Simultaneous Screening of Activities of Five Cytochrome P450 and Four Uridine 5’-Diphospho-glucuronosyltransferase Enzymes in Human Liver Microsomes Using Cocktail Incubation and Liquid Chromatography-Tandem Mass Spectrometry

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Running title: Simultaneous Evaluation of P450 and UGT Enzyme Activities

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ABBREVIATIONS:
HLM, human liver microsomes; P450, cytochrome P450; UDPGA, uridine 5'-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase.
ABSTRACT

Cytochrome P450 (P450) and uridine 5'-diphosphoglucuronosyltransferase (UGT) are major metabolizing enzymes in the biotransformation of most drugs. Altered P450 and UGT activities are a potential cause of adverse drug-drug interaction (DDI). A method for the simultaneous evaluation of the activities of five P450s (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A) and four UGTs (UGT1A1, UGT1A4, UGT1A9, and UGT2B7) was developed using in vitro cocktail incubation and tandem mass spectrometry. The nine probe substrates used in this assay were phenacetin (CYP1A2), diclofenac (CYP2C9), S-mephenytoin (CYP2C19), dextromethorphan (CYP2D6), midazolam (CYP3A4), SN-38 (UGT1A1), trifluoperazine (UGT1A4), mycophenolic acid (UGT1A9), and naloxone (UGT2B7). This new method involves incubation of two cocktail doses and single cassette analysis. The two cocktail doses and the concentration of each probe substrate in vitro were determined to minimize mutual drug interactions among substrates. Cocktail A comprised phenacetin, diclofenac, S-mephenytoin, dextromethorphan, and midazolam, while cocktail B comprised SN-38, trifluoperazine, mycophenolic acid, and naloxone. In the incubation study of these cocktails, the reaction mixtures were pooled and simultaneously analyzed using liquid chromatography-tandem mass spectrometry. The method was validated by comparing inhibition data obtained from the incubation of each probe substrate alone with data from the cocktail method. The IC₅₀ value obtained in both cocktail and individual incubations were in agreement with values previously reported in the literature. This cocktail method offers a rapid and robust way to simultaneously evaluate phase I and II enzyme inhibition profiles of many new chemical entities. This new method will also be useful in the drug discovery process and for advancing the mechanistic understanding of drug interactions.
Introduction

Drug–drug interaction (DDI) can cause intense clinical complications, either by increasing the toxicity or by weakening the therapeutic efficacy of drugs. DDIs are one of the major reasons for drug withdrawal from the market (Huang et al., 2008). Therefore, the early detection of DDIs is a critical factor of drug discovery that has led to the development of new screening methods for determining drug interactions. The present guidelines on pharmacokinetics and DDIs from the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) noted that phase I and phase II metabolizing enzymes are of clinical relevance.

Cytochrome P450 (P450) and uridine 5'-diphosphoglucuronosyltransferase (UGT) enzymes are the representative phase I and II enzymes, respectively, which play important roles in the metabolism of most drugs. In practice, P450s are the enzymes involved in the biotransformation of about 75% of all drugs metabolized by phase I enzymes (Guengerich, 2008). UGT enzymes are involved in the biotransformation of about 20 to 30% of all drugs (Meech et al., 2012; Stingl et al., 2014). Several in vitro screening methods for the simultaneous evaluation of potential P450-mediated DDIs have been developed, which include a mixture of P450 probe substrates in a cocktail incubation. The resultant P450-mediated metabolites are determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Kim et al., 2005; Na et al., 2011; Spaggiari et al., 2014). Recently, three screening methods for UGT enzyme activities using cocktail incubation and tandem mass spectrometry also have been reported (Gagez et al., 2012; Joo et al., 2014; Seo et al., 2014). A screening method for the simultaneous evaluation of P450 and UGT enzyme activities would accelerate the evaluation of the DDI potential of new chemical entities during drug development. To date, however, no such method has been developed.
In this study, we report a new screening method that allows the simultaneous evaluation of the activities of five human hepatic P450 and four UGT enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A, UGT1A1, UGT1A4, UGT1A9, and UGT2B7). Their established probe substrates are used for the screening of potential inhibitory interactions of test compounds. We explored the optimal incubation conditions to avoid potential interactions between the cocktail compounds. Furthermore, we developed an analytical method for the simultaneous determination of five P450-specific substrate metabolites and four UGT-specific glucuronide metabolites using LC-MS/MS. To validate our newly developed method, the IC\textsubscript{50} values of known P450 and UGT inhibitors from the new screening method were directly compared with the IC\textsubscript{50} values from the incubation of individual substrates.
Materials and Methods

