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High Throughput and Reliable Isotope Label-free approach for Profiling 24

Metabolic Enzymes in FVB Mice and Gender Differences

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Running title: The profile of 24 DMEs in male and female FVB mice.

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Manuscript metrics:

Number of text pages: 39

Number of tables: 6

Number of figures: 8

Number of references: 33

Number of words in the Abstract: 246

Number of words in the Introduction: 675

Number of words in the Discussion: 1046

Abbreviations: DME, drug-metabolizing enzyme; CYP/Cyp, cytochrome P450; UGT/Ugt, UDP-glucuronosyltransferase; SULT/Sult, sulfotransferase; UHPLC, ultrahigh performance liquid chromatography; MLS9, mouse liver S9; MRM, multiple reaction monitoring; CE, collision energy; DDI, drug-drug interaction.

Abstract

FVB mice are extensively used in transgenic and pharmacokinetic research. In this study, a validated isotope label-free method was constructed using ultrahigh-performance liquid chromatography (UHPLC)-MS/MS to quantify 24 drug-metabolizing enzymes (DMEs) in FVB mice. The DMEs include cytochrome P450s (CYPs/Cyps), UDP-glucuronsyltransferases (UGTs/Ugts), and sulfotransferases (SULTs/Sults), which catalyze a variety of reactions to detoxify xenobiotics and endobiotics. The proposed UHPLC-MS/MS method exhibited good range and high sensitivity for signature peptides, as well as acceptable accuracy, precision, and recovery. The protein expression profiles of the DMEs were determined in male and female mice. Overall, the major Cyps, Ugts, and Sults were expressed in male mice followed the rank order: Cyp2c29 > 2e1 > 3a11 > 1a2 > 2d22 > 27a1 > 2c39; Ugt2b5 > 2b1 > 1a6a > 1a9 > 1a1 > 2a3 > 1a2 > 1a5; and Sult1a1 > 3a > 1d1. In contrast, the rank order in female mice was Cyp2c29 > 2e1 > 2c39 > 2d22 > 3a11 > 1a2 > 27a1; Ugt1a6a > 2b5 > 1a1 > 2b1 > 2a3 > 1a9> 1a5 > 1a2; and Sult1a1 > 3a1 > 1d1. Cyp2c29, Cyp1a2, Cyp27a1, Ugt2b1, Ugt2b5 and Ugt2b36 were male-predominant, while Cyp2c39, Cyp2d22, Cyp7a1, Ugt1a1, Ugt1a5, Sult1a1, Sult3a1, and Sult1d1 were female-predominant. This work could

serve as a useful reference for the metabolic study of new drugs and for elucidating

the effectiveness and toxicity of drugs. And the method is a stable, simple, and rapid

for determining the expression of DMEs in animals.

Introduction

As the primary organ for metabolism due to high activity of CYPs, UGTs, and

SULTs, the liver converts both endogenous and exogenous substances into polar

products that are amenable for excretion (Yan et al., 2015). Metabolism and drug-drug

interactions (DDIs) involved in metabolism can influence the pharmacokinetics and

efficacy/toxicity of drugs (Zhang et al., 2015). Thus, knowledge on metabolism and

DDIs would provide useful information to design prodrugs and to predict the outcome

of therapy (Margaillan et al., 2015).

Many studies have quantified hepatic DMEs through targeted proteomics

(Sakamoto et al., 2011; Fallon et al., 2013a; Fallon et al., 2013b). These studies were

primarily performed in humans, and only limited data are available for mice. At

present, mouse is becoming an increasingly common laboratory species because of

the high sequence homology between mice and humans, and the availability of

transgenic and knockout mice (Longo et al., 2011). FVB mice offer many advantages

for transgenic research, such as defined inbred background, large litters, and

prominent pronuclei (Goelz et al., 1998; Girard et al., 2016). Several studies have

utilized wild-type FVB and efflux transporter knockout mice to investigate the effect

of these transporters on drug bioavailability (Zaher et al., 2006; Agarwal et al., 2012;

Ge et al., 2015). For this reason, we investigated DMEs in FVB mice in the current

study.

It has been proposed that the following DMEs are of clinical relevance in

humans: CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5; UGT1A1, 1A3,

2B7, and 2B15 (Gröer et al., 2014); SULT1A1, 1A3, 1B1, 1E1, and 2A1 (Riches et al.,

2009). Mouse phase I enzymes (Cyp1a2, 1b1, 2c29, 2c39, 2d22, 2e1, 3a11, 7a1, and

27a1) and phase II enzymes (Ugt1a1, 1a5, 1a6a, 1a9, 2b1, 2b5, and Sult1a1) are

orthologs of the corresponding human enzymes. Other isoforms, including Cyp3a25,

Ugt1a2, 2a3, 2b34, 2b35, 2b36; Sult1d1 and 3a1 are also important in mice. Several

epidemiological and clinical studies have evaluated the effects of sex differences in

the pharmacokinetics of drugs. The results have revealed that sex differences in

DMEs may influence drug efficacy and safety. Thus, clarifying the contribution of

these enzymes, particularly in terms of gender differences, is important for drug

efficacy and safety.

DMEs have been studied using bioanalytical methods, such as Western blot

(WB), enzyme-linked immunosorbent assay (ELISA), reverse-transcriptase

polymerase chain reaction (RT-PCR) and enzyme activity assays (Ohno and Nakajin,

2009; Joo et al., 2014; Shi et al., 2015). However, these methods intended to measure

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the abundance of DMEs have many limitations. For example, WB requires specific

antibodies that are rarely available due to highly homologous enzymes within the

DMEs. The application of RT-PCR to detect mRNA is unreliable because the mRNA

expression level may not always correlate with protein level. Enzymatic activities are

widely used for indirectly reflecting the protein amounts. However, a variety of DMEs

always have broad and often overlapping substrates specificities. Furthermore, these

methods are usually semi-quantitative and low throughput. Thus, development of an

accurate and high-throughput method for determining DMEs protein levels is highly

desirable. An alternative approach to accurately quantify DMEs using LC-MS/MS is

broadly used (Seibert et al., 2009). The present new approach is able to sensitively

(detection of fmol protein) and easily (quantification of multiple proteins) determine

the absolute amounts of proteins with a high degree of sequence homology

(distinction of molecular weight less than 1 Da) in complex biologic matrices, such as

quantifying CYP and UGT superfamilies in biologic tissues or cultured cell lines

(Schaefer et al., 2012; Achour et al., 2014). Ms-based protein quantification

approaches, which are characterized by selective, precise, and large dynamic range,

are highlighted as a promising method for basic research on drug development such as

DDI studies and PK/PD modeling.

In this study, we successfully developed an accurate and high throughput method to systematically quantify 24 DMEs in male and female FVB mice. The developed method and animal model can provide deep insights into the hepatic disposition and metabolism of drugs. Understanding gender differences may clarify the molecular basis for differences in drug disposition between males and females.

Materials and Methods

Chemicals and Reagents. Ammonium bicarbonate (ABC), dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), formic acid (FA), acetic acid, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Sequencing grade modified trypsin was provided by Promega (Madison, WI). All peptides and internal standard (IS) (purity>95%) were purchased from Your R&D Partner. Solid phase extraction (SPE) cartridges (C18 50 mg, 3 mL) were obtained from J.T. Baker (Philipsburg, NJ). Coomassie brilliant blue for protein measurement was purchased from Bio-Rad (Hercules, California, USA).

Instrumentation and Mass Spectrometry. An Agilent 1290 series UHPLC system and an Agilent 6490 triple quadrupole mass spectrometer interfaced with an electrospray ionization source (Agilent Technologies) was applied for quantifying all samples.

A Poroshell C18 column (2.1mm × 100 mm, 2.7μm; Agilent Technologies) with an injection volume of 10 μL was used for separation. The sample rack temperature was maintained at 10°C, and the analytical column temperature was set at 40°C. The mobile phase A was 0.1% FA in water and the mobile phase B was 100%

acetonitrile. In order to achieve chromatographic separation, a linear gradient was

set at a flow rate of 0.3 mL/min as follows: 0-1.8 min, 5-8% B; 1.8-2 min, 8-10% B;

2-3.5 min, 10-14% B; 3.5-10 min, 14-35% B; 10-12 min, 35-80% B; 12-14 min,

80-80% B ; 14-15 min, 80-5% B and post time was set to 2 min for column

equilibration.

