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Validated liquid chromatography–tandem mass spectrometry method for determination of totally nine probe metabolites of cytochrome P450 enzymes and UDP-glucuronosyltransferases

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ABSTRACT

A simplified, rapid, and selective liquid chromatography–tandem mass spectrometry method for the determination of the activities of cytochrome P450 (CYP) enzymes and UDP-glucuronosyltransferases (UGTs) in two separate settings was developed and successfully applied to 8 CYP isoenzymes and UGT2B7 enzyme activities in rat liver microsomes.

The triple-quadrupole mass spectrometric detection was operated in positive mode for the probe metabolites: CYP1A2 (resorufin), CYP2B6 (hydroxybupropion), CYP2C19 (5-hydroxyomeprazole), CYP2D6 (dextrophan), CYP3A4 (6β-hydroxytestosterone), and UGT2B7 (morphine-3-glucuronide); also in negative mode for CYP2C9 (4-hydroxytolbutamide), CYP2E1 (6-hydroxychloroxazone), and CYP4A (hydroxylauric acid). The metabolic reactions were terminated with acetonitrile, containing metoprolol and acetaminophen as the internal standard for positive and negative ion electrospray ionization, respectively.

The method was validated over the concentration range of 25–2500 ng/mL for 5-hydroxyomeprazole, dextrophan, hydroxylauric acid, and morphine-3-glucuronide; 5–500 ng/mL for resorufin; 3–300 ng/mL for hydroxybupropion; 10–1000 ng/mL for 4-hydroxytolbutamide; 40–4000 ng/mL for 6-hydroxychloroxazone; and 63–6300 ng/mL for 6β-hydroxytestosterone. All of the extraction recoveries of these analytes were greater than 85%, except for hydroxylauric acid at mid-concentration with a recovery of 83.2% ± 3.2%. The matrix effects were between 85.8% and 119.9%; the respective within- and between-run precisions were 0.9–12.0% and 2.0–13.9%; and the within- and between-run accuracy levels were 0.6–17.2% and 0.1–15.1%, respectively, for all these analytes. All of the analytes were stable during the assay and storage in the liver microsomes of Sprague-Dawley rats.

The measurement activity of multiple enzymes was feasible using a cocktail approach. This method proved to be a robust, fast, accurate, specific and sensitive assay, and was successfully used to investigate *in vivo* enzyme activities of 8 major CYP isoenzymes and UGT2B7 in Sprague-Dawley rats with fatty livers. By the end of the eighth week, the CD-fed induced fatty liver rats showed a significant decrease in the activities of CYP1A2 and UGT2B7 as compared to the standard diet group.

Abbreviations: CD, Choline-deficient; CYP, Cytochrome P450; ESI, Electrospray ionization; FDA, Food and Drug Administration; IACUC, Institutional Animal Care and Use Committee; IS, Internal standard; HPLC, High-performance liquid chromatography; LC-MS/MS, Liquid chromatography–tandem mass spectrometry; LLOQ, Lower limit of quantification; MRM, Multiple reaction monitoring; NASH, Nonalcoholic steatohepatitis; QC, Quality control; RE, Relative error; RLM, Rat liver microsomes; RSD, Relative standard deviation; SD, Sprague-Dawley; UGT2B7, UDP-glucuronosyltransferases 2B7

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1. Introduction

Cytochrome P450 (CYP) is involved in the metabolization of drugs, chemicals, and endogenous substrates. Drug metabolism is widely impaired in patients with liver diseases, particularly through the changes of CYP isoenzymes [1]. The content or activity of CYP1A, CYP 2C8/9/19, CYP2D6, CYP 2E1, and CYP 3A4 appears to be particularly vulnerable to the effects of liver disease [2–4]. A strong relationship between the activities of CYP isoenzymes and the severity of cirrhosis has been demonstrated [5]. Possible alterations of CYP isoenzymes activities in patients with fatty liver disease have not been studied extensively. Having appropriate detection ability is essential for investigating the possible alterations of CYP isoenzymes in these patients.

Several methods have already been applied to determine the activities of CYP isoenzymes in hepatic microsomes: radioactivity [6], fluorescence [6,7], high-performance liquid chromatography (HPLC) [8], gas chromatography/mass spectrometry (GC/MS) [6], liquid chromatography–mass or -tandem mass spectrometry (LC–MS, LC–MS/MS) [9–12], and monoclonal anti-CYP antibodies [9,10]. Approximately 97% of drugs in Phase I metabolism has been metabolized using P450 CYP1A, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 [13]. However, a study on *Cyp2e1*$^{-/-}$ mice with nonalcoholic steatohepatitis (NASH) indicated that CYP4A10 and CYP4A14 were up-regulated [14]. Similar to CYP2E1, CYP4A enzymes play an important role in the metabolism of various endogenous lipid substrates, such as fatty acid and arachidonic acid [15,16]. The present published literature excludes CYP4A from almost all published methods that focus on the determination of the activities of Phase I metabolic enzymes in liver microsomes [9–12].

The major metabolism of drugs undergoes both Phase I and Phase II biotransformation. The method for determining the activities of the Phase II metabolic enzymes is limited, unlike that of Cytochrome P450 reactions. Glucuronic acid conjugation catalyzed by UDP-glucuronosyltransferases (UGTs) is one of the major Phase II metabolic processes [17]. CYP isoenzymes and UGTs enzymes both play important roles in the absorption, distribution, metabolism, and excretion profile for most drugs, such as xenobiotics and endogenous compounds [18]. Simultaneous determination of the enzyme activities of CYPs and UGTs for Phase I and Phase II metabolism is frequently required in human metabolism studies. Morphine-3-glucuronide, the probe metabolite of UGT2B7 enzyme, was chosen in this study because glucuronidation is a major pathway for the elimination of morphine. Morphine is metabolized to 3-glucuronide (90%) and 6-glucuronide (10%) mainly by UGT2B7 [19]. Among all 15 enzymes, UGT2B7 is more active in the liver, whereas the other 14 enzymes show more activity in extrahepatic organs [13].

In this study, we selected 7 probe substrates for CYP isoenzymes based on a representative list of preferred and acceptable *in vitro* probe substrates recommended by the Food and Drug Administration (FDA) of the United States [20]. These probe substrates are hydroxybupropion (CYP 2B6), 4-hydroxytolbutamide (CYP 2C9), dextrophan (CYP 2D6), 6-hydroxychloroxazone (CYP 2E1), 6β-hydroxytestosterone (CYP 3A4), resorufin (CYP 1A2), and 5-hydroxyomeprazole (CYP 2C19). The probe substrates for CYP4A and UGT2B7 are hydroxylauric acid [21] and morphine-3-glucuronide [19,22], which was selected in accordance with published studies.

