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

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CYP450 Cocktail Inhibition Assay Using UHPLC-MS/MS

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Nonstandard abbreviations:
CYP: cytochrome P450
FDA: United States Food and Drug Administration
K_m: Michaelis constant
UHPLC-MS/MS: Ultrahigh pressure liquid chromatography-tandem mass spectrometry
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 (CYP) 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 CYP cocktail inhibition assay was developed that uses 10 substrates simultaneously against 9 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 CYP enzymes as previous assays while remaining among the fastest due to short incubation times and rapid analysis using UHPLC-MS/MS. The method was validated using known inhibitors of each CYP 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.
1. 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 (CYP) 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 CYP inhibition assays are widely used to study potential drug-drug and drug-botanical interactions. Although these assays typically evaluate inhibition of one CYP isoform at a time, the U.S. Food and Drug Administration (FDA) recommends that at least seven CYP 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 CYP isoforms simultaneously. Most of these assays test for inhibition of five to eight CYP isoforms and use a wide variety of experimental conditions and probe substrates (Dierks et al., 2001; Testino and Patonay, 2003; Smith et al., 2007; Workman and Raynaud, 2007; He et al., 2007; Li et al., 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 9 isoforms, they actually carry out separate incubations of subsets of probe substrates to
avoid CYP interactions before pooling the mixtures immediately prior to a quantitative analysis step, utilize CYP substrates which are not recommended by the FDA, and/or use long incubation times of up to 60 min (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 CYP cocktail inhibition assay using 10 substrates against 9 CYP enzymes. Simultaneously assessing the inhibition of 9 CYP 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 utilized a fast and sensitive UHPLC-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 CYP inhibitors, an extract of the botanical dietary supplement licorice (Glycyrrhiza glabra) was evaluated for CYP inhibition.
2. Materials and Methods

2.1 Materials and Chemicals

Phenacetin was purchased from Tokyo Chemical Industry (Tokyo, Japan). Acetaminophen, coumarin, bupropion hydrochloride, tolbutamide, dextrophan tartrate, chlorzoxazone, 6β-hydroxytestosterone, furafylline, ticlopidine hydrochloride, quercetin, sulfaphenazole, quinidine, ketoconazole, ammonium diethyldithiocarbamate, β-nicotinamide adenine dinucleotide phosphate (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), and 7-hydroxycoumarin was purchased from Indofine Chemical (Hillsborough, NJ). Hydroxybupropion and hydroxybupropion-d₆ were purchased from Santa Cruz Biotechnology (Dallas, TX), and 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, [d₅]-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). LC/MS-grade acetonitrile and methanol were purchased from
Thermo Fisher (Fair Lawn, NJ). Water was prepared using an Elga Purelab Ultra (Siemens Water Technologies, Woodridge, IL) water purification system. An extract of licorice roots (*Glycyrrhiza glabra*) was prepared from botanically authenticated plant material provided by the UIC/NIH Center for Botanical Dietary Supplements Research. The licorice root was extracted with 90% ethanol, 5% isopropanol, 5% water (v/v/v) (weight root powder (g)/volume solvent (mL): 1:15).

2.2 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\textsubscript{m}) were incubated at 37 °C for 10 min. 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 13000 x \textit{g} at 4 °C for 15 min prior to analysis using UHPLC-MS/MS.

2.2.1 Michaelis constant (K\textsubscript{m}) determination

Human liver microsomes (0.05-0.20 mg/mL) were incubated at 37 °C for 5-20 min with each CYP substrate at 8-10 different concentrations. After quantitative analysis using UHPLC-MS/MS, K\textsubscript{m} values were calculated.
2.2.2 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 min. After quenching and quantitative analysis, the linearity of metabolite formation was evaluated.

2.2.3 Comparison of cocktail and single substrate assays using known CYP inhibitors and licorice root extract

Known CYP 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 0.005-250 µg/mL were also evaluated for inhibition. After incubation with probe substrates as described above and quantitative analysis of metabolites as described below, IC₅₀ values were calculated.

2.3 UHPLC-MS/MS

All metabolites and surrogate standards were analyzed in a single run using UHPLC-MS/MS on a Shimadzu (Kyoto, Japan) Nexera UHPLC and LCMS-8050 triple quadrupole mass spectrometer. The 10 metabolites of the probe substrates and their corresponding isotope-labeled internal standards were separated on a Waters (Milford, MA) ACQUITY UPLC BEH C₁₈ column (2.1 x 100 mm, 1.7 µm) using a 3-min gradient from 20% to 75% acetonitrile in water containing 0.1% 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 (SRM) (Table 1).