Chemicals and Reagents. Acetaminophen, alamethicin, α-naphthoflavone, β-nicotinamide adenine dinucleotide phosphate (NADP\(^+\)), dextromethorphan, hecogenin, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), ketoconazole, magnolol, mefenamic acid, miconazole, mycophenolic acid, naloxone, naloxone 3-glucuronide, niflumic acid, phenacetin, quinidine, D-saccharic acid 1,4-lactone monohydrate, sulfaphenazole, trifluoperazine, trizma base, troleandomycin, and UDP-glucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SN-38 was provided by Santa Cruz Biotechnology (Dallas, TX, USA) and atazanavir, S-benzynirvanol, bilirubin, dextrorphan, diclofenac, furafylline, 4-hydroxydiclofenac, 4′-hydroxymephenytoin, 1′-hydroxymidazolam, ketoconazole, S-mephenytoin, midazolam, mycophenolic acid-β-D-glucuronide, SN-38 glucuronide, and omeprazole were obtained from Toronto Research Chemicals (Toronto, ON, Canada). Solvents were LC-MS grade (Fisher Scientific Co., Pittsburgh, PA, USA). Pooled human liver microsomes (HLMs, Catalog No. H2630, Mixed Gender) were purchased from XenoTech (Lenexa, KS, USA).

Microsomal Incubations. All microsomal incubations were done under linear incubation time and protein concentration conditions for the formation of metabolites. Incubations contained either each substrate cocktail set (A set: phenacetin, diclofenac, S-mephenytoin, dextromethorphan, and midazolam; B set: SN-38, trifluoperazine, mycophenolic acid, and naloxone) or an individual substrate. The final concentration of methanol in the cocktail incubation conditions was 1.0\% (v/v) (Easterbrook et al., 2001; Uchaipichat et al., 2004). The incubation mixtures (final volume, 100 μL) for the cocktail A set contained 0.25 mg/mL microsomal protein, 0.1 M potassium phosphate buffer (pH 7.4), and various P450 enzyme-
specific substrates or a substrate cocktail A set. After a 5-min pre-incubation at 37ºC, the reactions were initiated by adding a NADPH-generating system containing 3.3 mM G6P, 1.3 mM β-NADP+, 3.3 mM MgCl₂, and 500 unit/mL G6PDH, and further incubated for 15 min at 37ºC in a thermoshaker (Kim et al., 2005). The incubation mixtures (final volume, 100 μL) for the cocktail B set consisted of 0.25 mg/mL microsomal protein, 25 μg/mL alamethicin, 0.1 M Tris-HCl (pH 7.4), 10 mM MgCl₂, and various UGT enzyme-specific substrates or a substrate cocktail B set (Joo et al., 2014). After pre-incubation on ice for 15 min, the reactions were initiated by the addition of 5 mM UDPGA, and incubated for 1 h at 37ºC. All reactions were terminated by the addition of 50 μL cold acetonitrile containing 5 ng/mL terfenadine (internal standard, IS) to the mixtures, and centrifuging at 10,000 g for 5 min at 4 ºC. The supernatants of the individual incubation samples and pooled cocktail reaction samples (A set/B set, 1/1) were analyzed by LC-MS/MS.

**LC-MS/MS Analysis.** The samples were analyzed using a Thermo Vantage triple quadrupole mass spectrometer coupled with a high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, San Jose, CA, USA). Analytes separation was performed on a Phenomenex Kinetex XB-C18 HPLC column (2.6 μm, 100 Å, 100 mm × 2.10 mm). The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) that formed the following gradient: 0 min (0% A), 0 to 1 min (40% A), 1 to 5 min (50% A), 5 to 5.1 min (0% A), and 5.1 to 8 min (0% A) (Joo et al., 2014). Quantitation was performed by selected reaction monitoring (SRM) of the [M+H]⁺ (or [M–H]⁻) ion and the related product ion for each metabolite, using an IS to establish peak area ratios. The SRM transitions and collision energies determined for each metabolite and IS are listed in Table 1.
**Method Validation.** Four inter- and intra-day validations were performed to validate the LC-MS/MS method for the simultaneous quantification of the five P450 and four UGT probe metabolites in microsomal incubates. Calibration standards were prepared at six different concentrations from 20 to 5,000 nM (acetaminophen, 4-hydroxydiclofenac, 4’-hydroxymephenytoin, trifluoperazine N-glucuronide, and mycophenolic acid glucuronide) or 2 to 500 nM (dextrorphan, 1’-hydroxymidazolam, SN-38 glucuronide, and naloxone 3-glucuronide), in a blank microsomal mixture. Quality control (QC) samples were prepared separately at two different concentrations (5 and 20 nM for dextrorphan, 1’-hydroxymidazolam, SN-38 glucuronide, and naloxone 3-glucuronide; 50 and 200 nM for other metabolites) for assays. Inter- and intra-day precision and accuracy were determined by analyzing replicates of the QC samples ($n=4$).