No study has reported the simultaneous determination of the activities of CYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, 4A, and UGT2B7 in the liver microsomes of Sprague-Dawley (SD) rats with fatty livers by using LC-MS/MS. The purpose of this work was to develop a LC-MS/MS method that could be used to assess the alteration activities of these 9 isoenzymes in SD rats with fatty livers. In this paper, a fully validated, accurate, and sensitive LC-MS/MS method was established and successfully applied to determine *in vivo* the activities of 8 major CYP isoenzymes of Phase I and UGT2B7 of Phase II enzyme in rat liver microsomes. SD rats were placed on a choline-deficient (CD) diet for 8 weeks in accordance with a fatty liver model.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN) and methanol (MeOH), both of HPLC grade, were obtained from Tedia Company, Inc. (OH, USA). Water was prepared using the Milli-Q water purification system (Millipore, Bedford, MA, USA). Standard morphine and morphine-3-glucuronide were purchased from Cerillant Corp. (TX, USA). Losec (ompeprazole) and 6-hydroxychlorzoxazone were purchased from Astra Zeneca (Mölndal, Sweden) and Toronto Research Chemicals Inc. (ONT, Canada), respectively. Astra Zeneca (Mölndal, Sweden) generously donated the 5-hydroxyomeprazole. All other chemicals were obtained from Sigma-Aldrich (MO, USA). All these standard compounds possessed purity greater than 99%.

2.2. Rat liver microsomes (RLM)

The RLM samples used in this study were prepared based on the Testino et al. [23] study, with minor modifications. The rats were sacrificed and the livers were immediately harvested. The RLM samples were prepared using the following differential ultracentrifugation method: The liver tissue was minced and rinsed in a 1.15% (w/v) KCl solution. The tissue was weighed, washed in 2 volumes of 1.15% KCl and then homogenized in a motorized homogenizer (American Laboratory Trading, USA) with two 20 s bursts. The homogenate was centrifuged at approximately 9000 rpm for 20 min at 4 °C. The supernatant was transferred to fresh centrifuge tubes and then centrifuged at approximately 40,000 rpm for 2 h at 4 °C. The pellet was washed once in 1.15% KCl. The resulting pellet was resuspended in 1.5 volume of 0.1 M PBS (pH 7.4) by a tissue grinder, aliquoted (1 ml) into an eppendorf and stored at −80 °C until use. The RLM protein was assayed using Lowry et al.'s method [24].

2.3. Calibration standards and quality control samples

The stock solution of each metabolite of the probe substrate prepared was 100 μg/mL in MeOH; dextrophan was prepared at the same concentration in water, except for 6β-hydroxytestosterone, which was prepared at a concentration of 900 μg/mL in MeOH. All stock solutions were stored at −20 °C and protected from light. They were stable for at least 180 days. Standard working solutions for positive ion electrospray ionization (ESI) were made from 2 separate solutions. Resorufin (5 μg/mL), 6β-hydroxytestosterone (63 μg/mL), hydroxybupropion (3 μg/mL), dextrophan (25 μg/mL), and morphine-3-glucuronide (25 μg/mL) were prepared in MeOH: H_2O (50:50, v/v), and 5-hydroxyomeprazole (25 μg/mL) was prepared in MeOH. These 6 standard metabolites were serially diluted from working solutions with MeOH: H_2O (50:50, v/v). Standard working solutions for negative ion ESI [hydroxylauric acid (10 μg/mL), 4-hydroxytolbutamide (40 μg/mL), and 6-hydroxychloroxazone (25 μg/mL)] were prepared and diluted in ACN: H_2O (70:30, v/v).

Each RLM incubation mixture (500 μL) contained a 2 mg/mL protein concentration of RLM, 0.1 M PBS (pH 7.4), 2 mM $MgCl_2$, 5 μg/mL alamethicin, a NADPH regeneration system (2 mM $NADP^+$, 20 mM glucose-6-phosphate, and 4 U/mL glucose-6-phosphate dehydrogenase), and 2 mM UDPGA. Standard calibration and quality control (QC) samples were prepared by adding a 50 μL working

solution of each metabolite of the probe substrate in blank RLM incubation mixtures.

Calibration curves were made for 6 different concentrations and QC samples containing 4 different concentrations. Pooled (6-in-1) calibration standards of positive ion ESI containing a mixture of each analyte were fixed at concentrations of 5, 12.5, 50, 100, 250, and 500 ng/mL for resorufin; 63, 157.5, 630, 1260, 3150, and 6300 ng/mL for 6β-hydroxytestosterone; 3, 7.5, 30, 60, 150, and 300 ng/mL for hydroxybupropion; and 25, 62.5, 250, 500, 1250, and 2500 ng/mL for 5-hydroxyomeprazole, dextrophan, and morphine-3-glucuronide. Furthermore, pooled (3-in-1) calibration standards of negative ion ESI containing the mixture of each analyte were prescribed at concentrations of 10, 25, 100, 200, 500, and 1000 ng/mL for 4-hydroxytolbutamide; 40, 100, 400, 800, 2000, and 4000 ng/mL for 6-hydroxychloroxazone; and 25, 62.5, 250, 500, 1250, and 2500 ng/mL for hydroxylauric acid.

Pooled (6-in-1) QC samples of the positive ion ESI samples were prepared at the lower limit of quantification (LLOQ) with low, middle, and high concentrations, which were set at 5, 15, 200, and 400 ng/mL for resorufin; 63, 189, 2520, and 5040 ng/mL for 6β-hydroxytestosterone; 3, 9, 120, and 240 ng/mL for hydroxybupropion; and 25, 75, 1000, and 2000 ng/mL for 5-hydroxyomeprazole, dextrophan, and morphine-3-glucuronide. Pooled (3-in-1) QC samples of the negative ion ESI samples were prepared at 10, 30, 400, and 800 ng/mL for 4-hydroxytolbutamide; 40, 120, 1600, and 3200 ng/mL for 6-hydroxychloroxazone; and 25, 75, 1000, and 2000 ng/mL for hydroxylauric acid.

2.4. RLM incubation and sample preparation

RLM incubations were done separately with a positive cocktail [pooled (6-in-1)] and a negative cocktail [pooled (3-in-1)]. A description of the concentrations of the calibration standards and quality control samples used in the cocktails are detailed in Section 2.3. The reaction mixture was incubated at 37 °C for 30 min in a shaking incubator block. The reactions were terminated with 500 μL of ACN containing 160 ng/mL metoprolol (for positive ion ESI) or 1.6 μg/mL acetaminophen (for negative ion ESI) as an IS. The samples were vortexed briefly, placed on ice, and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant (100 μL) was then evaporated until dry under a N_2 stream, reconstituted with 100 μL of HPLC mobile phase and vortexed for 30 s. Finally, the solution was transferred to autosampler vials, and 5 μL was injected into the LC-MS/MS system.