2.4 Data analysis

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

The ten probe substrates specific to 9 CYP isoforms (Table 1) were selected based on U.S. FDA recommendation (Food and Drug Administration, 2006), 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 SRM transitions for all ten 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 Figure 1. Note that all compounds eluted within 3 min.

Although the metabolic transformation of most probe substrates in the cocktail was linear for at least 20 min, the rates of metabolism of coumarin by CYP2A6, of amodiaquine by CYP2C8 and of testosterone by CYP3A4/5 showed linearity for up to only 10-15 min. The formation of 1'-hydroxymidazolam was not linear under any incubation conditions but was most linear during the first 10 min (Figure 2). Therefore, an incubation time of 10 min 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 (Figure 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* (Figure 4). The licorice root extract inhibited CYP2B6 and the CYP2C family of enzymes (CYP2C8, CYP2C9 and CYP2C19) with IC$_{50}$ values <20 μg/mL. Note that good agreement was observed between the cocktail and single substrate approaches as indicated by the data in Table 3.
4. Discussion

4.1 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 to 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 (Figure 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 (Figure 1). For evaluation of CYP3A4/5 inhibition, the use of two
structurally unrelated substrates are recommended (Yuan et al., 2002; Food and Drug Administration, 2006). Therefore, midazolam 1-hydroxylation and testosterone 6β-hydroxylation were used to probe inhibition of CYP3A4/5 as recommended by the FDA. For [13C3]-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 while the other seven metabolites were measured in positive ion mode (Figure 1). Poor ionization efficiency of 6-hydroxychlorzoxazone during positive ion 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 (Figure 1), because the fast polarity-switching and high sensitivity of this generation triple quadrupole mass spectrometer enabled the measurement of all ten probes including 6-hydroxychlorzoxazone and their surrogate standards in a single analysis.

4.2 Optimization of Probe Substrate Concentrations and Incubation Conditions

The probe substrate concentrations in cocktail assays should be $\leq K_m$ of the corresponding cytochrome P450 enzymes. Although $K_m$ values from the literature have been utilized 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 1.7-152 µM, and those for
tolbutamide methyl-hydroxylation range from 67-838 µM (Food and Drug Administration,
2006). 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
utilized 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 CYP 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 CYP isoforms, 10 substrate mixtures containing varying
concentrations (0.05$K_m$, 0.1$K_m$, 0.2$K_m$, 0.4$K_m$, and 0.8$K_m$) 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
Therefore, the concentrations of bupropion and chlorzoxazone were set to 0.1Km 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, 6 of them showed linearity up to 20 min, while 4 substrates were linear for 15 min or less (Figure 2). Although a 10-min 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 (Ogilivie et al., 2008). Therefore, further studies on any compounds/extracts that show moderate/weak inhibition of CYP3A4/5 (midazolam) would be recommended.

4.3 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 GS, Gal-Edd N, Arkin M, 2004; Davis and Ward, 2014). The ratios of the IC50 values obtained using both approaches (Table 2) were compared and had ratios ranging from 1.03 to 1.82. Because these values were within a 2-fold range of each other, they were in good agreement. As additional validation of the cocktail method, the measured IC50 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; Testino and
Patonay, 2003; Patki et al., 2003; Walsky and Obach, 2004; Turpeinen et al., 2005; Kim et al., 2005; Workman and Raynaud, 2007; Zientek et al., 2008; Otten et al., 2011; Yao et al., 2012; Kozakai et al., 2012; Qin et al., 2014; Qiao et al., 2014; Liu et al., 2015).

IC\textsubscript{50} values are extrinsic constants and depend on experimental conditions, but Ki values are intrinsic constants. Therefore, researchers sometimes estimate K\textsubscript{i} from IC\textsubscript{50} values (Otten et al., 2011). Assuming competitive inhibition, K\textsubscript{i} values can be calculated using the Cheng-Prusoff equation (K\textsubscript{i}=IC\textsubscript{50}/2 when substrate concentration is \(\approx\)Km; and K\textsubscript{i}=IC\textsubscript{50}/1.1 when the substrate concentration is Km/10). Using the IC50 values in Table 2 and the Cheng-Prusoff equation, the calculated cocktail K\textsubscript{i} values are in good accordance with those obtained single substrate assays, except for ticlopidine (CYP2B6) and diethyldithiocarbamate (CYP2E1) for which the calculated Ki values for the cocktail assays (0.96 and 20.53 µM) were ~3-fold higher 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 IC\textsubscript{50} values are susceptible to changes of microsomal conditions (Turpeinen and Nieminen, 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.) 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 to minor assay variations.
In conclusion, an in vitro high-throughput cytochrome P450 cocktail inhibition assay containing 10 substrates for 9 CYP isoforms was developed using UHPLC-MS/MS and validated using known inhibitors of each CYP enzyme. This assay includes all 7 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 min compared with up to 60 min in some previous assays, and each UHPLC-MS/MS analysis required less than 5 min 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.
5. Acknowledgments

