail IC ₅₀ ± S.D.	Literature IC ₅₀	
		μM	μM	μM	
CYP1A2	Phenacetin	Furafylline	3.70 ± 1.00	3.58 ± 0.49	0.48–13.88 (Eagling et al., 1998; Testino and Patonay, 2003; Walsky and Obach, 2004; Zientek et al., 2008; Otten et al., 2011; Kozakai et al., 2012; Liu et al., 2015)
CYP2A6	Coumarin	Methoxsalen	0.40 ± 0.03	0.48 ± 0.05	0.21–2.11 (Kim et al., 2005; Workman and Raynaud, 2007; Kozakai et al., 2012)
CYP2B6	Bupropion	Ticlopidine	0.70 ± 0.08	1.06 ± 0.10	0.1–0.78 (Turpeinen et al., 2005; Otten et al., 2011)
CYP2C8	Amodiaquine	Quercetin	5.92 ± 0.54	3.40 ± 0.32	1.09–7.00 (Dierks et al., 2001; Walsky and Obach, 2004; Otten et al., 2011; Liu et al., 2015)
CYP2C9	Tolbutamide	Sulfaphenazole	0.53 ± 0.06	0.90 ± 0.10	0.069–1.30 (Eagling et al., 1998; Shader et al., 1999; Testino and Patonay, 2003; Workman and Raynaud, 2007; Zientek et al., 2008; Otten et al., 2011; Kozakai et al., 2012; Yao et al., 2012; Qin et al., 2014; Liu et al., 2015)
CYP2C19	(S)-mephenytoin	Ticlopidine	3.40 ± 0.32	5.81 ± 0.80	0.86–4.46 (Giancarlo et al., 2001; Zientek et al., 2008; Yao et al., 2012; Qin et al., 2014; Liu et al., 2015)
CYP2D6	Dextromethorphan	Quinidine	0.46 ± 0.02	0.84 ± 0.10	0.009–0.52 (Shader et al., 1999; Testino and Patonay, 2003; Walsky and Obach, 2004; Zientek et al., 2008; Otten et al., 2011; Kozakai et al., 2012; Yao et al., 2012; Qiao et al., 2014; Qin et al., 2014; Liu et al., 2015)
CYP3A4/5	Midazolam	Ketoconazole	0.094 ± 0.006	0.115 ± 0.056	0.01–0.13 (Patki et al., 2003; Walsky and Obach, 2004; Workman and Raynaud, 2007; Otten et al., 2011; Kozakai et al., 2012; Yao et al., 2012; Qiao et al., 2014; Qin et al., 2014; Liu et al., 2015)
	Testosterone		0.062 ± 0.004	0.054 ± 0.002	0.008–0.0477 (Eagling et al., 1998; Patki et al., 2003; Walsky and Obach, 2004; Kozakai et al., 2012; Yao et al., 2012; Qiao et al., 2014)
CYP2E1	Chlorzoxazone	Diethyldithiocarbamate	12.42 ± 1.70	22.58 ± 5.60	9.8–16.6 (Eagling et al., 1998; Shader et al., 1999; Workman and Raynaud, 2007)

in the design of most existing cocktail assays, the reported values can span a wide range (Turpeinen et al., 2005; Liu et al., 2015). For example, the K_m values reported for phenacetin *O*-deethylation range from 1.7 to 152 μM, and those for tolbutamide methyl-hydroxylation range from 67 to 838 μM (<http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm>). The variability of K_m values between laboratories is primarily caused by differences in experimental procedures and genetic variations in the enzymes being probed. To be certain that appropriate concentrations of each probe substrate were used in our application, we determined the K_m values for each probe substrate using the same experimental procedures, and the same batch of pooled human liver microsomes was used for all subsequent inhibition experiments (Table 1).

Possible interactions between the probe substrates of the P450 enzymes were evaluated, and substrate concentrations for the cocktail assay were adjusted to minimize these interactions. Phenacetin weakly inhibited CYP2B6 and CYP3A4/5 (midazolam); bupropion strongly inhibited the activities of CYP2C19 and CYP2D6 and weakly inhibited CYP3A4/5 (midazolam). Chlorzoxazone inhibited CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP3A4/5 (midazolam and testosterone), and testosterone moderately inhibited CYP2B6 and CYP3A4/5 (midazolam) while weakly inhibiting CYP2C9.

Lowering substrate concentrations was an effective strategy to decrease interactions in the cocktail assay. For example, because bupropion and chlorzoxazone strongly inhibited multiple P450 isoforms, 10 substrate mixtures containing varying concentrations (0.05K_m, 0.1K_m, 0.2K_m,

0.4K_m, and 0.8K_m) of bupropion and chlorzoxazone were tested. (S)-mephenytoin is a low-turnover substrate of CYP2C19 (Yao et al., 2012), and to ensure analytical detection of its metabolite 4'-hydroxymephenytoin, both bupropion and chlorzoxazone concentrations needed to be ≤0.1K_m (data not shown). Therefore, the concentrations of bupropion and chlorzoxazone were set to 0.1K_m in the cocktail assay.