2.5. Instruments

2.5.1. Liquid chromatography

The HPLC system consisted of a pump (SHIMADZU LC-10AD*VP*, Japan) and an autosampler (SHIMADZU SIL-10AD*VP*, Japan). A generic positive ion ESI method was used for resorufin, 6β-hydroxytestosterone, hydroxybupropion, 5-hydroxyomeprazole, dextrophan, morphine-3-glucuronide, and metoprolol (internal standard; IS). Samples (5 μL) were injected onto a Waters X Bridge® phenyl column (3.0×100 mm^2, i.d., 5 μm particle size) with an on-line precolumn filter and a column temperature of 40 °C. The mobile phase consisting of MeOH: H_2O: formic acid (70:30:0.2, v/v/v) was used. The flow rate was set at 0.3 mL/min. The runtime was 5 min. The generic negative ion ESI method was used for hydroxylauric acid, 4-hydroxytolbutamide, 6-hydroxychloroxazone, and acetaminophen (IS). Samples (5 μL) were injected onto a Waters X Bridge® phenyl column (3.0×50 mm, i.d., 5 μm particle size) and maintained at 40 °C. The mobile phase consisted of Solvent A (0.1% formic acid in water) and Solvent B (0.1% formic acid in ACN). The 12.5 min gradient was as follows: 90% A (0–0.5 min), 15% A (0.5–5 min), 15% A (5–7.5 min), 90% A (7.5–7.6 min), and 90% A (7.6–12.5 min). The flow rate was set at 0.25 mL/min. The autosampler was conditioned at 4 °C in both methods.

2.5.2. Mass spectrometry

An Applied Biosystems-Sciex API 3000 (Foster City, CA, USA) triple-quadrupole mass spectrometer equipped with an ESI interface was used in this study. The ESI was performed in either positive ion ESI (5500 V) or negative ion ESI (4500 V), and the turboionspray temperature was set at 400 °C. The multiple reaction monitoring (MRM) mode was used for quantification. Selected transitions of m/z of positive ion ESI were: 214.2→214.2 for resorufin, 305.3→269.3 for 6β-hydroxytestosterone, 256.2→238.1 for hydroxybupropion, 362.1→214.2 for 5-hydroxyomeprazole, 258.1→157.4 for dextrophan, 462.3→286.3 for morphine-3-glucuronide, and 268→116.2 for metoprolol (set as an IS). The selected transitions of m/z of negative ion ESI were 215.1→215.1 for hydroxylauric acid, 284.9→186.0 for 4-hydroxytolbutamide, 183.9→119.9 for 6-hydroxychloroxazone, and 149.9→107 acetaminophen (set as an IS). High-purity nitrogen gas was used as collision-induced dissociation gas (setting 12) and curtain gas (setting 9). Nebulizer gas was set at 12 in the positive and 10 in the negative ion ESI, respectively.

2.6. Method validation

The method validation assays were conducted based on the currently accepted U.S. FDA Bioanalytical Method Validation Guidance [25]. The following parameters were determined for the validation of the analytical method developed for resorufin, 6β-hydroxytestosterone, hydroxybupropion, 5-hydroxyomeprazole, dextrophan, morphine-3-glucuronide, hydroxylauric acid, 4-hydroxytolbutamide, and 6-hydroxychloroxazone in RLM: selectivity, linearity, LLOQ, precision, accuracy, extraction recovery, matrix effect, and stability.

Selectivity was evaluated by comparing chromatograms of 6 blank RLM from 6 rats to ensure that there was no significant interfering peak at retention time at LLOQ of the analytes. To assess linearity and the lower limit of quantification, a line was fitted through the standard curve range by a weighted linear regression (weight$=1/x$) of the peak area ratio of the analyte to IS (Y) *versus* the actual concentration of the analyte (X). The equation: $Y=aX+b$ was used to calculate the concentration of 8 major CYP isoenzymes of Phase I and UGT2B7 of Phase II in RLM from the peak area ratio obtained by LC-MS/MS. As defined in the present study, LLOQ is the lowest RLM concentration in the calibration curve, indicating that the analyte response at LLOQ was 5 times the baseline noise and could be determined with a precision of ≦20% and an accuracy of 80–120%.

The concentrations used for the evaluation of recovery and the matrix effect were performed in triplicate with 3 QC concentrations (low, middle, and high). The extraction recoveries were determined by comparing the response ratios of extracted RLM standards with those of extracted blank RLM spiked with corresponding concentrations. The response was defined as the peak area of analytes divided by the peak area of IS. Blank RLM from six rats were used to assess the matrix effect. The absolute and relative matrix effect was previously defined by Matuszewski et al. [26]. The absolute matrix effect was evaluated by comparing the peak areas of analytes added to extract blank RLM with those of the extracted water. The RSD of the mean peak areas of analytes in the extracted blank RLM indicated the relative matrix effect. To assess accuracy and precision, the within-run precision and accuracy were determined by analyzing QC samples ($n=6$). The between-run precision and accuracy were also verified by analyzing QC samples in 6 batches on different days. The precision was presented as relative standard deviation (RSD) and the accuracy was shown as relative error (RE). The acceptable within-

and between-run precision and accuracy were set at ≦15%, except for LLOQ, which was set at ≦20%.

The results were evaluated by comparing the calculated concentration with the initial values. The stability tests were performed in triplicate at 3 QC concentrations (low, middle, and high). The percentage of deviation in the concentrations was used as an indicator of stability. The analyte was considered stable when within 15%. Comparisons were made between the stock solutions (−20 °C for 180 days) with freshly made ones (stored at −80 °C) at 250 ng/mL ($n=3$). The working solutions were immediately diluted with either 1 mL of the mobile phase containing 80 ng/mL metoprolol (for positive ion ESI) or 0.8 μg/mL acetaminophen (for negative ion ESI) or maintained at ambient temperature (25 °C) for 4 h and then diluted with the mobile phase. Short-term temperature stability was performed to evaluate the analyte stability in the matrix. During the examination, samples were stored at room temperature (25 °C) for at least 4 h prior to extraction. To assess the injector stability of the processed samples, the QC samples were extracted and placed in the autosampler at 4 °C for 24 h, and then injected into the LC-MS/MS system for analysis. The freeze/thaw stability was determined after 3 freeze (−80 °C) and thaw (25 °C) cycles before being analyzed. The long-term stability was evaluated after storage: the QC samples stored in a freezer at −80 °C remained stable for a minimum of 90 days. All samples were subsequently thawed and analyzed together with freshly prepared calibration samples.

2.7. Applying analytical method to CYPs isoenzyme activities of fatty livers

The abovementioned analytical method was applied to compare the *in vivo* effects of 8 major hepatic CYP isoenzymes and UGT2B7 activities in SD rats with fatty livers with the *in vivo* effects on healthy rats. To induce fatty liver, the study group was given a CD diet for 8 weeks [27,28]. The intensity of the enzyme activity was obtained *in vitro* by removing the liver, isolating hepatic microsomes and determining the microsomal proteins. All samples were stored at −80 °C before analysis.

2.7.1. Experimental animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Defense Medical Center (IACUC: 10–082). Male SD rats, each weighing between 220 g and 250 g, and 7 weeks of age, were purchased from the National Applied Research Laboratories and National Laboratory Animal Center (Taiwan). They were placed in individual cages in a laboratory where the temperature, humidity, and light/dark (12 h/12 h) cycles were controlled. Tap water (*ad libitum*) was available throughout the study period. Following a 2-week acclimation period on a basal diet (Young Li Trading Co. LTD., Taiwan), the SD rats were randomly divided into 2 groups. The food supply (10–15 g/100 kg BW) was replaced daily and all rats were weighed weekly during the study period. A CD diet was given to the SD rats to induce fatty liver. In this study, 14 rats were randomly divided into 2 groups: the standard group ($n=7$) was provided with a standard diet for 8 week; and the study group ($n=7$) was given a CD diet for 8 weeks.