The mass spectrometer was operated in positive ion mode to monitor the m/z

transitions for all peptides. The detailed information of peptides was listed in

Supplemental Table 1. For all peptides, at least three transitions of each peptide

were selected for quantification. In order to gain high sensitivity, one of the most

abundant fragments was used for quantification, whereas at least two of the

fragments were used for qualitative analysis for high selectivity. In order to

optimize the collision energies, a scheduled MRM acquisition method was

developed. The following main mass working parameters were set as follows:

capillary voltage 3 kV; nozzle voltage 1500 V; gas temperature 200 °C; sheath gas

temperature 250 °C; sheath gas flow of 11 L/min; gas flow of 14 L/min. Data were

collected and analyzed using the Mass Hunter software (version B.06.00, Agilent

Technologies).

Animals. Male and female FVB mice (Eleven weeks) were obtained from

Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The animals

were housed in a controlled condition, with ambient temperature of 24-26℃ and

humidity of 50-60% and a 12h light/dark cycle. All animals were approved by the

Institutional Animal Care and Use Committee of the Guangzhou University of

Chinese Medicine. Prior to the experiment, the animals were fasted overnight with

free access to water.

Preparation of mouse liver S9 fractions. S9 fractions from livers were

isolated from male and female FVB mice. MLS9 were prepared as previously

described with minor modifications (Zhu et al., 2010; Tang et al., 2012). Mouse

livers were washed, perfused with solution B (8 mM KH_2PO_4, 5.6 mM Na_2HPO_4,

1.5 mM EDTA), and then minced. The minced livers were homogenized using a

motorized homogenizer in an ice-cold homogenization buffer (50 mM potassium

phosphate, 250 mM sucrose, 1 mM EDTA, pH 7.4) and then centrifuged at 12,000 g

for 15 min at 4°C. The fat layer and the pellet were discarded, and the supernatant

was collected and stored at -80°C until use. Protein concentrations (typically 5-30

mg/ml) of MLS9 were measured by Coomassie brilliant blue, and bovine serum

albumin was used as the standard.

Sample preparation and tryptic digestion. S9 protein (120 μg) from MLS9

was digested as previously described with minor modifications (Sridar et al., 2013).

Samples were denatured and reduced at 95°C for 10 min in ABC digestion buffer

(50 mM, 90 µL), which was spiked with DTT in 5 mM final concentration. After

cooling down, 10 µL IAA (10 mM final concentration) was added for alkylation at

least 30 min in the dark at room temperature. Subsequently, the mixture (final

volume, 124 µL) was incubated with trypsin at 37°C for 4 h (trypsin:protein=1:50).

The digested reaction was terminated with TFA (120 µL, 0.5% v/v), and 20 µL of IS

(200 nM) was added. After centrifugation at 12, 000 g for 10 min, the supernatants

were dried under nitrogen. 1 mL of TFA: acetonitrile (0.1:100, v/v) and 1 mL of

TFA: water (0.1:100, v/v) were used to prepare SPE for sample cleaning,

respectively. After the samples were loaded into the conditioned SPE and then

washed with 1 ml of 0.1% TFA. Finally, the analytes were eluted with 1 mL of

acetonitrile-water-TFA mixture (60:40:0.1, v/v/v). After the eluent was evaporated

to dryness under nitrogen, and the samples were reconstituted with 200 µL

methanol-water-acetic acid mixture (5:95:0.1, v/v/v) and then centrifuged for 30

min at 18,000 g. The supernatant was determined by UHPLC-MS/MS.

Method validation. According to the US FDA guidelines, the fundamental

parameters, including linearity, accuracy, precision, matrix effect, recovery and

stability should be evaluated in the present work.

To prepare the calibration curves and quality control (QC) samples, digested Insect Cell Control Supersomes[TM] was used as a blank matrix and was spiked with various of each peptide to obtain the following concentrations for calibration values: 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 200 nM and QC samples were prepared in 1.56 or 3.13, 12.5, and 100 nmol or 12.5, 25 and 100 nmol concentrations. The calibration curves were prepared in the same way as described in the sample preparation section.

The precision and accuracy of the method were evaluated by measuring the standard samples at three concentration levels (12.5, 25, and 100 nM for Cyp1a2, Cyp1b1, Cyp2d22, Cyp3a25, Cyp7a1, Cyp27a1, Ugt1a1, and Ugt1a2; 1.56, 12.5, and 100 nM for other signature peptides of DMEs). Intra-day precision was determined using 6 replicates QC samples in the same day. Inter-day precision was measured using 18 replicates QC samples for three days. According to each calibration curve, the concentration of target protein can be calculated. Matrix effects were investigated by spiking target peptide standards into trypsinized MLS9.

The extraction recovery was calculated by comparing the concentrations obtained from analytes before the extraction with those from samples to which

analytes were added after the extraction. Autosampler stability was investigated by

measuring sample extracts immediately after preparation and after storing them in a

cooled autosampler (10 °C) for 3 and 7 days.

Data Analysis. Independent sample *t* test was conducted using SPSS 17.0 to

evaluate statistical differences. *P* < 0.05 denotes statistical significance.

Results

Calibration Curves. To date, it has been hard to find validation data for quantification of DMEs that meet the requirements of current bioanalytical guidelines. Therefore, the present study developed and validated modified method. In order to avoid producing major interference, we used control insect Supersomes[TM] instead of mammalian liver S9 as the matrices to construct calibration curves. The developed method was selective for determining all proteospecific peptides in digested insect Supersome[TM] and in digested MLS9. In most cases, peptide concentration linearly correlated with the analytical signal among the overall validation range (1.56-200 nM). The correlation coefficients (r^2) for all targeted signature peptides were better than 0.980 (1/x weighting) (Supplemental Table 2). And the MRM chromatograms of the standards were shown in Fig. 1.

Accuracy and Precision. The accuracy and precision data were shown in Supplemental Table 3. The inaccuracy values of the majority of signature peptides were less than 25% at three tested concentration levels, except for Ugt1a1 (-28.6%, 43.1% and -10.2% at low, middle, high concentration). Intra-and inter-day imprecision were below 15% in samples at all three concentration levels.

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Matrix effect. It is common to use isotope-labeled peptides to overcome the matrix effects for protein quantification. However, the standard addition method is an alternative for determining the matrix effects. In the present work, eight samples were randomly selected as test samples. A known amount of external standard peptides was spiked into test samples, and the samples were evaluated by UHPLC-MS/MS. The added amount of the peptides was calculated by subtracting the amount of peptides originally contained in the MLS9 from that in the spiked samples. The matrix effect was assessed by comparing the calculated amounts of spiked peptides with the known (i.e. externally added) amounts. The inaccuracy value of most measured peptides were less than 20%, however, the inaccuracy value of Cyp2c39, Ugt1a6a, Ugt2b34, and Sult1a1 in one of eight samples were higher than 20% (24%, 26.9% , 25.2%, and -32.2%, respectively). Nevertheless, the accuracies of our determinations were within acceptable ranges, suggesting that the standard addition method could be applied to measure matrix effects.

Extraction recovery and stability. The recovery was calculated due to all samples were processed using SPE. As shown in Supplemental Table 5, the recovery of most signature peptides was higher than 80% at the three concentration levels. The extraction efficiency for Sult2a1 and Sult4a1 was slightly lower but

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within the acceptable range of 70-80%. The recovery values were within acceptable ranges suggesting that this SPE protocol could be applied to clean-up protein digestion. The analytes displayed varying properties for all signature peptides. Therefore, it was hard to obtain the ideal range of 70-130%.   Autosampler stability was measured for 3 and 7 days at 10 °C. As shown in Supplemental Table 5, all peptides demonstrated sufficient stability ($\pm$20% of the initial concentrations at low, medium and high concentration) for 3 days in the autosampler rack. However, acceptable stability was observed for the middle and high conditions. By contrast, at low concentrations, isoforms Ugt2a3, 2b1, 2b5, and Sult1d1 failed to meet the requirements of the current bioanalytical guidelines (greater than 25%) for 7 days.