**Comparison of the Cocktail and Individual Substrates for Inhibition Screening.** Known inhibitors of specific P450 and UGT enzymes ($\alpha$-naphthoflavone for CYP1A2; sulfaphenazole for CYP2C9; S-benzynirvanol for CYP2C19; quinidine for CYP2D6; ketoconazole for CYP3A4; atazanavir for UGT1A1; hecogenin for UGT1A4; niflumic acid UGT1A9; and mefenamic acid for UGT2B7) were incubated with each substrate cocktail set and with the individual substrates alone and the results were compared. The incubations were performed (as described above) with various inhibitor concentrations and all incubations were performed in triplicate. Furafylline, paroxetine and troleandomycin were pre-incubated for 10 min at 37 °C with HLMs and an NADPH-generating system (as described above). The reactions were initiated by the addition of the individual or cocktail substrate. With the exception of the addition of P450- or UGT-isoform-specific inhibitors, all other incubation conditions were as described above.
Data Analysis. The P450- or UGT-isoform-mediated activities in the presence of inhibitors were expressed as percentages of the corresponding control values. The percentage of inhibition was calculated by the ratio of the amounts of metabolites formed, in the presence and absence of the specific inhibitor. To calculate the enzyme inhibition IC₅₀ values, the relevant data were fitted to an inhibitory effect model [i.e. \( v = E_{\text{max}} \times (1 - [I]/(IC_{50} + [I])) \)] using the WinNonlin (Pharsight, Mountain View, CA, USA).
Results

Substrate Selection and Optimization of Microsomal Incubations. The structures of the P450 and UGT probe substrates, their metabolites, and the internal standard used in the assays are shown in Fig. 1. In this study, we selected a P450-isoform specific probe substrate based on the preferred and accepted P450 substrates for assessing activity in vitro (Tucker et al., 2001; Na et al., 2011). The UGT-isoform specific substrates were selected on the basis of their specificity and previously published data (Hanioka et al., 2001; Di Marco et al., 2005; Picard et al., 2005; Uchaipichat et al., 2006; Joo et al., 2014). The probe substrates for the P450 and UGT isoforms were as follows: phenacetin, CYP1A2; diclofenac, CYP2C9; S-mephenytoin, CYP2C19; dextromethorphan, CYP2D6; midazolam, CYP3A; SN-38, UGT1A1; trifluoperazine, UGT1A4; mycophenolic acid, UGT1A9; and naloxone, UGT2B7. The optimum microsomal incubation time was 15 min and 1 hr for phase I (cocktail A set) and phase II (cocktail B set), respectively, with 0.25 mg/mL microsomal protein. The concentration of each probe substrate was optimized to avoid interactions between them: 100 μM for phenacetin, 10 μM for diclofenac, 100 μM for S-mephenytoin, 5 μM for dextromethorphan, 5 μM for midazolam, 0.5 μM for SN-38, 0.5 μM for trifluoperazine, 0.2 μM for mycophenolic acid, and 1 μM for naloxone (Table 1). These concentrations were lower than their respective $K_m$ values.

Development of a Simultaneous Analytical Method using LC-MS/MS. We developed a method for simultaneously analyzing metabolites of five P450 and four UGT probe substrates, using LC-MS/MS. Among the nine metabolites, only mycophenolic acid glucuronide was detected in the negative mode because of its poor ionization property in the positive mode. The remaining eight metabolites and the IS were monitored in the positive mode. The SRM
transitions and optimal MS/MS collision energy are described in Table 1. The representative chromatograms for the five P450 and four UGT probe metabolites and the IS in microsomal incubation mixtures are presented in Fig. 2. As shown in Fig. 2, all the metabolites were eluted in less than 6.5 min and separated into their individual SRM channels. The retention times for acetaminophen, 4-hydroxydiclofenac, 4'-hydroxymephenytoin, dextrorphan, 1'-hydroxymidazolam, SN-38 glucuronide, trifluoperazine N-glucuronide, mycophenolic acid glucuronide, naloxone 3-glucuronide, and terfenadine were approximately 3.3, 6.2, 3.9, 3.4, 3.9, 3.6, 4.8, 4.0, 3.0, and 5.8 min, respectively. For acetaminophen, three peaks were observed in the microsomal incubation. Similar results have also been observed in other reported studies (Joo and Liu, 2013; Pillai et al., 2013; Song et al., 2013). The peak with a retention time of 3.3 min was identified as acetaminophen by comparing it with the retention time of an authentic standard.

**Cocktail Dose Selection.** The inhibition potential of each P450 or UGT substrate was evaluated by comparing the reaction of each metabolite in the single substrate incubations, to the response of the same metabolite formed in incubations with the two-substrate cocktail set. The change in each P450 and UGT enzyme activity level was less than 15% in each cocktail set, compared with the individual incubation (Fig. 3). The relative standard deviations (RSDs) ranged from 2.4–10.3% ($n = 3$) for the results of the cocktail incubation procedure.

**Method Validation.** The inter- and intra-day precision and accuracy of the method were determined by analyzing four QC replicates. The method accuracy was expressed as the percentage of the metabolite concentration measured in each sample relative to the known amount of metabolites added (Joo et al., 2014). Calibration curves produced good correlation
coefficients for nine metabolites in the mixture ($r > 0.999$). The inter-day and intra-day accuracy and precision data for the five P450 and four UGT probe metabolites in human liver microsomal incubates are summarized in Table 2 and 3. Overall, the inter- and intra-day accuracy was 88.2-110.9% with a precision of less than 15.3%.