2.7.2. Diets

The CD diet was in a soft-pellet form (Product no. 1812444; LLC/PMI® Nutrition International Company, USA). The detailed composition of this diet has been described in previous reports [29,30]. The standard group was given a standard diet (per weight basis: 18% protein, 7% fat, 5% fiber, 62.5% carbohydrates, 3.78% vitamin, 0.22% choline chloride, and 3.5% mineral content). A CD diet composed of protein (18%), fat (7%), fiber (5%), carbohydrates (62.5%), vitamins (4%), and mineral content (3.5%) was fed to the study group. All diets were stored at 4 °C immediately after arrival, and every batch was consumed within 180 days.

2.7.3. Microsomal CYP activity determination

The rats were sacrificed after 8 weeks and the livers were harvested immediately. The RLM samples were prepared as described in Section 2.2. Microsomal CYP isoenzymes activities of RLM were determined by incubation of the probe substrates with RLM incubation mixtures. The stock solutions of each probe drug were prepared in MeOH, except for bupropion HCl, dextromethorphan, and omeprazole, which were prepared in water. Working solutions of ethoxyresorufin, testosterone, morphine, lauric acid, tolbutamide, and chlorzoxazone were concocted in ACN: H_2O (20:80, v/v). Probe substrates were separated into 2 parts: the first part was a substrate cocktail for the incubation solution of positive ion ESI, which was composed of ethoxyresorufin (25 μM), testosterone (1.8 mM), morphine (0.5 mM), bupropion (1 mM), dextromethorphan (1 mM), and omeprazole Na (1 mM); and the second part was a substrate cocktail for the incubation solution of negative ion ESI, which was composed of lauric acid (50 μM), tolbutamide (0.3 mM), and chlorzoxazone (0.2 mM). Following the addition of probe substrates, the reaction mixture was incubated at 37 °C for 30 min in a shaking incubator block. Thereafter, the termination procedure was performed in the same manner described in Section 2.4.

2.8. Statistical analysis

All data were expressed as mean ± SD. The Independent-Samples *T*-test was conducted to evaluate the effect of CYP isoenzymes and UGT2B7 enzyme activities. The statistical calculations were performed using Sigma Stat Software Package Version 17 (SPSS, Chicago, IL).

3. Results and discussion

3.1. Method development

The ESI/MS response of the probe metabolites was examined using both positive and negative ion ESI with differing mobile phase compositions (H_2O/MeOH and H_2O/ACN). A broad and double-peak chromatogram of hydroxylauric acid in the MeOH: H_2O: formic acid system was observed. By changing to the ACN:H_2O: formic acid gradient system, a sharp and single-peak chromatogram, as well as improved chromatographic separation, was achieved.

Because hydroxylauric acid, 4-hydroxytolbutamide, and 6-hydroxychloroxazone have poor ionization properties in the positive ion mode electrospray, a negative ionization mode was used. Furthermore, a cross-reactivity of resorufin (m/z 214.2→214.2) and hydroxylauric acid (m/z 215.1→215.1) was observed in both mobile phase compositions. Therefore, the determination of the activities of the 9 probe substrates was achieved in 2 separate settings: one set with pooled (6-in-1) cocktail-incubations under a positive mode, and the other set with pooled (3-in-1) cocktail-incubations under a negative mode.

The concentration of probe metabolites was high in the present study, and the reason was possibly that the enzyme kinetics followed Michaelis–Menten kinetics (nonlinear). Specifically, it followed first order metabolism at low concentrations and zero order metabolisms (steady-state level) at high concentrations. With low concentration, the metabolism response would be concentration-dependent as it takes two or three concentrations to

reflect the rate of enzyme metabolism (V). The metabolism response of the high concentration is concentration-independent, in that it only takes one concentration to observe the rate of enzyme metabolism (V). Therefore, high concentration was considered the better option because the concentration change was less likely to be affected, so the differences between individual enzymes could be minimized. By comparing the metabolism rates after incubations with high concentrations, we found a consistency in the reactions of all enzyme metabolisms. The metabolism rates after cocktail incubations were lower than those after separate-incubations (The data were not shown). These results indicated that in the concentration used in the study, no conflict results were obtained in the metabolism rate of 8 CYP isoenzymes and UGT2B7 between cocktail-incubations and separated-incubations.

The precursor/product ions of resorufin are at m/z 214 -> 186 [31]. According to Bu et al., producing precursor ions (214) and product ions of resorufin (186) requires enormous declustering potential and impact, but the intensity of the product ion (186) was found to be weak in our system and resulted in relatively poor correlation coefficients of calibration curves (r^2 ≦0.9). The necessary changes have been made to improve the intense product ion. We used single-ion mode (m/z 214 -> 214) [32] instead, and observed better correlation coefficients ($r^2$0.9977 ± 0.0011). The most intense product ion at m/z of positive ion ESI indicated 214.2 for resorufin, 269.3 for 6β-hydroxytestosterone, 238.1 for hydroxybupropion, 214.2 for 5-hydroxyomeprazole, 157.4 for dextrophan, 286.3 for morphine-3-glucuronide, and 116.2 for the internal standard (metoprolol). Furthermore, the most intense product ion of m/z of negative ion ESI was 215.1 for hydroxylauric acid, 186.0 for 4-hydroxytolbutamide, 119.9 for 6-hydroxychloroxazone, and 107 for acetaminophen. The MS/MS settings were adjusted to maximize the response of each precursor-product ion combination. The parameters of mass spectrometry were optimized, as shown in Table 1. A full-scan mass spectrum of each analyte was acquired in both positive and negative ion modes by using ESI.

For examining Phase II metabolism, UGTs cofactor (UDPGA ≦2 mM and alamethicin ≦5 ug/mL) did not inhibit the marker activities of the 5 major CYP isoenzymes (CYP1A, CYP 2C9, CYP 2C19, CYP 2D6, and CYP 3A4) [33]. The results showed that the CYP cofactors NADP+, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, at the tested concentrations did not have significant effects on glucuronide formation under the experimental conditions. The results demonstrated that no significant inhibitory effects existed in the microsomal incubations between glucuronic conjugation catalyzed by UGTs and oxidative metabolism mediated by CYPs.

3.2. Selectivity

Representative chromatograms of the 9 metabolites of the probe substrates are shown in Fig. 1(a) positive ion ESI of pure compound mixture; (b) positive ion ESI of the extracted ion chromatograms of a spiked LLQC sample; (c) negative ion ESI of the pure compound mixture; and (d) negative ion ESI of the extracted ion chromatograms of a spiked LLQC sample.

The retention times of positive ion ESI and the retention times of negative ion ESI are shown in Table 1. No significant interfering peaks caused by endogenous compounds or reagents were observed at the retention times of these analytes and IS in the chromatogram of blank RLM.