Enzyme expression profiles of 24 DMEs in S9 fractions prepared from male FVB mice. As shown in Figs. 2A-4A, all isoforms of the 24 DMEs could be quantified in the same individual samples. Fig. 2B and Supplemental Table 6 showed that the most abundant Cyp was Cyp2c29, with an average protein concentration of 88.2 pmol/mg, followed by Cyp2e1, at 34.1 pmol/mg. The average protein concentrations of Cyp3a11, Cyp1a2, and Cyp2d22 were within a similar range at 15.9, 15.6, and 11.9 pmol/mg, respectively. The average protein concentrations of five other measurable Cyps were below 5 pmol/mg. Fig. 3B and

supplemental Table 6 showed that among the Ugts in the liver, Ugt2b5, Ugt2b1, and

Ugt1a6a consistently had the highest enzyme expression levels, with average

protein concentrations of 38.6, 27.8, and 25.7 pmol/mg. Moderate levels of Ugt2b36,

Ugt1a9, Ugt1a1, Ugt2a3, and Ugt2b34 were detected in the liver, at 9.6, 9.5, 8.9, 8.6,

and 8.2 pmol/mg proteins, respectively. Ugt2b35, Ugt1a2, and Ugt1a5 were readily

detectable, but their expression levels were generally lower (below 5 pmol/mg

proteins) compared to other Ugts enzymes. The protein expression levels of three

Sults isoforms were shown in Fig. 4B and Supplemental Table 6. Sult1a1 was the

most abundant enzyme in the liver, at 8.2 pmol/mg protein, accounting for

approximately half of the total Sults content, followed by Sult3a1 (5.3 pmol/mg

protein) and Sult1d1 was 3.5 pmol/mg protein. To estimate the percentage

contributions of individual DME isoforms to the total enzymes present in the liver,

the mean expression levels for each enzyme were calculated and expressed as the

percentage of sum of all the means of all Cyps or Ugts or Sults present in the

sample. The complete data were plotted in Figs. 2C-4C. Several recent studies have

addressed the absolute concentration of CYP or UGT isoforms in human liver

microsomes. The ranks of DMEs in the current study were in good agreement with

the published data in humans, suggesting that the FVB mouse may be an ideal

animal model for predicting drug kinetics and toxicity in humans.

Enzyme expression profiles of 24 DMEs in S9 fractions prepared from female FVB mice. As shown in Figs. 5A-7A, the protein expression levels of the individual isoforms of DMEs were also determined in five female mouse liver S9 fractions. Cyp2c29, Ugt1a6a, and Sult1a1 had the highest protein expression levels, at 48.7 (Fig. 5B or Supplemental Table 6), 24.4 (Fig. 6B or Supplemental Table 6), and 32.1 pmol/mg protein (Fig. 7B or Supplemental Table 6), respectively. Overall, in female mice, Cyps, Ugts, and Sults were expressed in the following rank order, respectively: Cyp2c29 > Cyp2e1 > Cyp2c39 > Cyp2d22 > Cyp3a11 > Cyp1a2 > Cyp3a25 > Cyp1b1 > Cyp27a1 > Cyp7a1; Ugt1a6a > Ugt2b5 > Ugt1a1 > Ugt2b1 > Ugt2a3 > Ugt2b34 > Ugt1a9 > Ugt2b36 > Ugt2b35 > Ugt1a5 > Ugt1a2; Sult1a1 > Sult3a1 > Sult1d1. In addition, when the expression levels of each Cyp, Ugt, or Sult isoforms were compared. Cyp1a2 was found to be expressed at a significant lower level than Cyp2c29 and Cyp2e1 (more than 3 or 4-fold). Meanwhile, the expression level of Cyp2c29 was significantly higher (> 2-fold) than those of Cyp2d22 and Cyp3a11. Ugt1a6a was expressed at a significant higher level than the other isoforms (except for Ugt2b5). The protein expression level of Sult1a1 was higher than that of Sult1d1 (> 3-fold).

Comparison of 24 DMEs protein amounts in male and female FVB mice.

The protein expression levels of 24 DME isoforms in male mouse liver S9 fractions were compared against those in females. As shown in Fig. 8A, males and females displayed no difference in the protein expression levels of Cyp2e1, Cyp3a11, Cyp1b1, and Cyp3a25, whereas the protein expression level of Cyp2c39, Cyp2d22, and Cyp7a1 protein was female-specific. Cyp2c39 expression was significant higher in females than in males (more than 12-fold). Three isoforms (i.e. Cyp2c29, Cyp1a2, and Cyp27a1) were male-predominant. As shown in Fig. 8B, Ugt1a1 and Ugt1a5 exhibited higher expression levels in females than in male mice. By contrast, Ugt2b1, Ugt2b5, and Ugt2b36 were predominantly expressed in the liver of male mice. No gender difference was noted in the protein amounts of Ugt1a2, Ugt1a6a, Ugt1a9，Ugt2a3, Ugt2b34, and Ugt2b35. In addition, female mice displayed higher protein expression levels of the measured Sult isoforms than male mice (Fig. 8C). Sult1a1 and Sult3a1 were higher in females (more than 4-fold, and 7-fold, respectively) than in males. All differences were statistically significant. These data suggested that marked differences in gender-specific expression of DME isoforms exist in mice, and these differences potentially influence drug metabolism and pharmacokinetics.

Discussion

In this study, we first simultaneously determined the absolute protein expression levels of 24 metabolic enzymes in FVB mice using a UHPLC-MS/MS approach. Our results showed that 10 Cyps had detectable expression in FVB mice. As depicted in Fig. 8A, the most abundant Cyp was Cyp2c29, followed by Cyp2e1, in both male and female mice. Moreover, the isoforms differed in rank between male and female mice, as shown in Fig. 2C and Fig. 5C. The rank order of hepatic enzymes in male FVB mice was accordance with in human. As previously reported, CYP2E1 is the most abundant CYP in the liver, accounting for 32.0% of the total content, followed by CYP2C9 (18.8%), CYP3A4 (16.5%), CYP2C8 (13.6%), CYP1A2 (6.7%), CYP2B6 (4.8%), CYP2D6 (4.7%), CYP2C19 (1.1%), and CYP3A5 (1.0%) (Gröer et al., 2014; Yan et al., 2015). In female mice, the rank order of Cyp isoforms including Cyp2c39 and Cyp2d22, which mainly attributed to gender difference, were drastically increased (ranks 3 and 4, respectively).

In humans, it has been reported that UGT2B7 was the most abundant UGT (28% of the total). UGT2B4 accounted for 13% of the total protein amount. UGT2B15 and UGT1A4 each accounted for 12% of the total UGT protein content, followed by UGT1A1 (10%), UGT1A6 (10%), UGT1A3 (7%), UGT1A9 (7%), UGT2B10 (4%),

and UGT2B17 (4%). In mice, eight, three and seven functional members have been

identified for the Ugt1a, Ugt2a, and Ugt2b subfamilies (namely, Ugt1a1, Ugt1a2,

Ugt1a5, Ugt1a6a, Ugt1a7c, Ugt1a8, Ugt1a9, Ugt1a10; Ugt2a1, Ugt2a2, Ugt2a3; and

Ugt2b1, Ugt2b5, Ugt2b34, Ugt2b35, Ugt2b36, Ugt2b37, and Ugt2b38), respectively

(Rowland et al., 2013; Oda et al., 2015). As shown in Fig. 3C, the protein amount of

Ugts in male FVB mice consisted of Ugt2b5 (26%), Ugt2b1 (19%), Ugt1a6a (18%),

Ugt2b36 (7%), Ugt1a1, Ugt1a9, Ugt2a3, Ugt2b34 (6% of total, respectively),

Ugt2b35 (3% of total), and Ugt1a2 (1% of total).Interestingly, Ugt1a1 exhibited

significant higher expression in female than in male mice (~2-fold). It is well known

that human UGT1A1 plays a vital role in the glucuronidation of bilirubin, which leads

to hyperbilirubinemia. Therefore, our results on expression level of Ugt1a1 in FVB

mice may predict the metabolism of bilirubin in human.