**Validation of the Screening Method using Selective Inhibitors.** The newly developed screening method used as a tool for determining P450 and UGT inhibition, was validated using known selective inhibitors of the isoforms (α-naphthoflavone, CYP1A2; sulfaphenazole, CYP2C9; S-benzylnirvanol, CYP2C19; quinidine, CYP2D6; ketoconazole, CYP3A; atazanavir, UGT1A1; hecogenin, UGT1A4; niflumic acid, UGT1A9; and mefenamic acid, UGT2B7) with their corresponding substrates. The IC$_{50}$ value of each P450- and UGT-isoform selective inhibitor was estimated in both the individual and cocktail incubations (Table 2). The inhibition curves (Fig. 4) show there was no substantial difference between the individual inhibitor profiles for the two different incubation methods (single vs. cocktail).
Discussion

Pharmacodynamic interactions between drugs alter the response to one or both drugs while also affecting their plasma concentrations (Singh, 2006). Therefore, the risk of metabolism-based DDIs is always a potential problem to consider during the drug development process. For this reason, in vitro drug interaction studies using HLMs are used for the early estimation and prediction of the in vivo drug interaction potential of new candidate drugs (Baranczewski et al., 2006). Several LC-MS/MS-based P450 (Dierks et al., 2001; Kim et al., 2005; Smith et al., 2007; Zambon et al., 2010; Na et al., 2011; Pillai et al., 2013) or UGT (Gagez et al., 2012; Joo et al., 2014; Seo et al., 2014) inhibition methods for the evaluation of in vitro drug interaction potential have been reported. To date, however, a simultaneous evaluation method for both P450 and UGT enzyme activities has not been developed. The aim of this study was to develop and validate a cocktail assay to simultaneously evaluate the activity of hepatic P450 and UGT isoforms in HLMs using LC-MS/MS.

P450 and UGT are representative phase I and II enzymes responsible for the biotransformation of a wide variety of drugs (Evans and Relling, 1999). Among the numerous P450 and UGT enzymes identified to date, five P450 (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A) and four UGT (UGT1A1, UGT1A4, UGT1A9, and UGT2B7) enzymes have been shown to play an important role in the metabolism of marketed drugs. P450 enzymes biotransform about 75% of all drugs, and the above mentioned five P450 human isoforms biotransform about 95% of marketed drugs (Baj-Rossi et al., 2011). In the case of UGT enzymes, UGT2B7 was suggested to be responsible for the hepatic glucuronidation of 40% of drugs, while UGT1A1, 1A4, and 1A9 contribute to additional 47% (Williams et al., 2004; Burchell et al., 2005). Based on these data, we attempted to develop a
method for the simultaneous evaluation of the activities of five P450 (P450s 1A2, 2C9, 2C19, 2D6, and 3A) and four UGT (UGTs 1A1, 1A4, 1A9, and 2B7) enzyme, which are primarily responsible for drug metabolism.

The optimum incubation conditions for the assessment of enzyme activities are different between the P450 and UGT enzymes. The microsomal incubation of P450 enzymes was conducted in phosphate buffer, whereas UGT enzymes were incubated in Tris-HCl buffer. UGT enzyme activity was assessed in the presence of alamethicin, a membrane permeabilizing agent (Fisher et al., 2000) and saccharolactone, a β-glucuronidase inhibitor (Oleson and Court, 2008) using a longer incubation time (1 hr). In addition, UDPGA also has inhibitory potential against CYP2C9 and CYP2C19 activity to some degree (Yan and Caldwell, 2003). Therefore, we used two separate microsomal incubations with P450 and UGT probe substrates. The substrates were divided into two cocktail groups (cocktail A for five P450 probe substrates and cocktail B for four UGT probe substrates). These two cocktail mixtures were pooled after incubation and analyzed together using LC-MS/MS to reduce the assay time.

The selection of specific probe substrates for each P450 and UGT enzyme is important because multiple enzymes are involved in the metabolism of a single drug. Therefore, in this study we chose specific probe substrates for the nine major P450 and UGT enzymes in the human liver based on previous reports (Table 1). Phenacetin, dextromethorphan, and midazolam are the most frequently employed probe substrate for in vitro activity assessment of CYP1A2, CYP2D6, and CYP3A enzymes, respectively, using the cocktail approach, and are also the preferred probes for regulatory authorities (Kim et al., 2005; Otten et al., 2011; Pillai et al., 2013). S-Mephenytoin (Dierks et al., 2001; Kim et al., 2005; Smith et al., 2007; Zambon et al., 2010; Otten et al., 2011; Pillai et al., 2013) and omeprazole (Testino and
Patonay, 2003; He et al., 2007; Tolonen et al., 2007; Song et al., 2013) are used as CYP2C19 substrates in cocktail incubation studies. In our preliminary study, omeprazole (20 μM) inhibited CYP3A-mediated midazolam hydroxylase activity (40% of control) and CYP1A2-mediated phenacetin O-deethylation activity (>20% of control) (Supplemental Figure 1).