After optimizing the substrate concentrations for the cocktail assay (Table 1), the linearity of metabolite formation was investigated. All 10 substrates were evaluated: six of them showed linearity up to 20 minutes, whereas four substrates were linear for 15 minutes or less (Fig. 2). Although a 10-minute incubation time was not optimal for midazolam, it was the minimum required to ensure sufficient metabolite formation for the low-turnover substrates. Under these conditions, the inhibition potency of moderate inhibitors for midazolam 1-hydroxylation might be underestimated (Ogilvie et al., 2008). Therefore, further studies on any compounds/extracts that show moderate/weak inhibition of CYP3A4/5 (midazolam) would be recommended.

Validation and Application of the Cocktail Assay. Ideally, the cocktail cytochrome P450 enzyme inhibition assay should yield the same results as would be obtained if each substrate were assayed separately. As quantitative measures of the potencies of enzyme inhibitors, the IC₅₀ values for inhibitors tested individually or in the cocktail assay should also be comparable (Sittampalam et al., 2004; Davis and Ward, 2014). The ratios of the IC₅₀ values obtained using both approaches (Table 2) were compared and ranged from 1.03 to 1.82. Because these values were within a 2-fold range of each other, they

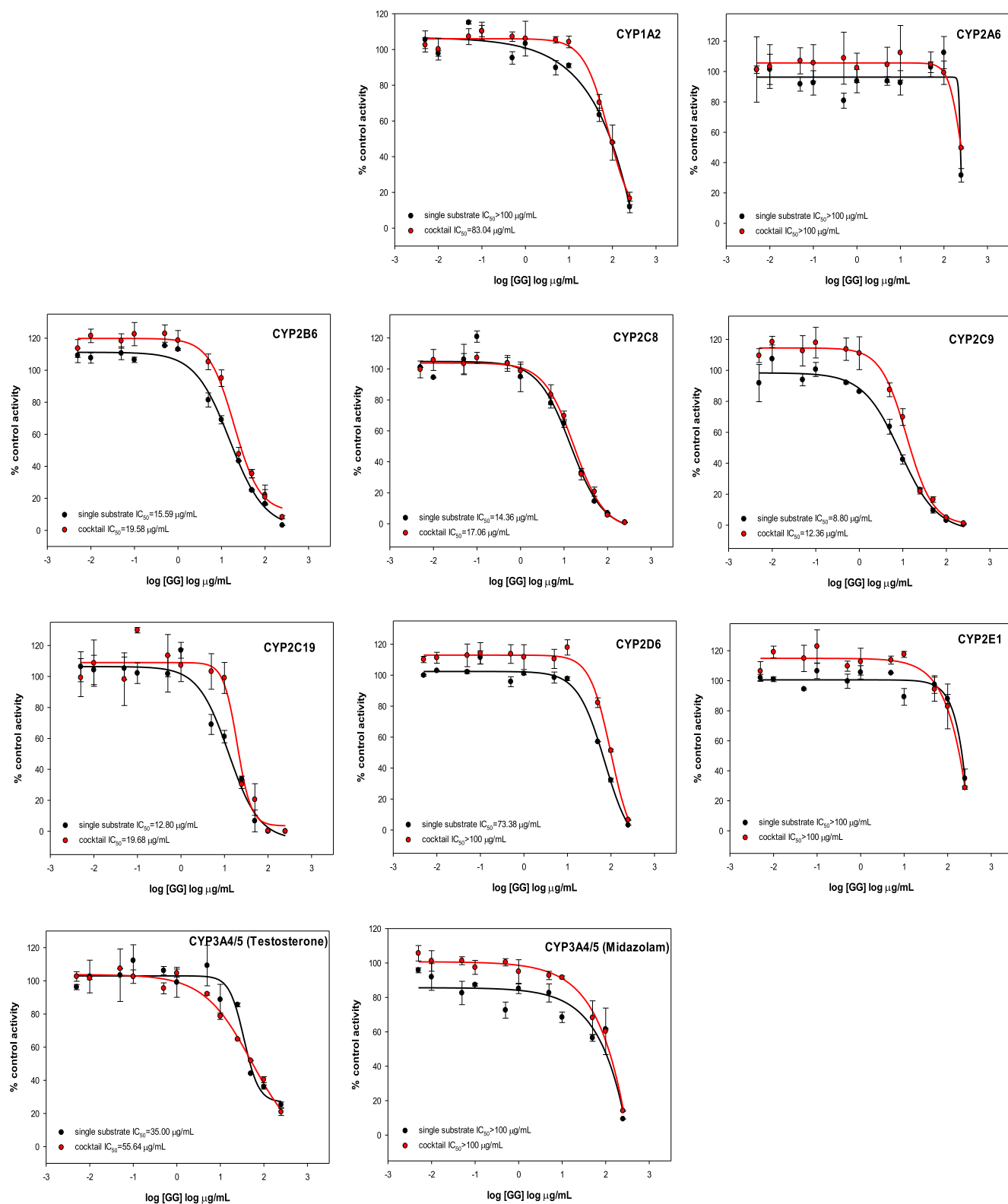


Fig. 4. Inhibition curves of a licorice root extract obtained using single substrates or the substrate cocktail. Each inhibitor was incubated in separate experiments with a single substrate or the substrate cocktail. The activity is shown as a percentage of remaining activity compared with the control when no inhibitor was added. Experiments were carried out three times. GG = *Glycyrrhiza glabra* extract.

