

DMD # 67538

TITLE PAGE

Quantification of Flavin-containing Monooxygenases 1, 3 and 5 in Human Liver Microsomes by
UPLC-MRM-based Targeted Quantitative Proteomics and Its Application to the Study of
Ontogeny

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DMD # 67538

Running Title Page

Running title: Targeted proteomic quantification of FMO enzymes (60 characters limit)

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Nonstandard abbreviations

CI, confidence interval; CYP, cytochrome P450; EPI, extracted product ion; FMO, flavin-containing monooxygenase; HLM, human liver microsomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; UGT, UDP-glucuronosyltransferase

DMD # 67538

Abstract

Flavin-containing monooxygenases (FMOs) have a significant role in the metabolism of small molecule pharmaceuticals. Among the five human FMOs, FMO1, FMO3 and FMO5 are the most relevant to hepatic drug metabolism. Although age-dependent hepatic protein expression, based on immunoquantification, has been reported previously for FMO1 and FMO3, there is very little information on hepatic FMO5 protein expression. To overcome the limitations of immunoquantification, a UPLC-MRM-based targeted quantitative proteomic method was developed and optimized for the quantification of FMO1, FMO3 and FMO5 in human liver microsomes (HLM). A post-*in silico* product ion screening process was incorporated to verify LC-MRM detection of potential signature peptides prior to their synthesis. The developed method was validated by correlating marker substrate activity and protein expression in a panel of adult individual donor HLM (age 39-67 years). The mean (range) protein expression of FMO3 and FMO5 was 46 (26 – 65) pmol/mg HLM protein and 27 (11.5 – 49) pmol/mg HLM protein, respectively. To demonstrate quantification of FMO1, a panel of fetal individual donor HLM (gestational age 14-20 weeks) was analyzed. The mean (range) FMO1 protein expression was 7.0 (4.9 – 9.7) pmol/mg HLM protein. Furthermore, the ontogenetic protein expression of FMO5 was evaluated in fetal, pediatric and adult HLM. The quantification of FMO proteins also was compared using two different calibration standards, recombinant proteins *vs.* synthetic signature peptides, to assess the ratio between holoprotein *vs.* total protein. In conclusion, a UPLC-MRM-based targeted quantitative proteomic method has been developed for the quantification of FMO enzymes in HLM.

DMD # 67538

Introduction

Flavin-containing monooxygenases (FMOs; EC 1.14.13.8) are FAD- and NADPH-dependent microsomal enzymes that have a significant role in the metabolism and detoxification of pharmaceutical, endogenous substances and environmental compounds. FMOs catalyze the oxygenation of soft nucleophilic heteroatom-containing (*e.g.*, N, S and P) organic substances, converting them to more readily excreted polar metabolites. Five functional human FMO isozymes have been discovered; among these, FMOs 1, 3 and 5 are relevant to hepatic drug metabolism (Krueger and Williams, 2005; Cashman and Zhang, 2006; Mitchell, 2008).

FMO1 and FMO3 are differentially expressed in the liver during development (*i.e.*, undergo a developmental transition). FMO1 expression, the major fetal isozyme, peaks early in gestation (first and second trimesters) and gradually decreases to undetectable at birth (Koukouritaki et al., 2002). In contrast, FMO3 expression, the major adult isozyme, turns on after birth and increases over time, reaching an adult level in the early teenage years (Koukouritaki et al., 2002). This differential enzyme expression has garnered much attention, specifically in terms of adjusting the dosage of FMO substrate drugs for infants and children (Yokoi, 2009; Yanni et al., 2010). *FMO5* mRNA expression in adult liver exceeds that of *FMO3* (Cashman and Zhang, 2006); however, earlier reports have suggested the opposite (Cashman, 1995; Cashman, 2000). In addition, *FMO5* mRNA expression in fetal livers is approximately one-sixth of that in adult livers (Cashman and Zhang, 2006). However, the ontogeny of hepatic FMO5 protein expression has not yet been characterized.