3.3. Method validation

3.3.1. Linearity and LLOQ

The standard calibration curves of 8 major CYP isoenzymes and UGT2B7 enzyme activities were determined using the 6-point concentration curve. The best-fit line of the calibration curve for each analyte was obtained using a weighting factor of 1/x. We obtained excellent correlation coefficients (r^2 ≧ 0.997; Table 2). Using this method, the LLOQ varied between 3 and 63 ng/mL for the drugs studied.

3.3.2. Extraction recovery and matrix effect

This study yielded a mean recovery greater than 83% for all analytes (Table 2) with good sensitivity, precision, and accuracy for the 8 major hepatic CYP isoenzymes and UGT2B7 in RLM. The absolute and relative matrix effects are shown in Table 2. No significant matrix effect was observed for the probe substrates. The matrix caused only relatively minor effects on the ionization efficiency of the analyte compounds, and the suppression or enhancement of the ionization was between 85.8% and 111.6% for each analyte in both approaches. The relative matrix effects were all less than 10.6%.

3.3.3. Accuracy and precision

The data on accuracy and precision are shown in Table 3. The precision of within- and between-run was 0.9–12.0% and 2.0–13.9%, respectively. The accuracy of within- and between-run was 0.6–17.2% and 0.1–15.1%, respectively. The RE of the LLOQ values of 6β-hydroxytestosterone, dextrophan, and morphine-3-glucuronide were −17.1%, −17.2%, and 15.9%. Consequently,

Table 1
Multiple reaction monitoring transitions and fragmentation parameters for the analytes and internal standards.

Probe substrates	CYP	Metabolite	Molecular weight	Polarity	Precursor	Product	DP (V)	FP (V)	CEP (V)	CE (eV)	CXP (V)	Retention time (min)
Ethoxyresorufin	1A2	Resorufin	235	ES+	214.2	214.2	66	310	9	20	17	4.07
Bupropion	2B6	OH bupropion	255	ES+	256.2	238.1	20	90	7	17	12	4.32
Omeprazole	2C19	5-OH omeprazole	383	ES+	362.1	214.2	40	400	9	11	6	3.49
Dexmethotrophan	2D6	Dextrophan	257	ES+	258.1	157.4	89	357	13	41	27	3.71
Testosterone	3A4	6β-OH testosterone	304	ES+	305.3	269.3	61	345	8	22	21	3.69
Morphine	UGT2B7	Morphine-3-glucuronide	461	ES+	462.3	286.3	90	370	12	41	18	2.69
Tolbutamide	2C9	4-OH tolbutamide	286	ES−	284.9	186.0	−44	−200	−10	−23	−9	5.71
Chloroxazone	2E1	6-OH chloroxazone	185	ES−	183.9	119.9	−42	−150	−10	−23	−5	4.69
Lauric acid	4A	OH lauric acid	216	ES−	215.1	215.1	−50	−200	−10	−20	−13	6.50
Internal standard												
Metoprolol (IS of positiveion ESI)			685	ES+	268.0	116.2	80	400	10	28	9	3.84
Acetaminophen (IS of negative ion ESI)			151	ES−	149.9	107.0	−45	−230	−14	−25	−4	2.28

DP: declustering potential; *FP*: focusing potential; *CEP*: cell entrance potential; *CE*: collision energy; *CXP*: cell exit potential.