Diclofenac (Dierks et al., 2001; Smith et al., 2007; Pillai et al., 2013) and tolbutamide (Bu et al., 2001; Testino and Patonay, 2003; Kim et al., 2005; He et al., 2007; Tolonen et al., 2007; Otten et al., 2011; Joo and Liu, 2013) are generally used as CYP2C9 probe substrates. In this study, diclofenac was selected as the CYP2C9 probe substrate, because it showed higher detection intensity than tolbutamide in the proposed LC-MS/MS method. Based our preliminary findings, phenacetin, diclofenac, S-mephenytoin, dextromethorphan, and midazolam were selected as the CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A probe substrates, respectively (cocktail A set). For the cocktail B set (UGT enzymes) SN-38, trifluoperazine, mycophenolic acid, and naloxone were selected as probe substrates for UGT1A1, UGT1A4, UGT1A9, and UGT2B7, respectively, based on previously reported data (Uchaipichat et al., 2004; Di Marco et al., 2005; Picard et al., 2005) and our recently published data (Joo et al., 2014).

Following the selection of the P450 and UGT probe substrates for the cocktail incubation study, the potential metabolic interactions among the five P450 metabolites and four UGT metabolites of the selected probe substrates were evaluated. The simultaneous incubation of five or four substrates may lead to interactions. As shown in Fig. 3, P450 and UGT enzyme activities in the cocktail incubations were in accordance with the results from individual incubations, suggesting that drug interactions among probe substrates in the present cocktail set were negligible.

P450 and UGT-isoform selective inhibitors represent the most powerful tools available for
metabolizing enzyme phenotyping. The well-known P450 and UGT inhibitors include α-naphthoflavone for CYP1A2 (von Moltke et al., 1996), sulfaphenazole for CYP2C9 (Khojasteh et al., 2011), S-benzylirivanol for CYP2C19 (Suzuki et al., 2002), quinidine for CYP2D6 (Khojasteh et al., 2011), ketoconazole for CYP3A (Khojasteh et al., 2011), hecogenin for UGT1A4 (Uchaipichat et al., 2006), and niflumic acid for UGT1A9 (Miners et al., 2011). Atazanavir (Zhang et al., 2005) and mefenamic acid (Mano et al., 2007) are known inhibitors of UGT1A1 and UGT2B7, respectively. Our results demonstrated that α-naphthoflavone, sulfaphenazole, S-benzylirivanol, quinidine, hecogenin, and niflumic acid strongly and selectively inhibited CYP1A2, CYP2C9, CYP2C19, CYP2D6, UGT1A4, and UGT1A9, respectively (Table 3 and Fig. 5). Liu et al. (2011) also reported that selective P450 inhibitors such as α-naphthoflavone, sulfaphenazole, and quinidine have negligible inhibitory potential against UGT isoforms. Ketoconazole also selectively inhibits CYP3A with an IC₅₀ value of 0.03 μM, which is at least 100-fold more selective compared to its effects against all other P450s and UGTs tested (Fig. 5E) (Ren et al., 2013). The inhibitory potential of ketoconazole on UGT1A1-mediated SN-38 glucuronidation (IC₅₀ = 4.8 μM) concurred with previous findings in which ketoconazole had inhibitory potential on UGT1A1-catalyzed β-estradiol glucuronidation (IC₅₀ = 4.1 μM) (Liu et al., 2011). Hecogenin and niflumic acid are UGT1A4 and UGT1A9 selective inhibitor, respectively, and they have negligible inhibitory effect on the P450 isoforms tested (IC₅₀ > 20 μM, Table 3). Atazanavir strongly inhibited UGT1A1 activity with an IC₅₀ value of 0.4 μM, however it also inhibited CYP3A activity (IC₅₀ = 1.8 μM, Fig. 5F). Mefenamic acid inhibited UGT2B7 activity (IC₅₀ = 5.0 μM), but also weakly inhibited CYP1A2 and CYP2C9 activity with IC₅₀ values of 8.3 and 12 μM, respectively (Fig. 5I).

We also evaluated the IC₅₀ values of the characterized inhibitors for each P450 and UGT
enzymes using both the individual and the cocktail substrates (Fig. 4). The IC\textsubscript{50} value of each cocktail set using this approach was comparable to those of the individual substrates and were in agreement with those previously reported (Table 2). This confirms that the IC\textsubscript{50} values of P450 and UGT inhibitors can be accurately determined using the cocktail assay instead of individual substrate incubations, which would save considerable time in the screening process for new chemical entities. One exception observed was in the inhibition of CYP2C19 by S-benzynirvanol, which showed a slight difference compared with previously published values. The published IC\textsubscript{50} value of 0.41 \textmu M for S-benzynirvanol against CYP2C19-mediated S-mephenytoin hydroxylaiton activity (Walsky and Obach, 2003), was lower than our present data (1.2 \textmu M, Table 2). Such a difference is not unusual and could be due to the use of different microsomal incubation conditions (Dierks et al., 2001). Nonetheless, our results demonstrate that the IC\textsubscript{50} values of inhibitors against the nine enzymes can be determined accurately by cocktail incubation and cassette analysis thereby reducing the time required.