In humans, the data showed SULT1A1, SULT2A1, SULT1B1, and SULT1E

were detected in human liver, accounting for 53%, 27%, 14%, and 6% of five Sults

isoforms, respectively. However, SULT1A3 was not detectable (Riches et al., 2009).

Alnouti and Klaassen conducted a branched DNA signal amplification assay and

found that Sult1a1, Sult1c1, Sult1c2, Sult1d1, Sult2a1, and Sult3a1 were expressed in

mouse liver. However, we only detected the expression of Sult1a1, Sult1d1, and

Sult3a1 at the protein level in mouse liver.

Similar to the findings of other studies that analyzed the mRNA level and activity of DMEs, the protein expression of several DMEs showed gender-specificity. For example, Cyp1a2 and Cyp2c29 exhibited higher expression in males, whereas Cyp2d22 and Cyp2c39 exhibited higher expression in females. And no gender difference was observed for Cyp3a11 and Cyp2e1 in mice. A previous study showed CYP1A2 activity is slightly lower in women than in men (Hrycay and Bandiera, 2009). Benzene and chlorzoxazone, which are the substrates of CYP2E1, were found to be eliminated slowly in women than in men in an experimental study on human exposures. Erythromycin, midazolam, verapamil, which are substrates of CYP3A4 considered as the human ortholog of mouse Cyp3a11, had higher clearance in women (Franconi et al., 2007; Hrycay and Bandiera, 2009). Cyp3a25 is a female-specific member of the CYP3A gene subfamily expressed in the mouse liver (Sakuma et al., 2000). Cyp2d22 has been posited to be the mouse ortholog of human CYP2D6 (Blume et al., 2000). Faster clearance of dextrometorphan, sertraline, desipramine, and metoprolol has been observed in men than in women, suggesting that CYP2D6 activity may be lower in women (Franconi et al., 2007). It has been recognized that mouse Cyp2c37,

Cyp2c38, Cyp2c39, and Cyp2c40 are all female-specific (Lofgren et al., 2008).

Consistent with the findings of a previous report revealed that Ugt1a1 and Ugt1a5 displayed a female-predominant mRNA expression. By contrast, Ugt2b1 exhibited male-predominant mRNA expression. Our results also demonstrated the female predominance of Ugt1a1 and Ugt1a5 protein expression. In comparison, Ugt2b1 and Ugt2b5 exhibited significantly higher protein amounts in male than in female mice. In general, members of the UGT1A gene family are largely conserved among species. Hence, special attention may be put on the gender differences of Ugt1a family.

Sulfotransferases belong to the major phase II DMEs that sulfoconjugate various substance (e.g. biogenic amines, steroid hormones, bile acids, and drugs). Previous studies indicated that the mRNA expression of Sult1a1, Sult1d1, Sult2a1/a2, and Sult3a1 in the liver and Sult4a1 in the brain displayed gender differences, with higher mRNA levels in females compared to males (Alnouti and Klaassen, 2006). Hence, we developed a method for quantifying the protein expression Sult1 (Sult1a1, Sult1b1, and Sult1d1), Sult3 (Sult3a1), and Sult4 (Sult4a1) superfamilies. However, it has been identified that Sult1b1 and Sult4a1 were mainly distributed in the digestive track and brain, respectively (Alnouti and Klaassen, 2006; Butcher et al., 2010). Consistently,

Sult1a1, Sult1d1, and Sult3a1 were also detectable in the liver in current work, with

higher protein expression in females than in males.

Immunoquantification methods have been extensively applied for quantifying the

amount of DMEs. Although data on gene expression can indicate the presence of

DMEs, these data cannot be applied to PBPK modeling because of the poor

correlation between mRNA and protein levels. Meanwhile, protein quantification by

WB is semiquantitative. To date, MS-based quantification has become main approach

for qualitative and quantitative assays of protein. In the present study, the developed

method could be used to quantify multiple enzymes with good linear range, stability,

and matrix effect. Although the inaccuracy values of some isoforms were somewhat

higher due to the complex sample preparation and analytical technique, our results

were comparable to those of previous studies. In addition, the similar rank order and

the gender differences of DMEs in protein levels with mRNA and activity levels

suggested the approach was accurate and precise.

The present study is the first to employ an isotope label-free UHPLC-MS/MS

approach to systematically and comprehensively perform studies for profiling 24

DMEs in FVB mice. Our findings suggested that the FVB animal model can be well

applied for the metabolism and subsequent pharmacokinetics of substrates of these

24 enzymes.

Acknowledgments

Authorship Contributions

Participated in research design: Jiamei Chen, Zhongqiu Liu, Tongmeng Yan.

Conducted experiments: Jiamei Chen, Xiaoyan Li，Haihui Zheng , Cong Xie, Sijing Zeng

Contributed new reagents or analytic tools: Lijun Zhu, Ming Hu, Zhongqiu Liu, Linlin Lu, Xiaoxiao Qi, Ying Wang,

Performed data analysis: Jiamei Chen, Xiaoyan Li, Lijun Zhu, Haihui Zheng, Jia Yu, Huangyu Jiang

Wrote or contributed to the writing of the manuscript: Jiamei Chen, Zhongqiu Liu Lijun Zhu.

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Footnotes

Figure Legends

Figure 1: Dynamic MRM chromatograms for the developed LC–MS/MS methods applied to measure proteotypic peptides. A, B, and C represent the standards of Cyps, Ugts, and Sults, respectively.

Figure 2: Protein expression levels of Cyp isoforms in the liver S9 fractions of male FVB mice (n=5). (A) Protein expression amounts of 10 Cyps, namely, Cyp1a2, Cyp1b1, Cyp2c29, Cyp2c39, Cyp2d22, Cyp2e1, Cyp3a11, Cyp3a25, Cyp7a1, and Cyp27a1. NO.1-NO.5 is the label of the mouse. All experiments were performed in triplicates, and the data were presented as mean ± SD. (B) Average expression levels of 10 Cyps in five FVB mouse liver samples. (C) The mean expression values for each enzyme are displayed as percentages of the total sum of the quantified 10 Cyps present in the liver.
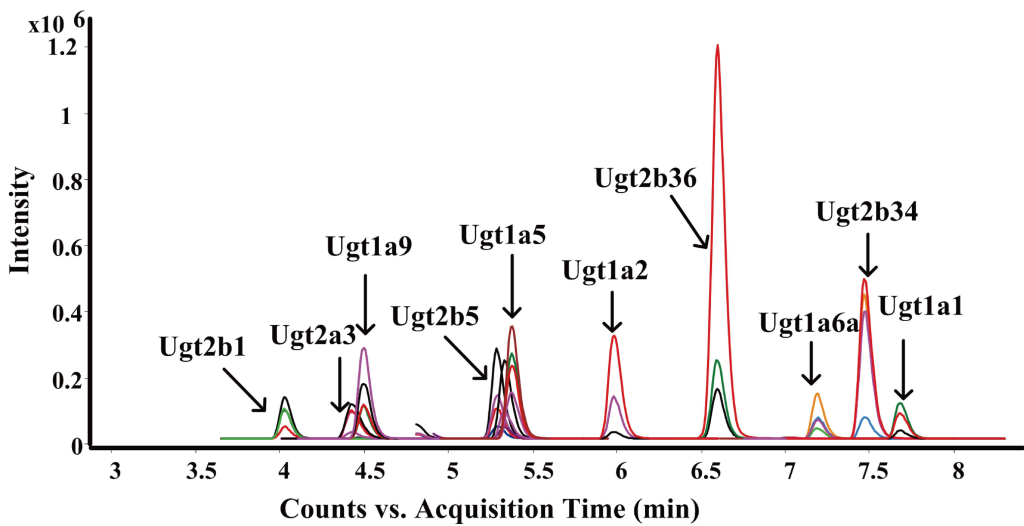
Figure 3: Protein expression levels of Ugt isoforms in male FVB mice (n=5) liver S9 fractions. (A) Protein expression amounts of 11 Ugts including Ugt1a1, Ugt1a2, Ugt1a5, Ugt1a6a, Ugt1a9, Ugt2a3, Ugt2b1, Ugt2b5, Ugt2b34, Ugt2b35, and Ugt2b36. All the experiments were performed in triplicate, and data were presented as mean ± SD. (B) Average expression levels of 11 Ugts in the five male FVB mice. (C) The mean expression values for each enzyme are displayed as percentages of the total sum

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of the quantified 11 Ugts present in the liver.