DMD # 67538

Traditionally, FMO enzyme quantification has relied on isozyme-specific antibody-based immunoquantification via Western blots. For absolute quantification, FMO content has been determined based on FAD content, the tightly-bound prosthetic group required for the catalytic activity of FMO holoproteins (Lang et al., 1998). Recombinant FMOs (*e.g.*, heterologously expressed in baculovirus-infected insect cells or Supersomes) have served as calibration standards (Yeung et al., 2000; Koukouritaki et al., 2002). Thus, previous studies have reported the quantification of FMO holoproteins, rather than total FMO proteins (*i.e.*, holoprotein + apoprotein).

To overcome the common limitations of immunoquantification (*i.e.*, cross-reactivity, dynamic range, reproducibility and multiplexity), liquid chromatography-tandem mass spectrometry (LC-MS/MS)- and multiple reaction monitoring (MRM)-based targeted quantitative proteomic methods have been developed for the absolute quantification of cytochrome P450s (CYPs), UDP-glucuronosyltransferases (UGTs) and membrane drug transporters (Fallon et al., 2008; Kamiie et al., 2008; Li et al., 2008; Wang et al., 2008). However, targeted quantitative proteomic methods for FMOs have yet to be reported. The term “absolute” quantification in these publications and the report herein refers to a type of proteomic quantification that produces protein concentration or amount, rather than “relative” protein expression profiles. A targeted quantitative proteomic method for absolute protein quantification relies on the use of either synthetic signature peptides of known concentration or signature peptides derived from the tryptic digest of target proteins of known concentration as calibration standards. The selection of appropriate signature peptides involves the *in silico* tryptic digestion of target proteins, followed by evaluation of the resulting candidate peptides based on several selection criteria to ensure

DMD # 67538

specificity, stability and digestion efficiency (Wang et al., 2008; Michaels and Wang, 2014; Peng et al., 2015). Candidate signature peptides (usually at least two for each protein) then can be synthesized and used to tune the MS (typically, a triple-quadrupole MS) for optimal MRM detection. However, some candidate signature peptides may not perform optimally due to poor digestion efficiency, chromatography or ionization during MS analysis, therefore rendering expensive signature peptides useless. Hence, it is desirable to incorporate an additional process(es) to verify candidate signature peptides following *in silico* prediction but prior to their synthesis.

The primary objective of the current study was to develop a UPLC-MRM-based targeted quantitative proteomic method for the absolute quantification of FMO1, FMO3 and FMO5 in human liver microsomes (HLM). The secondary objective was to evaluate post-*in silico* product ion screening of the target protein tryptic digest as a way to verify candidate signature peptides prior to their synthesis.

DMD # 67538

Materials and Methods

Chemicals, Enzymes, and Liver Tissues. Optima-grade acetonitrile, water, formic acid, and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA). Ammonium bicarbonate, dimethylsulfoxide, dithiothreitol, iodoacetamide, and cimetidine were purchased from Sigma-Aldrich (Saint Louis, MO). Cimetidine sulfoxide was acquired from Abcam Biochemicals (Cambridge, UK). Famotidine sulfoxide was acquired from Toronto Research Chemicals (Toronto, Canada). Recombinant human FMO1, FMO3 and FMO5 Supersomes, prepared from baculovirus-infected insect cells expressing human FMO enzymes, were purchased from Corning Gentest (Woburn, MA). The FMO concentration (pmol/mL and pmol/mg protein) of each Supersomes, based on the FAD content determined by an HPLC-fluorescence method (Lang et al., 1998), was provided by the supplier. Control Supersomes (Corning Gentest) contained microsomes from insect cells infected with wild-type baculovirus. Synthetic unlabeled AQUA Ultimate-grade signature peptides (5 pmol/ μ L \pm 5% by amino acid analysis) were ordered from Thermo Scientific (Ulm, Germany). Peptide purity (>97%), determined by RP-HPLC UV (detection wavelength of 215 nm) and MALDI-TOF MS, was provided by the manufacturer. Synthetic ^{13}C and ^{15}N stable isotope-labeled crude signature peptides also were acquired from Thermo Scientific. All synthetic peptide sequences were confirmed by MS/MS fragmentation analysis using a Waters Xevo TQ-S triple-quadrupole MS (Milford, MA). Sequencing-grade modified trypsin (cat. # V5113, lot 104493) was purchased from Promega (Madison, WI). Pooled HLM (XTreme 200) and nine individual adult donor HLM (Supplemental Table 1) were purchased from XenoTech, LLC (Lenexa, KS). Liver tissues from seven fetal (14-20 weeks gestation) donors and sixteen pediatric (aged 5 months-10 years) donors were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders

DMD # 67538

(Contract #HHSN275200900011C, Ref. No. NO1-HD-9-0011; Baltimore, MD) under an approved UNC-Chapel Hill IRB and were used to prepare fetal and pediatric HLM (Supplemental Table 1).

***In Silico* Selection of FMO Signature Peptides.** Candidate tryptic signature peptides for FMO quantification were selected *in silico* using criteria described previously (Wang et al., 2008; Michaels and Wang, 2014; Peng et al., 2015). The selected candidate peptides for each FMO protein are listed in Supplemental Table 2.

Trypsin Digestion. The tryptic digestion of FMO Supersomes and HLM was performed as described previously with minor modifications (Wang et al., 2008; Michaels and Wang, 2014). Briefly, protein samples (30 µg) were reduced in ammonium bicarbonate buffer (pH 8.0, 50 mM final concentration) containing dithiothreitol (4 mM final concentration) and heated at 60°C for 60 min to denature the proteins. After cooling to room temperature, the samples (90 µL total volume) were alkylated with iodoacetamide (10 mM final concentration) for 20 min in the dark prior to digestion with 1 µg trypsin at 37°C for 4 h unless stated otherwise. All reactions were carried out in Eppendorf Protein LoBind microcentrifuge tubes (Hamburg, Germany) to minimize protein and peptide loss due to binding. Solvent evaporation during the incubations was minimized by sealing the capped tubes with parafilm and applying pressure with an aluminum block. To optimize the trypsin digestion protocol, different digestion times (0.5, 1, 2, 4, 6, 8, 12 and 24 h) and protein-to-trypsin ratios (10, 20, 30, 40, 50, 60, 80 and 100:1) were examined. Reactions were cold-quenched with storage at -80°C. A mixture of stable isotope-

DMD # 67538

labeled signature peptides (1 μ L; internal standards) was spiked into the thawed samples prior to loading into a 6°C autosampler.

Signature Peptide Verification by Post-*In Silico* Product Ion Screening. After vortexing and centrifugation (16,000 g for 10 min at 4°C), the supernatants (10 μ L) of the quenched digestion mixtures underwent UPLC-MS/MS analysis. The UPLC-MS/MS instrument, consisting of a Waters Acquity UPLC I-class binary solvent manager coupled with a Waters Xevo TQ-S triple-quadrupole MS, was operated under positive electrospray ion mode. Chromatographic separation of the peptides was carried out on a reversed-phase column (Waters UPLC BEH-C18, 1.7 μ m, 2.1 x 100 mm), fitted with an in-line column filter and a VanGuardTM guard-column (Waters). The mobile phases consisted of (A) water containing 0.1% (v/v) formic acid and (B) acetonitrile containing 0.1% (v/v) formic acid. A 13.5 min gradient (0.4 mL/min) began with 2% B held for 1 min, followed by an increase to 15% B over 2 min, and to 30% B over the next 7 min. The column was washed with 95% B for 1.5 min and then re-equilibrated with 2% B for 2 min prior to the next injection.