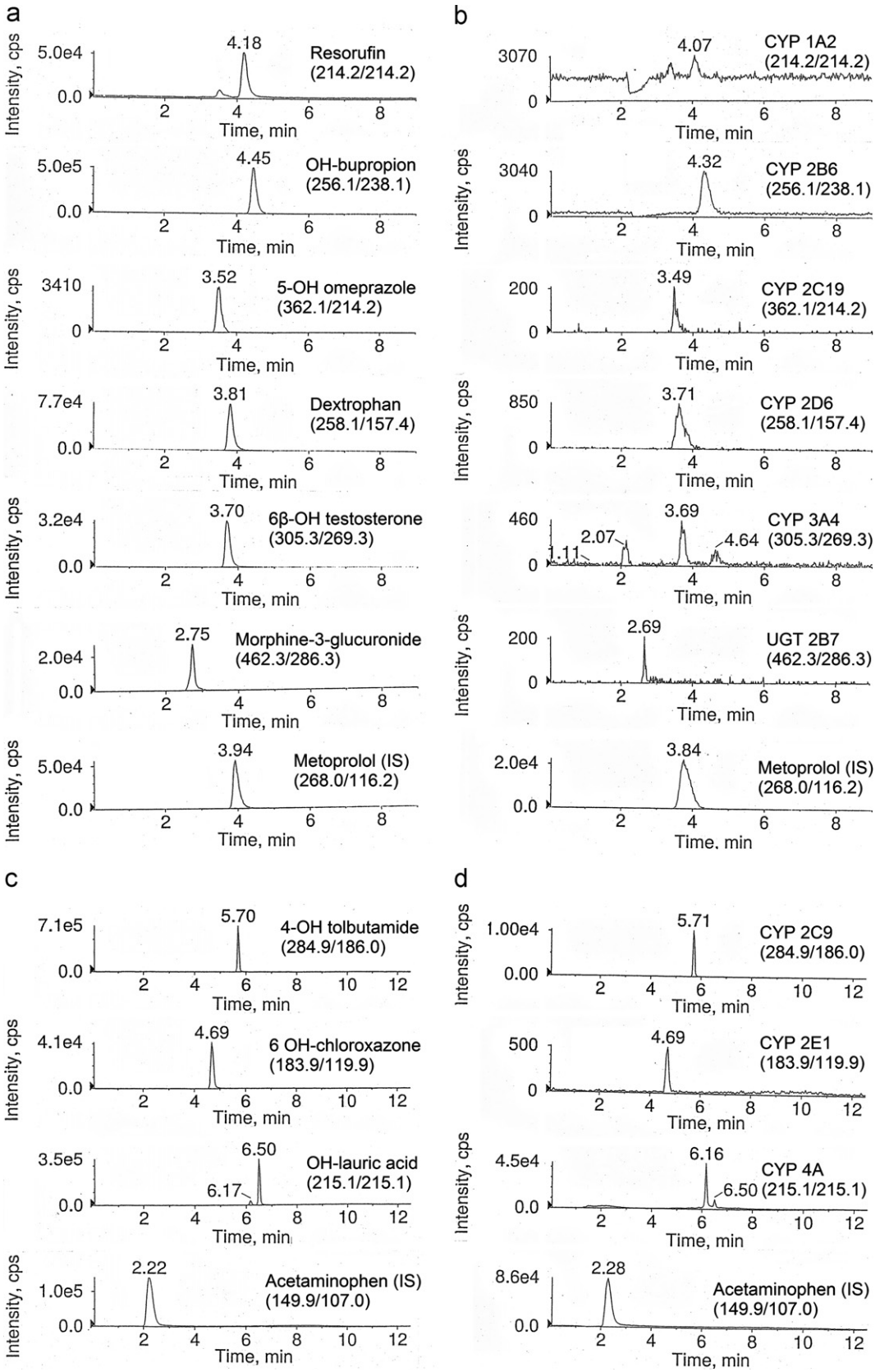

Fig. 1. Multiple reaction monitoring ion chromatograms of CYP isoform oxygenated products of probe substrates and internal standard. (a) Positive ion analytes of pure compound mixture: resorufin (channel A), hydroxybupropion (channel B), 5-hydroxyomeprazole (channel C), dextrophan (channel D), 6β-hydroxytestosterone (channel E), morphine-3-glucuronide (channel F), and channel G (metoprolol) for internal standard. (b) Positive ion ESI of the extracted ion chromatograms of a spiked LLQC sample: resorufin 5 ng/mL (channel A), hydroxybupropion 3 ng/mL (channel B), 5-hydroxyomeprazole 25 ng/mL (channel C), dextrophan 25 ng/mL (channel D), 6β-hydroxytestosterone 63 ng/mL (channel E), morphine-3-glucuronide 75 ng/mL (channel F), and channel G (metoprolol) for internal standard at low concentration. (c) Negative ion analytes of pure compound mixture: 4-hydroxytolbutamide (channel A), 6-hydroxychloroxazone (channel B), hydroxylauric acid (channel C), and channel D (acetaminophen) for internal standard. (d) Negative ion ESI of the extracted ion chromatograms of a spiked QC sample: 4-hydroxytolbutamide 10 ng/mL (channel A), 6-hydroxychloroxazone 40 ng/mL (channel B), hydroxylauric acid 25 ng/mL (channel C), and channel D (acetaminophen) for internal standard at low concentration. CYP, Cytochrome P450; IS, Internal standard.

Table 2
Calibration curves, sensitivity, extraction recoveries, and absolute and relative matrix effect of the assay.

Analyte	Calibration range	r^2	Recovery (%)[c] $n=3$			Absolute and relative matrix effect (%)[d] $n=6$		
	(ng/mL)	$n=6$	LOQ	MOQ	HOQ	LOQ	MOQ	HOQ
Resorufin[a]	5–500	0.9977 ± 0.0011	85.1 ± 7.6	86.7 ± 10.1	94.2 ± 3.2	87.8 (10.6)	89.4 (8.4)	86.2 (4.4)
OH Bupropion[a]	3–300	0.9990 ± 0.0007	90.6 ± 6.3	102.5 ± 10.8	103.8 ± 7.8	100.0 (7.4)	93.5 (6.3)	99.0 (5.5)
4-OH tolbutamide[b]	10–1000	0.9979 ± 0.0021	93.3 ± 6.5	85.3 ± 14.9	88.4 ± 6.4	108.2 (4.2)	104.2 (2.7)	111.6 (3.0)
5-OH omeprazole[a]	25–2500	0.9978 ± 0.0011	88.2 ± 10.2	94.2 ± 7.4	95.0 ± 7.5	85.8 (5.8)	86.9 (6.7)	87.1 (7.6)
Dextrophan[a]	25–2500	0.9993 ± 0.0006	92.6 ± 8.9	96.7 ± 11.9	96.3 ± 1.9	93.7 (8.0)	89.2 (6.3)	99.0 (8.5)
6-OH chloroxazone[b]	40–4000	0.9979 ± 0.0018	94.0 ± 9.4	96.5 ± 7.5	95.0 ± 1.6	90.9 (2.7)	93.3 (2.9)	97.8 (4.8)
6β-OH testosterone[a]	63–6300	0.9993 ± 0.0004	100.3 ± 9.0	103.9 ± 12.8	107.2 ± 3.2	94.6 (7.2)	99.4 (9.4)	100.9 (7.6)
OH lauric acid[b]	25–2500	0.9983 ± 0.0018	85.4 ± 2.8	83.2 ± 3.6	89.3 ± 0.9	108.5 (6.5)	104.4 (2.3)	104.9 (4.6)
Morphine-3-glucuronide[a]	25–2500	0.9991 ± 0.0006	87.2 ± 4.2	98.0 ± 15.4	97.5 ± 5.4	104.8 (4.3)	97.4 (6.1)	110.4 (3.9)
Metoprolol (IS of positiveion ESI)						108.1 (9.1)	102.6 (8.6)	103.3 (4.6)
Acetaminophen (IS of negative ion ESI)						119.9 (3.8)	111.2 (5.1)	115.6 (7.5)

[a] Metoprolol (160 ng/mL) was added into solutions at 3 concentrations: 5-OH omeprazole, dextrophan and morphine-3-glucuronide (75, 1000, and 2000 ng/mL), resorufin (15, 200, and 400 ng/mL), 6β-OH testosterone (189, 2520, and 5040 ng/mL), and OH-bupropion (9, 120, and 240 ng/mL).
[b] Acetaminophen (1.6 μg/mL) was added into solutions at 3 concentrations: OH lauric acid (75, 1000, and 2000 ng/mL), 4-OH tolbutamide (30, 400, and 800 ng/mL), and 6-OH chloroxazone (120, 1600, and 3200 ng/mL).
[c] The results of recovery are expressed as mean ± SD.
[d] The results are expressed as an absolute matrix effect percentage (relative matrix effect percentage).

Table 3
Intra- and inter-day precision and determination accuracy of 8 major hepatic CYP isoenzymes and UGT2B7 in rat microsomes.