Figure 4: Protein expression levels of Sult isoforms in male FVB mice (n=5) liver S9

fractions. (A) Protein expression amounts of three Sults including Sult1a1, Sult1d1

and Sult3a1. All the experiments were performed in triplicate, and data were

presented as mean ± SD. (B) Average expression levels of 3 Sults in five male FVB

mouse liver. (C) The mean expression values for each enzyme were displayed as

percentages of the total sum quantified 3 Sults present in liver.

Figure 5: Hepatic expression profiles of 10 Cyp proteins in 5 female FVB mice. (A)

Protein expression amounts of 10 Cyps including Cyp1a2, Cyp1b1, Cyp2c29,

Cyp2c39, Cyp2d22, Cyp2e1, Cyp3a11, Cyp3a25, Cyp7a1, and Cyp27a1. All the

experiments were performed in triplicate, and data are presented as mean ± SD. (B)

Average expression levels of 10 Cyps in five FVB mice. (C) The relative abundance

of hepatic 10 Cyps based on average concentrations.

Figure 6: Hepatic expression profiles of 11 Ugts isoforms in female FVB mice (n=5)

liver S9 fractions. (A) Protein expression amounts of 11 Ugts including Ugt1a1,

Ugt1a2, Ugt1a5, Ugt1a6a, Ugt1a9, Ugt2a3, Ugt2b1, Ugt2b5, Ugt2b34, Ugt2b35 and

Ugt2b36. All the experiments were performed in triplicate, and data were presented as

mean ± SD. (B) Average expression levels of 11 Ugts in five FVB mice. (C) The

relative abundance of hepatic 11 Ugts based on average concentrations.

Figure 7：Protein expression levels of DME isoforms in female FVB mice (n=5) liver S9 fractions. (A) Protein expression amounts of 3 Sults including Sult1a1, Sult1d1 and Sult3a1. All the experiments were performed in triplicate, and data were presented as mean ± SD. (B) Average expression levels of 3 Sults in five FVB mice. (C) The relative abundance of hepatic 3 Sults based on average concentrations.

Figure 8: Protein expression levels of DME isoforms in the liver S9 fractions of male FVB mice compared to female FVB mice (n=5, respectively). (A) Protein expression amounts of 10 Cyps in male FVB mice compared to female FVB mice, including Cyp1a2, Cyp1b1, Cyp2c29, Cyp2c39, Cyp2d22, Cyp2e1, Cyp3a11, Cyp3a25, Cyp7a1, and Cyp27a1. (B) Protein expression amounts of 11 Ugts including Ugt1a1, Ugt1a2, Ugt1a5, Ugt1a6, Ugt1a9, Ugt2a3, Ugt2b1, Ugt2b5, Ugt2b34, Ugt2b35, and Ugt2b36, in male FVB mice compared to female FVB mice. (C) Protein expression amounts of 3 Sults in male FVB mice compared to female FVB mice, including Sult1a1, Sult1d1, and Sult3a1. Data were presented as mean ± SD. Data analysis was performed by the way of independent sample *t* test. "*" denotes statistical significance ($p < 0.05$)
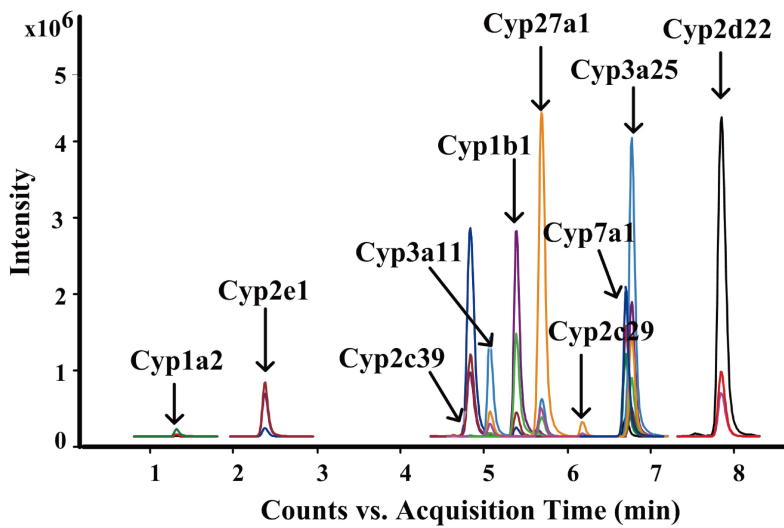
Figure 1