To detect the *in silico*-selected candidate signature peptides, product ion scans were set up using selected precursor ions corresponding to the doubly protonated ions of the candidate peptides in the Q1 quadrupole, fragmenting these precursor ions with a collision energy ramp (15-40 V) in the Q2 quadrupole, and mass analysis of the product ions in the Q3 quadrupole mass analyzer under a scan rate of 5000 amu/s. Extracted product ion (EPI) chromatograms of all the possible y ions of each candidate peptide were generated using Masslynx (Version 4.1; Waters) to allow visual inspection for product ion screening. A salient peak shared by most or all y ion EPI

DMD # 67538

chromatograms verified the detection of the corresponding signature peptide. Upon detection verification, the signature peptide sequences were sent for synthesis (Thermo Scientific).

UPLC-MRM Analysis. Lyophilized stable isotope-labeled signature peptides were dissolved in 1 mL of 1:1 (v/v) acetonitrile:water solution. The solution was diluted further to approximately 2-4 $\mu\text{g/mL}$ and then infused into the Xevo TQ-S MS at 5 $\mu\text{L/min}$ with an LC flow of 50% B at 0.4 mL/min. MRM parameters were optimized using IntelliStart (Waters) under positive electrospray ion mode: capillary voltage, 1.5 kV; cone voltage, 40 V; source offset, 40 V; dissolution temperature, 500°C; dissolution gas, 1000 L/h; nebulizer gas, 7 bar. The optimum collision energy and precursor/product masses for the signature peptides are summarized in Table 1. UPLC-MRM quantification was performed using the peak area ratios of signature peptides to corresponding stable isotope-labeled signature peptides (internal standards).

Preparation of Calibration Standards. Two types of calibration standards were prepared for the absolute quantification of FMOs in HLM. First, recombinant FMO1, FMO3 and FMO5 Supersomes of known concentrations (based on FAD content) were used to build calibration standards (0.005 to 20 pmol/digestion). Quality controls (QCs), consisting of FMO Supersomes at 0.2, 1 and 10 pmol/digestion, were prepared in triplicate. All recombinant protein standards and QCs were denatured, alkylated and trypsin-digested as described above, prior to UPLC-MRM analysis. Due to the varying amount of total proteins in the standards, additional trypsin (2 μg total) was used to keep the protein:trypsin ratio $\leq 30:1$ in the high concentration standards. Second, synthetic signature peptides of known concentrations (based on amino acid analysis) were used to build calibration standards (0.02 to 20 pmol/digestion). To normalize total protein

DMD # 67538

loading, control Supersomes (30 μ g) were spiked into the peptide standards. The spiked peptide standards also were denatured, alkylated and trypsin-digested prior to UPLC-MRM analysis. The lower limit of quantification was defined as the lowest standard concentration with signal-to-noise ratio > 5 and acceptable precision and accuracy (within 20%).

FMO Marker Substrate Activity Assay. Cimetidine sulfoxidation was used to measure FMO functional activity as described previously (Cashman et al., 1993; Overby et al., 1997). Cimetidine (1 mM; reported K_m values are 4 mM for FMO3 and >10 mM for FMO5) was pre-incubated with HLM (0.1 mg/mL) in a phosphate buffer (pH 7.4, 100 mM) containing 3.3 mM $MgCl_2$ for 5 min at 33°C. Although these conditions were different from what was used by Zane et al. (the companion paper), *i.e.*, substrate concentration and incubation temperature, they served the purpose of validating protein quantification by correlating marker substrate activity and protein expression. Reactions (200 μ L final volume) were initiated by the addition of NADPH (1 mM final concentration). Aliquots (10 μ L each) were removed from each reaction at 1 and 5 min and transferred to tubes containing ice-cold acetonitrile (300 μ L) and famotidine sulfoxide (10 nM; internal standard). Quenched reaction mixtures were centrifuged (2250 g for 20 min at 4°C) and the resulting supernatants (100 μ L) dried under nitrogen at 50°C. The dried samples were reconstituted in water (150 μ L) prior to UPLC-MS/MS quantification of cimetidine sulfoxide using the Xevo TQ-S triple-quadrupole MS operated under positive electrospray ion mode. Analytes were separated on a reversed-phase analytical column (Thermo Scientific Aquasil C18, 2.1 \times 50 mm, 3 μ m; Bellefonte, PA). The gradient (0.4 mL/min) began at 0% B for 0.5 min, then quickly increased to 5% B and was held there for 3 min. The column was washed with 100% B for 1 min and re-equilibrated at 0% B for 0.5 min prior to the next injection. UPLC-MS/MS