In conclusion, we developed an LC-MS/MS method for the simultaneous determination of five P450 and four UGT enzyme activities. Nine substrates were divided into two cocktail sets for incubation and pooled for LC-MS/MS analysis in a single run. This process allowed us to simultaneously evaluate the activity of five P450 and four UGT enzyme activities, using well-known P450- and UGT-isoform specific inhibitors. In addition, this cocktail method involving multiple substrates, provided inhibition profiles similar to those obtained from single substrate incubations. Therefore, these results suggest that this newly developed assay can be a useful tool for robust and rapid screening, which accelerates the prediction of the P450 and UGT inhibitory potential of new chemical entities.
**Authorship Contributions**

Participated in research design: B Lee, T Lee, Liu

Conducted experiments: B Lee, Ji, Liu

Contributed new reagents or analytic tools: B Lee, Liu

Performed data analysis: B Lee, T Lee, Liu

Wrote or contributed to the writing of the manuscript: B Lee, T Lee, Liu
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solvents, and inhibition by diclofenac and probenecid. Drug Metab Dispos 32:413-423.


Footnotes

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Figure legends.

**Fig. 1.** Structures of P450 and UGT-isoform specific probe substrates their metabolites, and internal standard (IS).

**Fig. 2.** Representative selected reaction monitoring chromatograms of P450- and UGT-mediated metabolites of probe substrate and internal standard: Acetaminophen (A), 4-hydroxydiclofenac (B), 4′-hydroxymephenytoin (C), dextrorphan (D), 1′-hydroxymidazolam (E), SN-38 glucuronide (F), trifluoperazine N-glucuronide (G), mycophenolic acid glucuronide (H), naloxone 3-glucuronide (I), and terfenadine (internal standard, J).

**Fig. 3.** Comparison of the results from the cocktail incubation with the individual incubations for each of five P450 (A) and four UGT (B) enzyme activities. Incubations were performed with the individual substrate (■), and each substrate cocktail set (□) in pooled human liver microsomes (HLMs). The activities are the means of triplicate incubations.

**Fig. 4.** Inhibition curves determined using individual substrates and the substrate cocktails. Each P450- and UGT-selective inhibitor was incubated in a separate experiment with cocktail substrates (●) or an individual substrate set (○). The activity is expressed as the percentage of the remaining activity compared with a control sample containing no inhibitor. Inhibition of (A) phenacetin O-deethylation by α-naphthoflavone; (B) diclofenac 4-hydroxylation by sulfaphenazole; (C) S-mephenytoin 4′-hydroxylation by S-benzynirvanol; (D) dextromethorphan O-demethylation by quinidine; (E) midazolam 1′-hydroxylation by ketoconazole, (F) SN-38 glucuronidation by atazanavir; (G) trifluoperazine N-glucuronidation by hecogenin; (H) mycophenolic acid glucuronidation by niflumic acid; and (I) naloxone 3-glucuronidation by mefenamic acid.
Fig. 5. Inhibitory effects of (A) α-naphthoflavone, (B) sulfaphenazole, (C) S-benzynirvanol, (D) quinidine, (E) ketoconazole, (F) atazanavir, (G) hecogenin, (H) niflumic acid, and (I) mefenamic acid on phenacetin O-deethylation (●), diclofenac 4-hydroxylation (○), S-mephenytoin 4′-hydroxylation (▼), dextromethorphan O-demethylation (▲), midazolam 1′-hydroxylation (■), SN-38 glucuronidation (□), trifluoperazine N-glucuronidation (◆), mycophenolic acid glucuronidation (◇), and naloxone 3-glucuronidation (▲) on incubation with pooled human liver microsomes (HLMs). Data are the means of triplicate experiments.
Table 1. Selected reaction monitoring (SRM) conditions for the major metabolites of the nine substrates used in all assays.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Conc. (μM)</th>
<th>Metabolite</th>
<th>Transition (m/z)</th>
<th>Polarity</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>100</td>
<td>Acetaminophen</td>
<td>152 &gt; 110</td>
<td>ESI⁺</td>
<td>25</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>10</td>
<td>4-Hydroxydiclofenac</td>
<td>312 &gt; 231</td>
<td>ESI⁺</td>
<td>23</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Mephenytoin</td>
<td>100</td>
<td>4'-Hydroxymephenytoin</td>
<td>235 &gt; 150</td>
<td>ESI⁺</td>
<td>27</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>5</td>
<td>Dextrophan</td>
<td>258 &gt; 157</td>
<td>ESI⁺</td>
<td>50</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Midazolam</td>
<td>5</td>
<td>1'-Hydroxymidazolam</td>
<td>342 &gt; 203</td>
<td>ESI⁺</td>
<td>25</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>SN-38</td>
<td>0.5</td>
<td>SN-38-glucuronide</td>
<td>569 &gt; 393.4</td>
<td>ESI⁺</td>
<td>30</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Trifluoperazine</td>
<td>0.5</td>
<td>Trifluoperazine N-glucuronide</td>
<td>584.5 &gt; 408.5</td>
<td>ESI⁺</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Mycophenolic acid</td>
<td>0.2</td>
<td>Mycophenolic acid-glucuronide</td>
<td>495 &gt; 319</td>
<td>ESI⁺</td>
<td>25</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>Naloxone</td>
<td>1</td>
<td>Naloxone 3-glucuronide</td>
<td>504 &gt; 310</td>
<td>ESI⁺</td>
<td>30</td>
</tr>
<tr>
<td>IS</td>
<td>Terfenadine</td>
<td></td>
<td></td>
<td>472 &gt; 436</td>
<td>ESI⁺</td>
<td>25</td>
</tr>
</tbody>
</table>