A

Cyp1a2

Cyp1b1

Cyp2c29

Cyp2c39

Cyp2d22

Cyp2e1

Cyp3a11

Cyp3a25

Cyp7a1

Cyp27a1

B

C

8.7% Cyp1a2
1.4% Cyp1b1
49.0% Cyp2c29
1.6% Cyp2c39
6.6% Cyp2d22
0.9% Cyp7a1
18.9% Cyp2e1
2.5% Cyp27a1
8.8% Cyp3a11
1.6% Cyp3a25
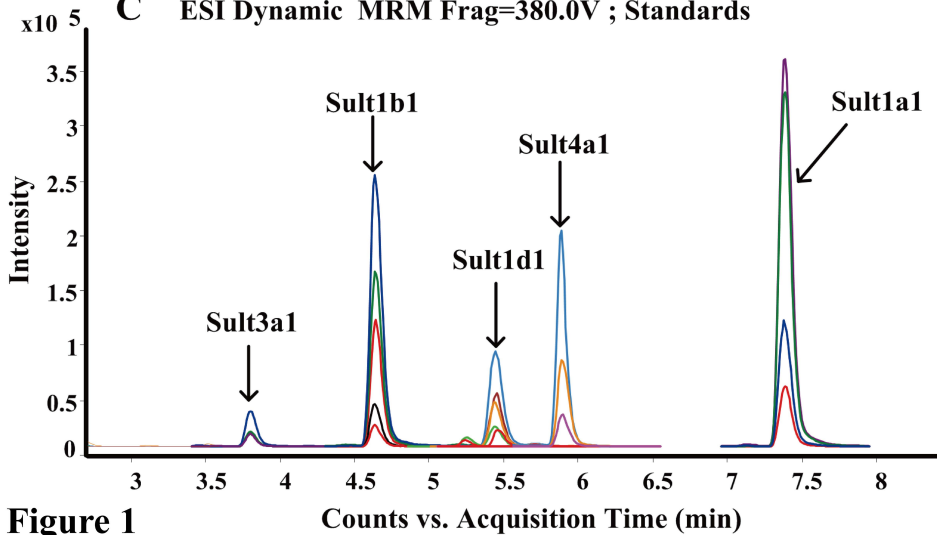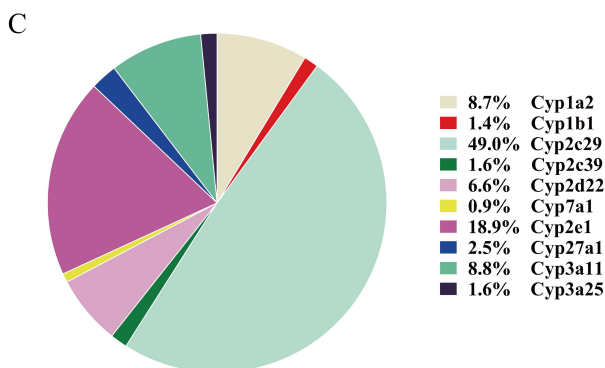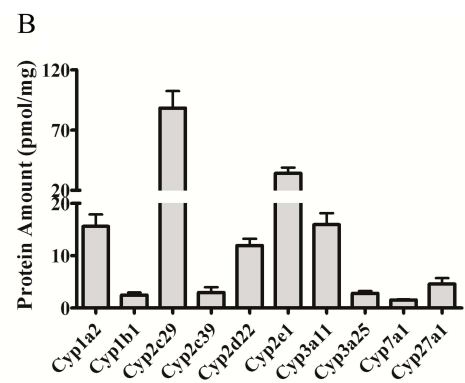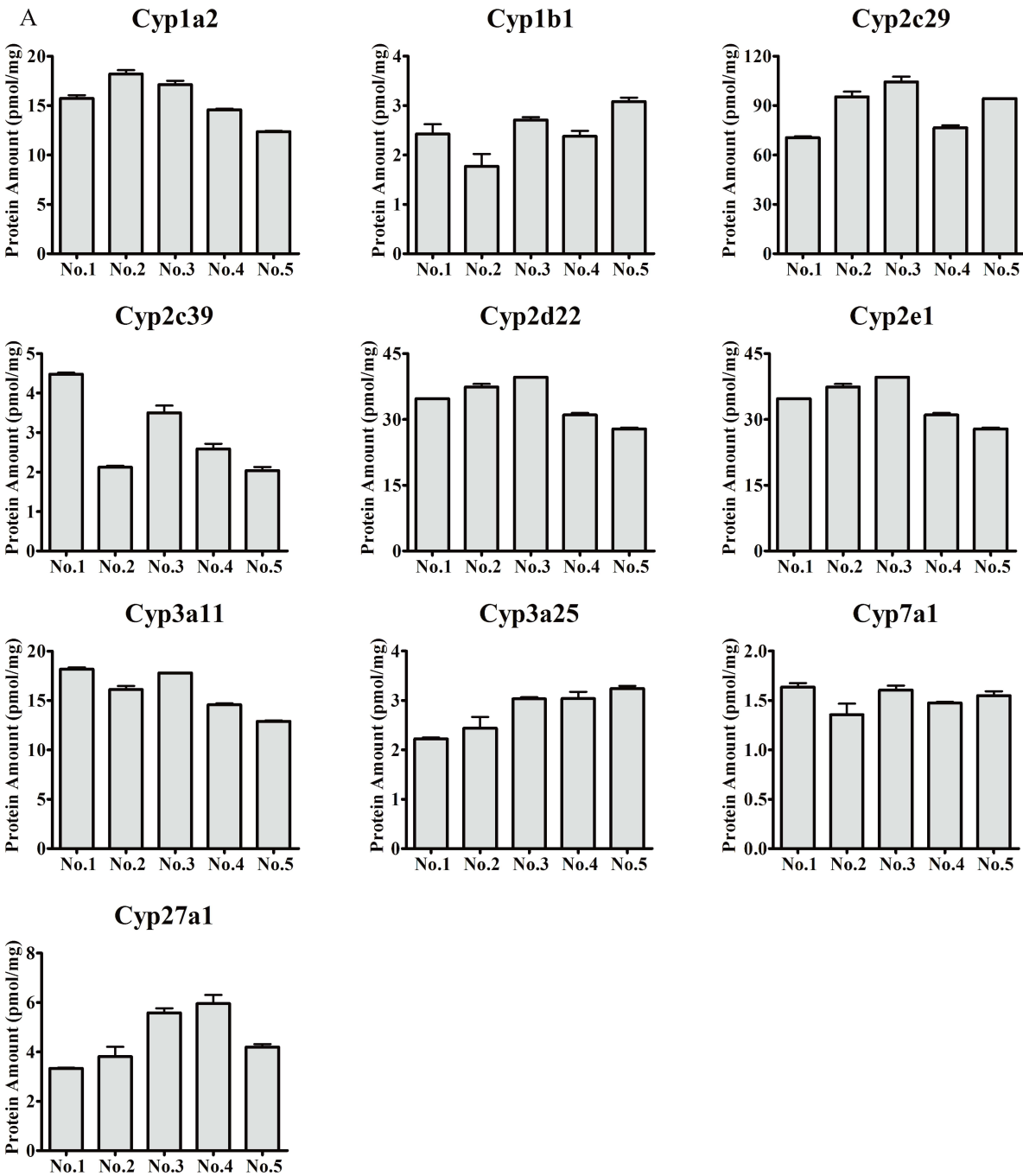
Figure 2