The authors thank Dr. Charlotte Simmler and Dr. Guido F. Pauli of the UIC/NIH Center for Botanical Dietary Supplements for kindly providing extracts of *Glycyrrhiza glabra*. We also thank Shimadzu Instruments for providing the UHPLC-MS/MS system used during this investigation.
6. 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.
7. References


8. Footnotes

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Figure Legends

Figure 1. UHPLC-MS/MS chromatograms of probe substrate metabolites (100 nM) and their corresponding stable isotope–labeled internal standards (IS).

Figure 2. Linearity of substrate metabolite formation during the cocktail assay. Metabolite formation is expressed as a percentage of the amount of metabolite at 20 min.

Figure 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.

Figure 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.
Table 1. $K_m$ values and concentrations of CYP-specific probe substrates, and the MS/MS SRM transitions and collision energies (CE) for substrate metabolites and stable isotope-labeled internal standards used in the cocktail assays.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>Conc (µM)</th>
<th>Metabolite</th>
<th>SRM transition (polarity)</th>
<th>CE (V)</th>
<th>Internal standard</th>
<th>SRM transition (polarity)</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>phenacetin</td>
<td>112.7 ± 10.9</td>
<td>100</td>
<td>acetaminophen</td>
<td>152.2&gt;110.0 (+)</td>
<td>-17</td>
<td>[d$_4$]-acetaminophen</td>
<td>156.2&gt;114.1 (+)</td>
<td>-19</td>
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<tr>
<td>CYP2A6</td>
<td>coumarin</td>
<td>1.5 ± 0.2</td>
<td>1.5</td>
<td>7-hydroxycoumarin</td>
<td>161.1&gt;133.1 (-)</td>
<td>23</td>
<td>[d$_5$]-7-hydroxycoumarin</td>
<td>166.1&gt;138.1 (-)</td>
<td>22</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>bupropion</td>
<td>125.2 ± 14.0</td>
<td>12</td>
<td>hydroxybupropion</td>
<td>256.2&gt;139.1 (+)</td>
<td>-26</td>
<td>[d$_5$]-hydroxybupropion</td>
<td>262.2&gt;139.0 (+)</td>
<td>-26</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>amodiaquine</td>
<td>1.0 ± 0.1</td>
<td>1</td>
<td>N-desethylamodiaquine</td>
<td>328.1&gt;283.0 (+)</td>
<td>-21</td>
<td>[d$_5$]-N-desethylamodiaque</td>
<td>333.1&gt;283.1 (+)</td>
<td>-29</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>tolbutamide</td>
<td>110.7 ± 11.6</td>
<td>100</td>
<td>hydroxytolbutamide</td>
<td>285.1&gt;186.0 (-)</td>
<td>18</td>
<td>[d$_5$]-hydroxytolbutamide</td>
<td>294.2&gt;186.0 (-)</td>
<td>18</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-mephenytoin</td>
<td>52.5 ± 10.6</td>
<td>50</td>
<td>(±)-4'-hydroxymephenytoin</td>
<td>235.1&gt;133.1 (+)</td>
<td>-18</td>
<td>[d$_3$]-(±)-4'-hydroxymephenytoin</td>
<td>238.1&gt;131.1 (+)</td>
<td>-22</td>
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<tr>
<td>CYP2D6</td>
<td>dextromethorphan</td>
<td>2.9 ± 0.5</td>
<td>2.5</td>
<td>dextrorphan</td>
<td>258.1&gt;157.0 (+)</td>
<td>-37</td>
<td>[d$_5$]-dextrorphan</td>
<td>261.2&gt;157.1 (+)</td>
<td>-40</td>
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<tr>
<td>CYP2E1</td>
<td>chlorzoxazone</td>
<td>149.8 ± 12.6</td>
<td>15</td>
<td>6-hydroxychlorzoxazone</td>
<td>184.0&gt;120.0 (-)</td>
<td>18</td>
<td>[d$_5$]-6-hydroxychlorzoxazone</td>
<td>186.2&gt;122.0 (-)</td>
<td>18</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>midazolam</td>
<td>2.7 ± 0.1</td>
<td>2.5</td>
<td>1'-hydroxymidazolam</td>
<td>342.1&gt;324.0 (+)</td>
<td>-21</td>
<td>[13C$_3$]-1'-hydroxymidazolam</td>
<td>347.2&gt;329.0 (+)</td>
<td>-22</td>
</tr>
<tr>
<td></td>
<td>testosterone</td>
<td>50.5 ± 5.6</td>
<td>50</td>
<td>6β-hydroxytestosterone</td>
<td>305.2&gt;269.2 (+)</td>
<td>-18</td>
<td>[d$_5$]-6β-hydroxytestosterone</td>
<td>312.2&gt;276.2 (+)</td>
<td>-16</td>
</tr>
</tbody>
</table>