Conc., concentration; IS, internal standard
Table 2. Intra-day \((n=4)\) validation data for the simultaneous determination of five P450 and four UGT-specific probe metabolites in human liver microsomal incubations using the LC-MS/MS method.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>50 nM</th>
<th></th>
<th>200 nM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. found</td>
<td>Accuracy</td>
<td>RSD</td>
<td>Conc. found</td>
</tr>
<tr>
<td></td>
<td>(nM)(^b)</td>
<td>(%)</td>
<td>(%)</td>
<td>(nM)(^b)</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>49.3</td>
<td>98.6</td>
<td>8.1</td>
<td>202.1</td>
</tr>
<tr>
<td>4-Hydroxydiclofenac</td>
<td>45.8</td>
<td>91.7</td>
<td>6.4</td>
<td>182.0</td>
</tr>
<tr>
<td>4’-Hydroxymephenytoin</td>
<td>51.5</td>
<td>103.0</td>
<td>13.4</td>
<td>202.9</td>
</tr>
<tr>
<td>Dextrophan(^a)</td>
<td>4.41</td>
<td>88.2</td>
<td>15.3</td>
<td>20.0</td>
</tr>
<tr>
<td>1’-Hydroxymidazolam(^a)</td>
<td>4.85</td>
<td>97.1</td>
<td>7.2</td>
<td>19.2</td>
</tr>
<tr>
<td>SN-38 glucuronide(^a)</td>
<td>5.30</td>
<td>106.0</td>
<td>10.8</td>
<td>19.1</td>
</tr>
<tr>
<td>Trifluoperazine (N)-glucuronide</td>
<td>45.4</td>
<td>90.8</td>
<td>1.9</td>
<td>179.9</td>
</tr>
<tr>
<td>Mycophenolic acid glucuronide</td>
<td>47.2</td>
<td>94.4</td>
<td>6.3</td>
<td>182.5</td>
</tr>
<tr>
<td>Naloxone 3-glucuronide(^a)</td>
<td>5.55</td>
<td>110.9</td>
<td>3.8</td>
<td>19.4</td>
</tr>
</tbody>
</table>

\(^a\)Dextrophan, 1’-hydroxymidazolam, SN-38 glucuronide, and naloxone 3-glucuronide concentrations were 5 and 20 nM.

\(^b\)Each value represents the mean of four replicates

Intra-day accuracy and precision were determined at two different concentration levels \((n = 4)\).
**Table 3.** Inter-day \((n = 4)\) validation data for the simultaneous determination of five P450 and four UGT-specific probe metabolites in human liver microsomal incubations using LC-MS/MS method

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>50 nM</th>
<th>200 nM</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. found (nM)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Accuracy (%)</td>
<td>RSD (%)</td>
<td>Conc. found (nM)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Accuracy (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>52.0</td>
<td>104.1</td>
<td>4.9</td>
<td>202.0</td>
<td>101.0</td>
<td>3.0</td>
</tr>
<tr>
<td>4-Hydroxydicolfenac</td>
<td>50.1</td>
<td>100.1</td>
<td>9.6</td>
<td>198.5</td>
<td>99.2</td>
<td>8.4</td>
</tr>
<tr>
<td>4’-Hydroxymephenytoin</td>
<td>51.3</td>
<td>102.7</td>
<td>3.4</td>
<td>203.8</td>
<td>101.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Dextrophan&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.61</td>
<td>92.1</td>
<td>8.5</td>
<td>20.6</td>
<td>103.0</td>
<td>7.1</td>
</tr>
<tr>
<td>1’-Hydroxymidazolam&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.35</td>
<td>107.0</td>
<td>5.7</td>
<td>20.2</td>
<td>100.7</td>
<td>8.7</td>
</tr>
<tr>
<td>SN-38 glucuronide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.81</td>
<td>96.2</td>
<td>9.9</td>
<td>19.9</td>
<td>99.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Trifluoperazine N-glucuronide</td>
<td>50.7</td>
<td>101.5</td>
<td>3.3</td>
<td>201.4</td>
<td>100.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Mycophenolic acid glucuronide</td>
<td>46.0</td>
<td>92.9</td>
<td>8.0</td>
<td>188.8</td>
<td>94.4</td>
<td>4.2</td>
</tr>
<tr>
<td>Naloxone 3-glucuronide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.46</td>
<td>109.2</td>
<td>4.9</td>
<td>20.2</td>
<td>101.0</td>
<td>3.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dextrophan, 1’-hydroxymidazolam, SN-38 glucuronide, and naloxone 3-glucuronide concentrations were 5 and 20 nM.