Cyp isoform	Metabolite	Added conc. (ng/mL)	Within run ($n=6$)			Between run ($n=6$)		
			Calculated Conc. (ng/mL)	RSD (%)	RE (%)	Calculated conc. (ng/mL)	RSD (%)	RE (%)
1A2	Resorufin	5	4.7 ± 0.1	2.5	−6.8	4.6 ± 0.2	3.8	−8.7
		15	14.3 ± 0.7	5.2	−4.9	13.8 ± 0.9	6.2	−8.0
		200	212.1 ± 13.4	6.3	6.1	190.8 ± 5.4	2.8	−4.6
		400	437.7 ± 19.7	4.5	9.4	379.8 ± 19.6	5.2	−5.0
2B6	OH Bupropion	3	2.9 ± 0.1	4.4	−2.6	3.1 ± 0.2	7.1	3.5
		9	8.4 ± 0.3	3.8	−6.6	8.5 ± 0.3	3.8	−5.1
		120	119.3 ± 4.3	3.6	−0.6	126.6 ± 4.2	3.3	5.5
		240	249.8 ± 6.7	2.7	4.1	270.5 ± 5.4	2.0	12.7
2C9	4-OH tolbutamide	10	9.4 ± 0.3	2.9	−5.9	11.0 ± 0.5	4.9	10.2
		30	32.0 ± 0.9	2.7	6.7	34.3 ± 2.6	7.7	14.3
		400	447.5 ± 13.1	2.9	11.9	410.6 ± 36.7	8.9	2.6
		800	915.6 ± 12.8	1.4	14.5	882.4 ± 73.3	8.3	10.3
2C19	5-OH omeprazole	25	26.5 ± 2.0	7.7	6.0	27.4 ± 0.9	3.4	9.7
		75	73.1 ± 3.0	4.2	−2.5	72.6 ± 2.3	3.1	−3.2
		1000	1075.7 ± 50.3	4.7	7.6	1001.2 ± 28.1	2.8	0.1
		2000	2214.7 ± 83.2	3.8	10.7	2154.2 ± 102.8	4.8	7.7
2D6	Dextrophan	25	20.7 ± 2.3	11.2	−17.2	25.7 ± 1.3	5.0	3.0
		75	67.9 ± 2.3	3.4	−9.5	68.0 ± 3.2	4.8	−9.4
		1000	1068.3 ± 30.0	2.8	6.8	1093.0 ± 32.8	3.0	9.3
		2000	2136.0 ± 99.7	4.7	6.8	2161.3 ± 68.1	3.2	8.1
2E1	6-OH chloroxazone	40	44.2 ± 0.8	1.6	10.5	43.2 ± 2.1	4.9	7.9
		120	124.3 ± 1.8	1.4	3.6	135.4 ± 9.0	6.6	12.8
		1600	1499.9 ± 47.5	3.2	−6.3	1571.9 ± 173.6	11.0	−1.8
		3200	3011.1 ± 26.4	0.9	−5.9	3391.8 ± 226.2	6.7	6.0
3A4	6β-OH testosterone	63	52.3 ± 4.2	8.0	−17.1	63.4 ± 4.2	6.6	0.6
		189	184.2 ± 10.9	5.9	−2.6	180.8 ± 8.0	4.4	−4.3
		2520	2727.8 ± 52.1	1.9	8.2	2825.5 ± 66.9	2.4	12.1
		5040	5725.8 ± 318.6	5.6	13.6	5376.0 ± 184.0	3.4	6.7
4A	OH lauric acid	25	28.8 ± 2.9	10.1	15.2	24.3 ± 1.0	4.1	−2.7
		75	80.9 ± 6.6	8.2	7.9	80.5 ± 4.7	5.8	7.3
		1000	926.7 ± 85.8	9.3	−7.3	892.6 ± 73.2	8.2	−10.7
		2000	2242.2 ± 63.3	2.8	12.1	1860.0 ± 179.1	9.6	−7.0
UGT2B7	Morphine-3-glucuronide	25	29.0 ± 1.7	5.9	15.9	25.4 ± 1.3	5.1	1.8
		75	74.0 ± 8.9	12.0	−1.3	73.1 ± 10.2	13.9	−2.5
		1000	976.6 ± 79.6	8.2	−2.3	849.3 ± 109.0	12.8	−15.1
		2000	2038.9 ± 166.0	8.1	1.9	1946.2 ± 246.3	12.6	−2.7

Results are expressed as mean ± SD.
RSD: relative standard deviation; *RE*: relative error.

we were able to conclude that the QC standards were acceptable as the values did not deviate by more than ± 15% (± 20% at LLOQ) from their nominal values, indicating that the method was precise and accurate within the range.

3.4. Stability

As shown in Table 4, the stability tests were all higher than 85.0%. No significant degradation was found in the analysis

Table 4
Summary of stability tests of 8 major hepatic CYP isoenzymes and UGT2B7 in rat microsomes.

Stability	Stock solutions (−20 °C for 180 days)	Working solution (25 °C for 4 h)	Short term (25 °C for 4 h)	Autosampler (4 °C for 24 h)	Freeze and thraw (3 cycle)	Long term (−80 °C for 90 days)
Resorufin	94.7–108.0	86.7–102.2	97.2–113.1	88.2–114.2	108.5–114.2	96.2–111.5
OH Bupropion	90.8–94.7	88.3–98.2	92.9–108.5	94.5–110.4	96.2–104.1	96.1–99.9
4-OH tolbutamide	86.2–89.5	97.5–99.6	104.1–113.6	102.4–112.8	104.6–114.6	91.2–104.2
5-OH omeprazole	92.3–110.7	85.4–98.0	89.1–114.1	86.9–100.1	85.6–90.7	90.4–104.9
Dextrophan	86.9–91.7	100.8–105.2	99.4–112.2	96.1–102.2	95.7–112.0	93.9–96.7
6-OH chloroxazone	87.0–99.1	94.6–103.9	92.2–99.9	91.3–97.2	86.5–98.8	85.9–87.1
6β-OH testosterone	89.8–105.5	95.1–101.6	105.7–114.1	102.0–111.4	85.2–97.1	94.6–107.1
OH lauric acid	90.3–113.2	92.6–101.7	99.4–109.5	96.6–102.1	103.3–109.3	96.1–101.7
Morphine-3-glucuronide	92.1–98.5	94.7–98.6	97.7–99.6	99.6–103.3	92.2–105.5	97.0–104.0

Results are expressed as % of control.

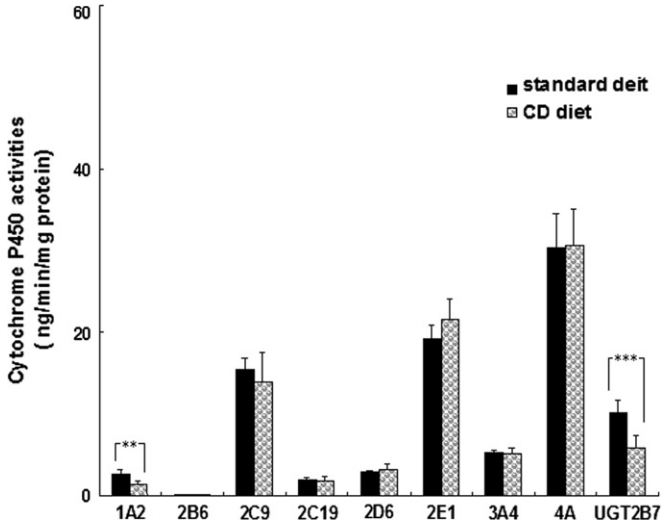

Fig. 2. Effect of CYP isoenzymes and UGT2B7 enzyme activities in Sprague-Dawley rats fed with a choline-deficient diet for 8 weeks. This study was *in vivo* interaction cocktail assay for 8 major hepatic CYP isoenzymes and UGT2B7 in rat microsomes. CD, Choline-deficient. All data are presented as mean ± standard deviation of triplicate determination.

concentrations when extracts were kept in stock solutions stored at −20 °C for 180 days, working solutions were kept at 25 °C for 4 h, the autosampler at 4 °C for 24 h, and QC samples were placed at 25 °C for 4 h. All analytes were found to be stable in the RLM samples when stored at −80 °C for 90 days or after 3 freeze-thaw cycles. If the analyte was not stable after 3 cycles, adequate amounts of aliquots needed to be stored to allow repeats without having to freeze and thaw the sample more than once. The results indicated that this analytical method could be applied for RLM sample analysis.