DMD # 67538

quantification was performed using the peak area ratios of cimetidine sulfoxide to famotidine sulfoxide. Cimetidine sulfoxide calibration standards ranged from 0.1 to 100 μ M. Cimetidine sulfoxidation rates were determined from the amount of metabolite generated between the 1 and 5 min reaction times. Cimetidine sulfoxide formation was linear for a minimum of 30 min under the described conditions (data not shown). Since FMO enzymes are heat labile in the absence of NADPH, their stability was examined during the pre-incubation (5 min at 33°C) with substrate only. Results showed no significant difference in cimetidine sulfoxidation activities of recombinant FMO1, FMO3, and the pooled HLM between pre-incubation with substrate cimetidine only and pre-incubation with NADPH (data not shown), indicating stability of FMO enzymes during the pre-incubation with substrate only.

Data Analysis. The final FMO protein concentration was the average value determined using two signature peptides for each FMO protein. All average values were calculated as the mean. For correlation analysis, measured cimetidine sulfoxide formation rates in HLM were plotted versus FMO protein concentration in the same sample and the Pearson r and P values were reported since all relevant data passed normality test (Supplemental Table 3). The slope and Y -intercept values were determined by least-square linear regression analysis. Student's t tests (two-tailed, unpaired) were used to compare the pairs of signature peptides. One-way analysis of variance (ANOVA) followed by post hoc test using Tukey's adjustment was used to compare FMO5 expression in the fetal, pediatric and adult HLM. $P < 0.05$ was considered significant. All data analyses were performed using GraphPad Prism (v. 5.0; San Diego, CA).

DMD # 67538

Results

Verification of Signature Peptides by Post-*In Silico* Product Ion Screening. After the initial *in silico* selection of human FMO3 signature peptides, eight candidate peptides (Supplemental Table 2) satisfied every selection criteria described previously (Wang et al., 2008; Peng et al., 2015). To select the final signature peptides (two for each protein) from the candidate peptides, recombinant FMO3 was reduced, alkylated and trypsinized, and the resulting digest separated on a UPLC analytical column. Analysis was completed through product ion screening of the doubly charged ions of the candidate peptides. Representative EPI chromatograms of predicted y ions for the two final FMO3 signature peptides selected for use in this study (FMO3_pep1_L and FMO3_pep4_L; Table 1) are shown in Figures 1A and 1B, respectively. The signature peptides produced salient peaks in each EPI chromatogram (2.5 min peak for FMO3_pep1_L and 5.5 min peak for FMO3_pep4_L) and the product ion mass spectra integrated across the peaks matched each peptide sequence (Figures 1C and 1D). In addition, EPI chromatograms of predicted y ions for the remaining six FMO3 candidate signature peptides are shown in Supplemental Figure 1. Likewise, EPI chromatograms of predicted y ions for the final FMO1 and FMO5 signature peptides (Table 1) also were examined and verified for optimal UPLC-MRM detection (data not shown).

After identification and verification of the predicted signature peptides, unlabeled signature peptides and corresponding ^{13}C and ^{15}N stable isotope-labeled signature peptides (Table 1) were synthesized and used for the development and optimization of a UPLC-MRM method. This method allows for the multiplexed detection and quantification of FMO1, FMO3 and FMO5 in

DMD # 67538

HLM. Representative UPLC-MRM chromatograms of signature peptides in tryptic digests of adult HLM and fetal HLM are shown in Figure 2.