Table 2. Comparison of IC₅₀ values of known inhibitors obtained using the single substrate approach, the new cocktail approach and literature values.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>single IC₅₀ (µM) ± SD</th>
<th>Cocktail IC₅₀ (µM) ± SD</th>
<th>literature IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>phenacetin</td>
<td>3.70 ± 1.00</td>
<td>3.58 ± 0.49</td>
<td>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)</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>coumarin</td>
<td>0.40 ± 0.03</td>
<td>0.48 ± 0.05</td>
<td>0.21-2.11 (Kim et al., 2005; Workman and Raynaud, 2007; Kozakai et al., 2012)</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>bupropion</td>
<td>0.70 ± 0.08</td>
<td>1.06 ± 0.10</td>
<td>0.1-0.78 (Turpeinen et al., 2005; Otten et al., 2011)</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>amodiaquine</td>
<td>5.92 ± 0.54</td>
<td>3.40 ± 0.32</td>
<td>1.09-7.00 (Dierks et al., 2001; Walsky and Obach, 2004; Otten et al., 2011; Liu et al., 2015)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>tolbutamide</td>
<td>0.53 ± 0.06</td>
<td>0.90 ± 0.10</td>
<td>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., 2017)</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-mephenytoin</td>
<td>ticlopidine</td>
<td>3.40 ± 0.32</td>
<td>5.81 ± 0.80</td>
</tr>
<tr>
<td>---------</td>
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<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>dextromethorphan</td>
<td>quinidine</td>
<td>0.46 ± 0.02</td>
<td>0.84 ± 0.10</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>midazolam</td>
<td>ketoconazole</td>
<td>0.094 ± 0.006</td>
<td>0.115 ± 0.056</td>
</tr>
<tr>
<td></td>
<td>testosterone</td>
<td>0.062 ± 0.004</td>
<td>0.054 ± 0.002</td>
<td>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)</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>chlorzoxazone</td>
<td>diethyl-dithiocarbamate</td>
<td>12.42 ± 1.70</td>
<td>22.58 ± 5.60</td>
</tr>
</tbody>
</table>
Table 3. IC₅₀ values for inhibition of CYP enzymes by a licorice extract (*G. glabra*) obtained using the single substrate approach or the new cocktail approach and comparison with literature values.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Single substrate IC₅₀ (µg/mL)</th>
<th>Cocktail IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>phenacetin</td>
<td>&gt; 100</td>
<td>83.04 ± 16.96</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>coumarin</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>bupropion</td>
<td>15.59 ± 1.79</td>
<td>19.58 ± 1.80</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>amodiaquine</td>
<td>14.36 ± 2.62</td>
<td>17.06 ± 1.54</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>tolbutamide</td>
<td>8.80 ± 1.20</td>
<td>12.36 ± 1.14</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-mephenytoin</td>
<td>12.80 ± 2.34</td>
<td>19.68 ± 2.70</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>dextromethorphan</td>
<td>73.38 ± 11.72</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>midazolam</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td></td>
<td>testosterone</td>
<td>35.00 ± 4.81</td>
<td>55.64 ± 18.49</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>chlorzoxazone</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>
Figure 1

CYP1A2
m/z 152.2>110.0 (+)
m/z 156.2>114.1 (+)

CYP2A6
m/z 161.1>133.1 (-)
m/z 166.1>138.1 (-)

CYP2B6
m/z 256.2>139.1 (+)
m/z 262.2>139.1 (+)

CYP2C8
m/z 328.1>283.0 (+)
m/z 333.1>283.1 (+)

CYP2C9
m/z 285.1>186.0 (-)
m/z 294.2>186.0 (-)

CYP2C19
m/z 235.1>133.1 (+)
m/z 238.1>133.1 (+)

CYP2D6
m/z 258.1>157.0 (+)
m/z 261.2>157.1 (+)

CYP2E1
m/z 184.0>120.0 (-)
m/z 186.2>122.0 (-)

CYP3A4/5 5β-hydroxytestosterone
m/z 305.20>269.20 (+)
m/z 312.20>276.20 (+)

CYP3A4/5 1'-hydroxymidazolam
m/z 342.1>324.1 (+)
m/z 347.2>329.0 (+)
Figure 2
Figure 3
Figure 4