3.5. Enzyme activities of 8 major CYP isoenzymes and UGT2B7 study

Fig. 2 shows an illustration of the effect of CYP isoenzymes and UGT2B7 enzyme activities in SD rats which were fed a CD diet for 8 weeks. The results indicated that the activities of CYP1A2 and UGT2B7 were significantly lower in CD-fed rats than in the standard diet group. The activity of CYP2E1 was inclined to increase in CD-fed rats as compared to the standard diet group. The activity of CYP2C9 tended to decrease in the CD-fed rats as compared to the standard diet group. These results were similar to the findings of Robertson et al. (CYP2E1 activity was increased in the rats of NASH) [34], and Fisher et al. (CYP1A2 was low in humans with fatty liver disease) [35]. UDP-glucuronyltransferase activity was not affected in human steatotic livers [36], but the individual enzymes have not as yet been identified.

4. Conclusion

This study described and a validated the LC-MS/MS method for determining 8 CYP isoenzymes and UGT2B7 enzyme activities. CYP4A, an important enzyme related to fatty liver, was included for the first time in this kind of study. This method was successfully not only in analyzing the enzyme activities of 8 CYP isoenzymes and UGT2B7 in SD rats with normal livers, but also in SD rats with fatty livers. Furthermore, the significant decrease in the CYP1A2 and UGT2B7 activities were observed in rats with fatty livers. In the future, this simplified, rapid, and accurate cocktail assay could be applied to measure CYP enzyme activities for patients suffering from liver disease.

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References

[1] A.M. Hoyumpa, S. Schenker, Hepatology 13 (1991) 786–795.
[2] J. George, M. Murray, K. Byth, G.C. Farrell, Hepatology 21 (1995) 120–128.
[3] K. Lown, J. Kolars, K. Turgeon, R. Merion, S.A. Wrighton, P.B. Watkins, Clin. Pharmacol. Ther. 51 (1992) 229–238.
[4] R.F. Frye, N.K. Zaheib, G.R. Matzke, Clin. Pharmacol. Ther. 80 (2006) 235–245.
[5] J.P. Villeneuve, V. Pichette, Curr. Drug Metab. 5 (204) 273–282.
[6] P. Lemaire, M. Lafaurie, D. Weissbart, F. Durst, P. Pflieger, C. Mioskowski, J.P. Salaun, Lipids 27 (1992) 187–191.
[7] C.L. Crespi, V.P. Miller, B.W. Penman, Anal. Biochem. 248 (1997) 188–190.
[8] Y. Liu, J. Jiao, C. Zhang, J. Lou, Biol. Pharm. Bull. 32 (2009) 717–720.
[9] R.L. Walsky, R.S. Obach, Drug Metab. Dispos. 32 (2004) 647.
[10] A. Tolonen, A. Petsalo, M. Turpeinen, J. Uusitalo, O. Pelkonen, J. Mass Spectrom. 42 (2007) 960–966.
[11] M. Turpeinen, U. Jouko, J. Jorma, P. Olavi, Eur. J. Pharm. Sci. 24 (2005) 123–132.
[12] A. Petsalo, M. Turpeinen, O. Pelkonen, A. Tolonen, J. Chromatogr. A 1215 (2008) 107–115.
[13] M.E. Burton, L.M. Shaw, J.J. Schentag, W.E. Evans, Drug Metabolism, Transport, and the influence of hepatic disease, Lippincott Williams & Wilkins, Maryland, 2006, pp. 121-164.
[14] I.A. Leclercq, G.C. Farrell, J. Field, D.R. Bell, F.J. Gonzalez, G.R. Robertson, J. Clin. Invest. 105 (2000) 1067–1075.

[15] M.J.J. Ronis, K.O. Lindros, M. Ingelman-Sundberg, in CRC press, Boca Raton, FL, pp. 211–239.
[16] Q. Chen, M. Galleano, A.I. Cederbaum, J. Biol. Chem. 272 (1997) 14532–14541.
[17] M.B. Fisher, M.F. Paine, T.J. Strelevitz, S.A. Wrighton, Drug Metab. Rev. 33 (2001) 273–297.
[18] D.F. Lewis, Pharmacogenomics 5 (2004) 305–318.
[19] S.D. Gregori, M.D. Gregori, G.N. Ranzani, M. Allegri, C. Minella, M. Regazzi, Metab. Brain Dis. 27 (2012) 1–5.
[20] Food and Drug Administration. Guidance for industry. Drug Interaction Studies—study design, data analysis, and implications for dosing and labeling. 2006. 〈http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072123.pdf〉, (accessed 03.11).
[21] S.M. Poloyac, M.A. Tortorici, D.I. Przychodzin, R.B. Reynolds, W. Xie, R.F. Frye, M.A. Zemaitis, Drug Metab. Dispos. 32 (2004) 727–733.
[22] A.A. Somogyi, M. Larsen, R.M. Abadi, J. Jittiwutikarn, R. Ali, J.M. White, Br. J. Clin. Pharmacol. 66 (2008) 640–647.
[23] S.A. Jr. Testino, G. Patonay, J. Pharm. Biomed. Anal. 30 (2003) 1459–1467.
[24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 35 (1951) 1817–1824.
[25] Guidance for Industry/Bioanalytical Method Validation, US Food and Drug Administration, Rockville, Maryland, 2001.
[26] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[27] J. George, N. Pera, N. Phung, I. Leclercq, J.Y. Hou, G. Farrel, J. Hepatol. 39 (2003) 756–764.
[28] K. Hironaka, I. Sakaida, K. Uchida, K. Okita, Dig. Dis. Sci. 45 (2000) 1935–1943.
[29] M. Murray, L. Zaluzny, G.A. Dannan, F.P. Guengerich, G.C. Farrell, Mol. Pharmacol. 31 (1987) 117–121.
[30] C.P. Oliveira, L.C. da Costa Gayotto, C. Tatai, B.I. Della Bina, M. Janiszewski, E.S. Lima, D.S. Abdalla, F.R. Laurindo, A.A. Laudanna, J. Cell. Mol. Med. 6 (2002) 399–406.
[31] H.Z. Bu, K. Knuth, L. Magis, P. Teitelbaum, Eur. J. Pharm. Sci. 12 (2001) 447–452.
[32] E.A. Dierks, K.R. Stams, H.K. Lim, G. Cornelius, H. Zhang, S.E. Ball, Drug Metab. Dispos. 29 (2001) 23–29.
[33] Z. Yan, G.W. Caldwell, Eur. J. Drug Metab. Pharmacokinet. 28 (2003) 223–232.
[34] G. Robertson, I. Leclereq, G.C. Farrell, Am. J. Physiol. Gastrointest. Liver Physiol. 281 (2001) G1135–G1139.
[35] C.D. Fisher, A.J. Lickteig, L.M. Augustine, J. Ranger-Moore, J.P. Jackson, S.S. Ferguson, N.J. Cherrington, Drug Metab. Dispos. 37 (2009) 2087–2094.
[36] M.T. Donato, A. Lahoz, N. Jimenez, G. Perez, A. Serralta, J. Mir, J.V. Castell, M.J. Gomez-Lechon, Drug Metab. Dispos. 34 (2006) 1556–1562.