<sup>b</sup>Each value represents the mean of four replicates.

Inter-day accuracy and precision were determined at two different concentration levels \((n = 4)\).
Table 4. Comparison of IC$_{50}$ values estimated using the individual substrate and the substrate cocktails of well-known P450- and UGT-selective inhibitors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Inhibitors</th>
<th>IC$_{50}$ (μM±SD)$^a$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Individual substrate</td>
<td>Cocktail substrate</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>α-Naphthoflavone</td>
<td>0.014 ± 0.003</td>
<td>0.013 ± 0.004</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>Sulfaphenazole</td>
<td>0.70 ± 0.08</td>
<td>0.68 ± 0.08</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Mephytoin</td>
<td>S-Benzynirvanol</td>
<td>1.02 ± 0.13</td>
<td>1.20 ± 0.06</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Quinidine</td>
<td>0.11 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Midazolam</td>
<td>Ketoconazole</td>
<td>0.038 ± 0.001</td>
<td>0.032 ± 0.0004</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>SN-38</td>
<td>Atazanavir</td>
<td>0.37 ± 0.11</td>
<td>0.38 ± 0.13</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Trifluoperazine</td>
<td>Hecogenin</td>
<td>0.89 ± 0.21</td>
<td>0.86 ± 0.11</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Mycophenolic acid</td>
<td>Niflumic acid</td>
<td>0.78 ± 0.10</td>
<td>0.83 ± 0.18</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>Naloxone</td>
<td>Mefenamic acid</td>
<td>4.18 ± 1.01</td>
<td>5.04 ± 1.34</td>
</tr>
</tbody>
</table>

$^a$Values represent the mean (±SD) of experiments performed in triplicate.

Inhibitors of P450 and UGT enzymes were incubated with each substrates cocktail set and with individual substrate alone. IC$_{50}$ values were calculated using a nonlinear least-squares regression analysis.
Table 5. Effects of specific enzyme inhibitors on cytochrome P450 and uridine 5’-diphosphoglucuronosyltransferase metabolic activities in pooled human liver microsomes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitors</th>
<th>P450s</th>
<th>UGTs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1A2</td>
<td>2C9</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>α-Naphthoflavone</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Sulfaphenazole</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Benzylnirvanol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Quinidine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Ketoconazole</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>Atazanavir</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Hecogenin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Niflumic acid</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>Mefenamic acid</td>
<td>8.3</td>
<td>12</td>
</tr>
</tbody>
</table>

*Values represent the mean of experiments performed in triplicate.

‘-’ means IC₅₀ > 50 μM.
Fig. 1

Phenacetin $\rightarrow$ CYP 1A2 $\rightarrow$ Acetaminophen

Diclofenac $\rightarrow$ CYP 2C9 $\rightarrow$ 4-Hydroxydiclofenac

(5)-Mephentoin $\rightarrow$ CYP 2C19 $\rightarrow$ 4'-Hydroxymephentoin

Dextromethorphan $\rightarrow$ CYP 2D6 $\rightarrow$ Dextrophan

Midazolam $\rightarrow$ CYP 3A $\rightarrow$ 1'-Hydroxymidazolam

SN-38 $\rightarrow$ UGT1A1 $\rightarrow$ SN-38-glucuronide

Trifluoperazine $\rightarrow$ UGT1A4 $\rightarrow$ Trifluoperazine-N-glucuronide

Mycophenolic acid $\rightarrow$ UGT1A9 $\rightarrow$ Mycophenolic acid glucuronide

Naloxone $\rightarrow$ UGT2B7 $\rightarrow$ Naloxone glucuronide

Terfenadine (B)

Fig. 2

(A) Acetaminophen

(B) 4-Hydroxydiclofenac

(C) 4'-Hydroxymephenytoin

(D) Dextrorphan

(E) 1'-Hydroxymidazolam

(F) SN-38-glucuronide

(G) Trifluoperazine N-glucuronide

(H) Mycophenolic acid-glucuronide

(I) Naloxone 3-glucuronide

(J) Terfenadine (IS)
Fig. 3
Fig. 4

(A) CYP1A2

(B) CYP2C9

(C) CYP2C19

(D) CYP2D6

(E) CYP3A

(F) UGT1A1

(G) UGT1A4

(H) UGT1A9

(I) UGT2B7
Fig. 5

(A) CYP1A2  
(B) CYP2C9  
(C) CYP2C19

(D) CYP2D6  
(E) CYP3A  
(F) UGT1A1

(G) UGT1A4  
(H) UGT1A9  
(I) UGT2B7

% of Control activity

- α-Naphthoflavone (μM)  
- Sulfaphenazole (μM)  
- S-Benzylmirvanol (μM)  
- Quinidine (μM)  
- Ketoconazole (μM)  
- Atazanavir (μM)  
- Hecogenin (μM)  
- Niflumic acid (μM)  
- Mefenamic acid (μM)

- CYP 1A2  
- CYP 2C9  
- CYP 2C19  
- CYP 2D6  
- CYP 3A  
- UGT 1A1  
- UGT 1A4  
- UGT 1A9  
- UGT 2B7