Figure 3

Figure 4

A

Cyp1a2

Cyp1b1

Cyp2c29

Cyp2c39

Cyp2d22

Cyp2e1

Cyp3a11

Cyp3a25

Cyp7a1

Cyp27a1

B

C

7.4% Cyp1a2
1.7% Cyp1b1
30.4% Cyp2c29
15.1% Cyp2c39
11.0% Cyp2d22
1.3% Cyp7a1
20.6% Cyp2e1
1.6% Cyp27a1
8.9% Cyp3a11
2.1% Cyp3a25
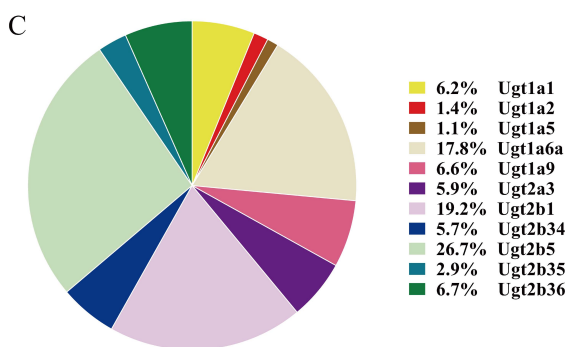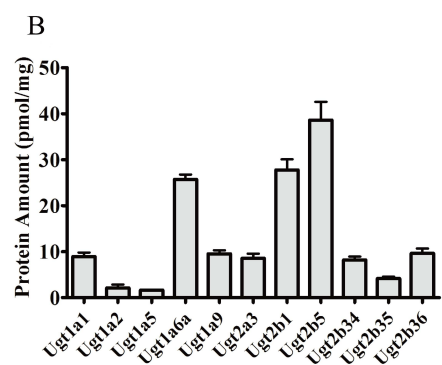