were in good agreement. As additional validation of the cocktail method, the measured IC_{50} values were consistent with values in the literature (Table 2) (Eagling et al., 1998; Shader et al., 1999; Dierks et al., 2001; Giancarlo et al., 2001; Patki et al., 2003; Testino and

Patonay, 2003; Walsky and Obach, 2004; Kim et al., 2005; Turpeinen et al., 2005; Workman and Raynaud, 2007; Zientek et al., 2008; Otten et al., 2011; Kozakai et al., 2012; Yao et al., 2012; Qiao et al., 2014; Qin et al., 2014; Liu et al., 2015).

TABLE 3

IC₅₀ values for inhibition of P450 enzymes by a licorice extract (*G. glabra*) obtained using the single-substrate approach or the new cocktail approach and comparison with literature values

Enzyme	Substrate	Single-Substrate IC ₅₀	Cocktail IC ₅₀
		μg/ml	μg/ml
CYP1A2	Phenacetin	>100	83.04 ± 16.96
CYP2A6	Coumarin	>100	>100
CYP2B6	Bupropion	15.59 ± 1.79	19.58 ± 1.80
CYP2C8	Amodiaquine	14.36 ± 2.62	17.06 ± 1.54
CYP2C9	Tolbutamide	8.80 ± 1.20	12.36 ± 1.14
CYP2C19	(S)-mephenytoin	12.80 ± 2.34	19.68 ± 2.70
CYP2D6	Dextromethorphan	73.38 ± 11.72	>100
CYP3A4/5	Midazolam	>100	>100
	Testosterone	35.00 ± 4.81	55.64 ± 18.49
CYP2E1	Chlorzoxazone	>100	>100

IC₅₀ values are extrinsic constants and depend on experimental conditions, but K_i values are intrinsic constants. Therefore, researchers sometimes estimate K_i from IC₅₀ values (Otten et al., 2011). Assuming competitive inhibition, K_i values can be calculated using the Cheng-Prusoff equation (K_i = IC₅₀/2 when substrate concentration is ≈K_m; and K_i = IC₅₀/1.1 when the substrate concentration is K_m/10). Using the IC₅₀ values in Table 2 and the Cheng-Prusoff equation, the calculated cocktail K_i values are in good accordance with those obtained for single-substrate assays, except for ticlopidine (CYP2B6) and diethyldithiocarbamate (CYP2E1), for which the calculated K_i values for the cocktail assays (0.96 and 20.53 μM) were ~3-fold higher than those for the single-substrate assays (0.35 and 6.21 μM). One possible explanation might be that ticlopidine and diethyldithiocarbamate are mechanism-based inhibitors of CYP2B6 and CYP2E1, respectively, and their IC₅₀ values are susceptible to changes of microsomal conditions (Turpeinen et al., 2004; Ogilvie et al., 2008; Pratt-Hyatt et al., 2010).

Kent et al. (2002) reported that an alcoholic extract of licorice root (*G. glabra*) moderately inhibited CYP3A4, and its flavonoid glabridin inhibited the activities of CYP2B6, CYP2C9, and CYP3A4. Our *G. glabra* extract showed moderate inhibition of CYP2B6, CYP2C9, CYP2C8, and CYP2C19 (which were not tested by Kent et al., 2000) but only weak inhibition of CYP3A4/5. The slight difference between inhibition potencies of CYP3A4/5 for these two assays is probably due to the different preparations of licorice extracts used or minor assay variations.

In conclusion, an in vitro high-throughput cytochrome P450 cocktail inhibition assay containing 10 substrates for nine P450 isoforms was developed using UHPLC-MS/MS and validated using known inhibitors of each P450 enzyme. This assay includes all seven cytochrome P450 enzymes recommended for testing by the U.S. FDA as well as two additional isoforms, CYP2A6 and CYP2E1, which are also important in the metabolism of xenobiotic compounds such as nicotine (CYP2A6) and ethanol (CYP2E1) (Pelkonen et al., 2000; Dey, 2013). Besides the obvious time efficiency and resource-saving advantages of combining 10 substrates into a single assay, each incubation was only 10 minutes compared with up to 60 minutes in some previous assays, and each UHPLC-MS/MS analysis required less than 5 minutes per sample, which is up to 5-fold faster than comparable HPLC-MS/MS approaches. The assay was applied to the prediction of drug-botanical interactions for a licorice root dietary supplement. As a complement to drug-drug interaction studies, drug-botanical interactions are understudied and yet can cause drug toxicity and therapeutic failure.

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Authorship Contributions

Participated in research design: Li, Huang, Nikolic, van Breemen.

Conducted experiments: Li.

Performed data analysis: Li.

Wrote or contributed to the writing of the manuscript: Li, van Breemen.

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