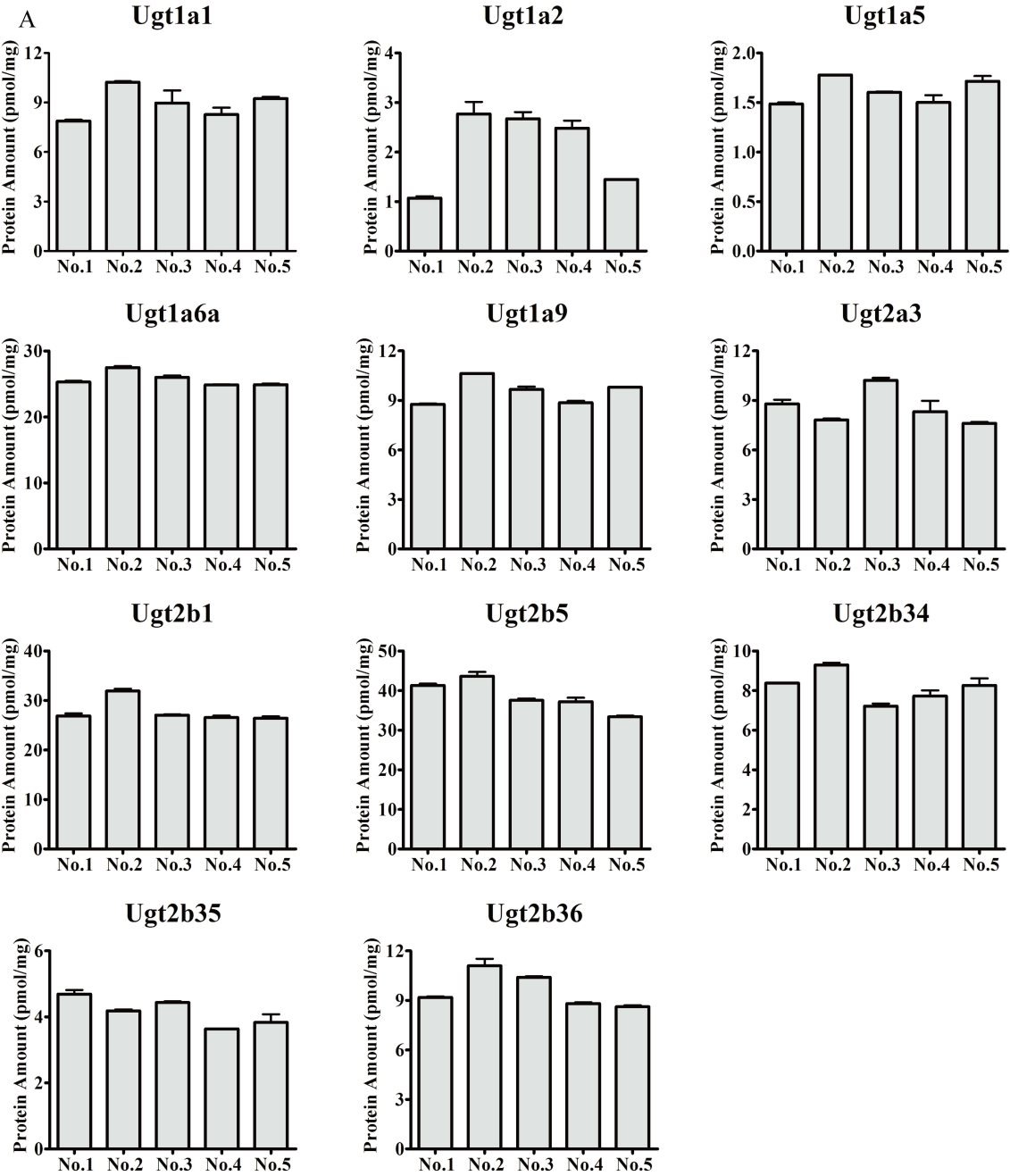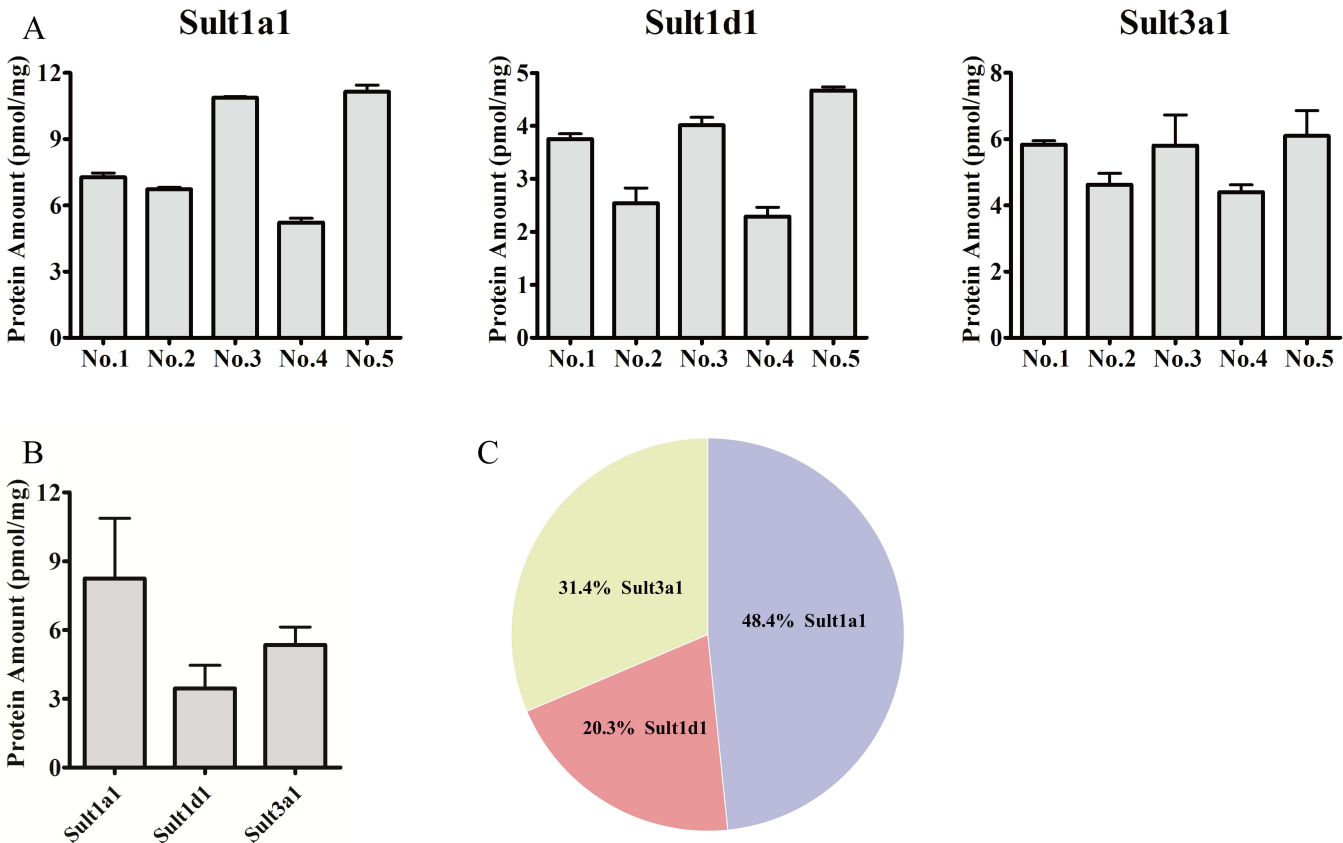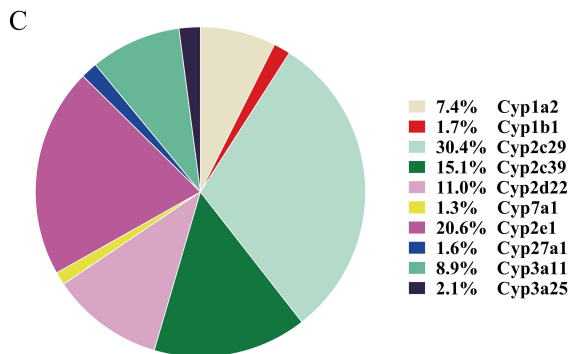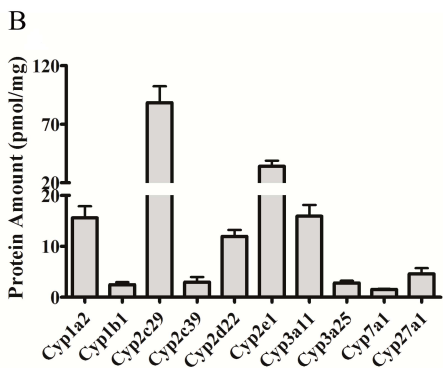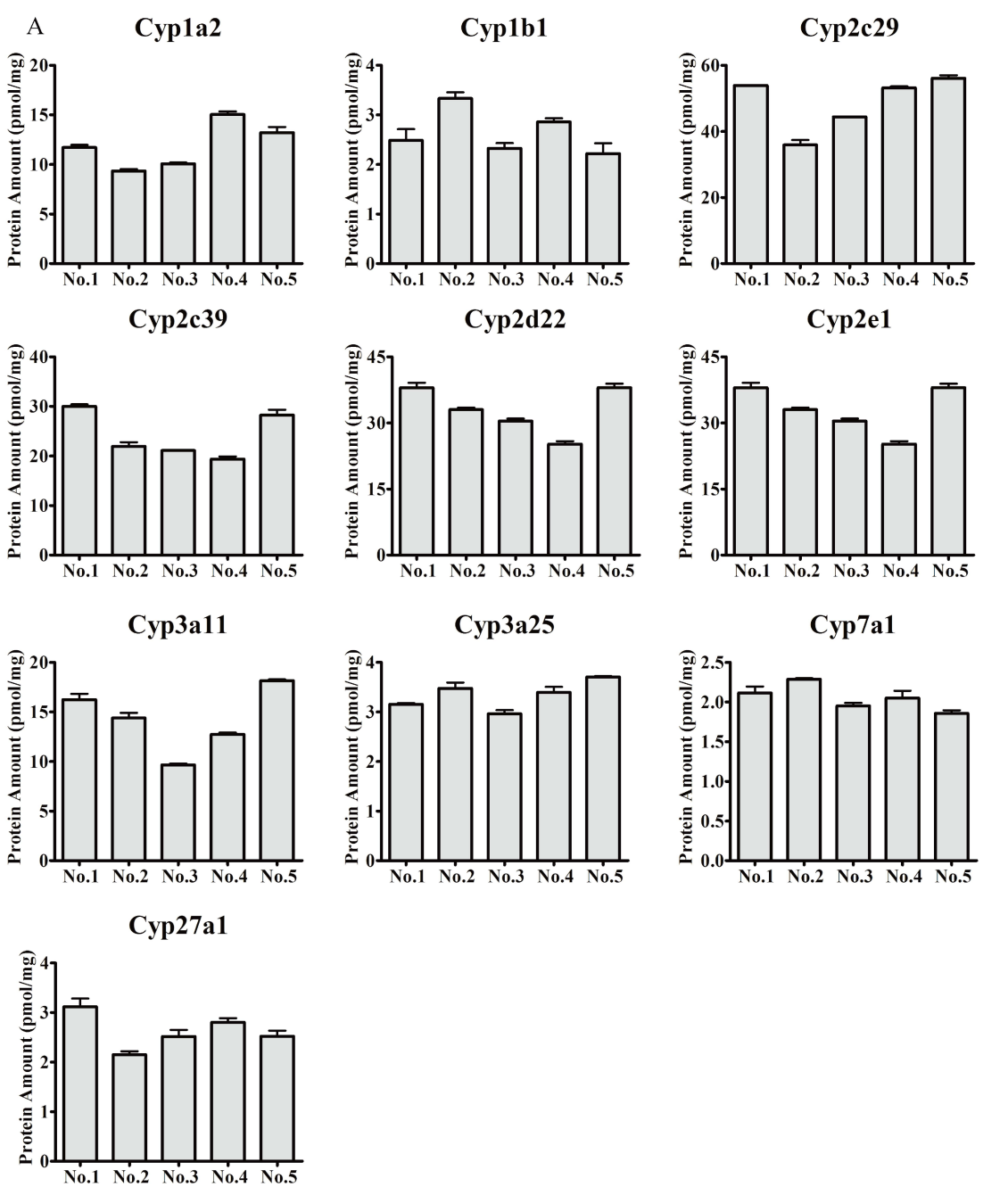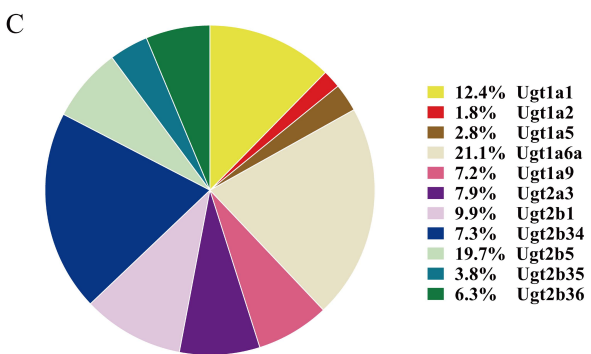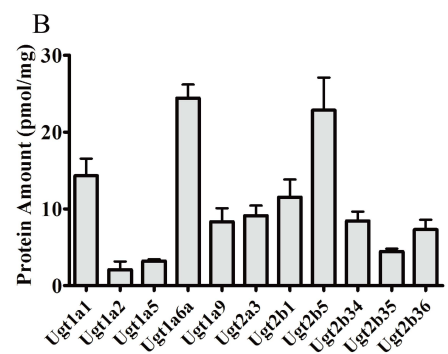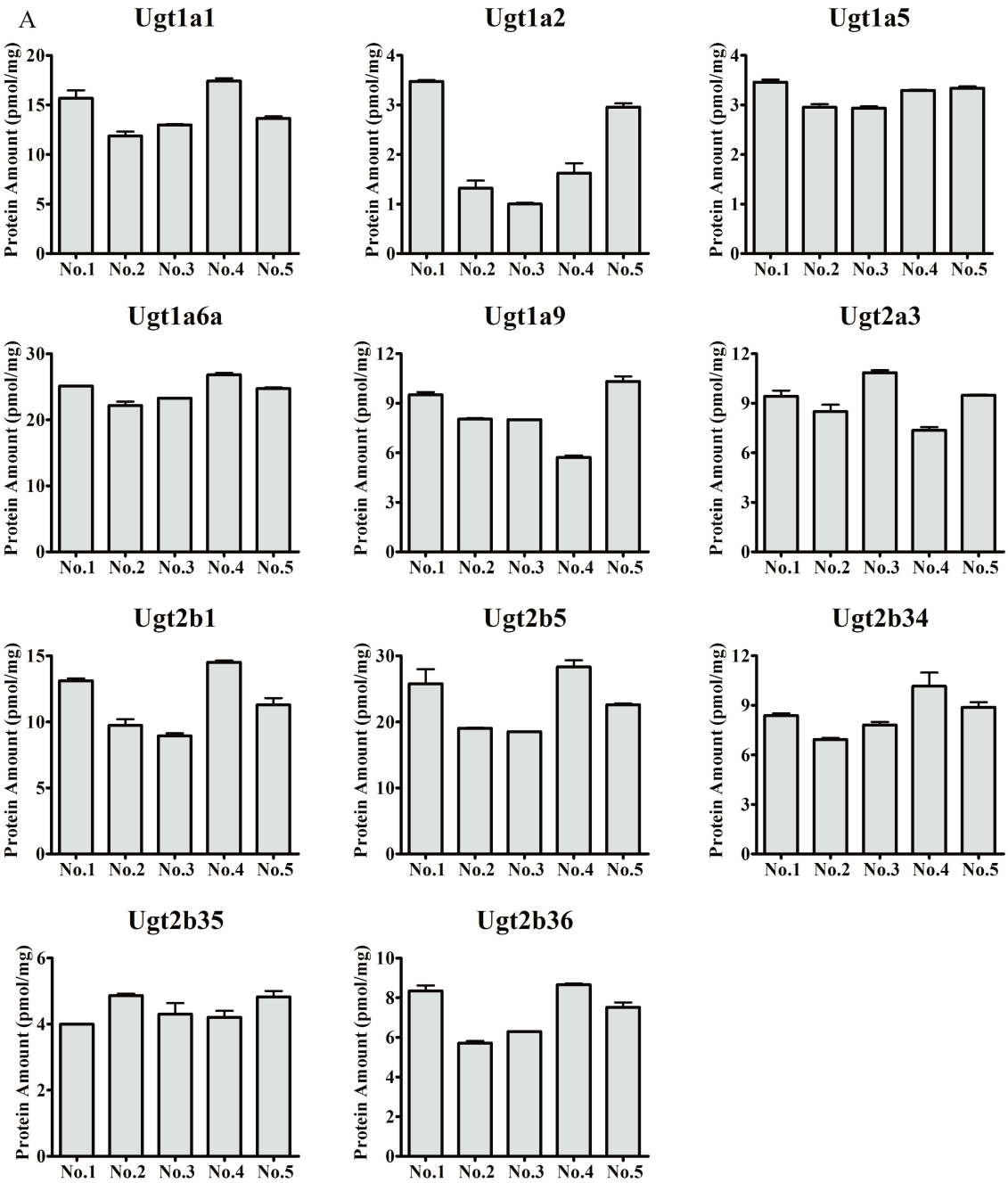
Figure 5

Figure 6

Figure 7

Figure 8