Effects of Trypsin Digestion Time and Protein:Trypsin Ratio. To optimize trypsin digestion conditions and determine the dynamic range, the effects of digestion time and protein:trypsin ratio on the absolute quantification of FMOs in pooled HLM were evaluated using the developed UPLC-MRM method. The relative UPLC-MRM signals of the signature peptides reached a maximum after 4 h of digestion and plateaued (or decreased slightly in some cases) thereafter (Figures 3A and 3B). Due to low expression of FMO1 in pooled HLM, only one of the two FMO1 signature peptides was detected and evaluated (Figure 3B). As a result, tryptic digestion was carried out for 4 h for the remainder of the study. In addition, the relative UPLC-MRM signals of the signature peptides increased linearly with respect to HLM protein loading between 10 μ g to 100 μ g when 1 μ g trypsin was used (Figures 3C and 3D); however, a slight downward deviation was noticed above 50 μ g of HLM protein. Thus, optimized trypsin digestion conditions, 4 h digestion and 30:1 protein:trypsin ratio, were selected and utilized for the absolute quantification of FMO1, FMO3 and FMO5 in HLMs.

Absolute Quantification of FMO3 and FMO5 in Adult HLM and Correlation to Marker Substrate Activity. Similar to the immunoquantification and targeted proteomic quantification of CYPs (Wang et al., 2008; Michaels and Wang, 2014), recombinant FMO Supersomes of known concentrations were used initially to create calibration standards. The concentrations of the recombinant FMO Supersomes, based on FAD content, were provided by the vendor. The calibration curves for each recombinant FMO Supersome (0.01 to 4 pmol/ digestion; 10-12

DMD # 67538

concentrations) demonstrated good linearity ($r^2 > 0.99$). Using 30 μg of HLM, the observed lower limit of quantification for the three FMOs was 0.33 pmol/mg HLM protein. The intraday accuracy (percent deviation) and precision (CV) of the analytical method, based on QC samples, were within 15%.

Method coherence was evaluated by comparing protein quantification results from two different signature peptides of the same protein (*i.e.*, FMO3_pep1_L vs. FMO3_pep4_L and FMO5_pep1_L vs. FMO5_pep6_L). In each case, a strong correlation, near-unity slope and near-zero Y-intercept were observed (Figures 4B and 4C), indicating consistent protein quantification results between the different signature peptides. In addition, good coherence was observed for two FMO1 signature peptides when fetal HLM were analyzed (Figure 4A; described below). As a result, final protein concentrations were calculated as the average of the quantification results from the two signature peptides.

Using a panel of adult HLM ($n = 9$ individual donors and 1 pooled), the protein concentrations of the three FMOs were determined using the developed targeted quantitative proteomic method. The FMO1 concentration in adult HLM was below the lower limit of quantification (<0.33 pmol/mg HLM protein). The final FMO3 and FMO5 average protein concentrations (range and 95% confidence interval [CI]) were 46 (26 – 65 and 36 – 56) and 27 (11.5 – 49 and 18.5 – 36) pmol/mg HLM protein, respectively. Furthermore, cimetidine sulfoxidation activities were measured in the HLM panel and compared to FMO protein concentrations. A strong correlation was observed between cimetidine sulfoxidation activity and FMO3 protein concentration ($r^2 =$

DMD # 67538

0.86, $P = 0.0001$; Figure 5A), but not FMO5 protein concentration ($r^2 = 0.30$, $P = 0.103$; Figure 5B).

Absolute Quantification of FMO1 and FMO5 in Fetal HLM and Correlation to Marker

Substrate Activity. To evaluate the method for absolute quantification of FMO1, a panel of fetal HLM ($n = 7$ individual donors) was analyzed; the adult HLM panel lacked FMO1 expression. The final FMO1 average protein concentration (range and 95% CI) in the fetal HLM panel was 7.0 (4.9 – 9.7 and 5.2 – 8.7) pmol/mg HLM protein. In addition, there were appreciable amounts of FMO5, which averaged 21 (14 – 32 and 14 – 29) pmol/mg HLM protein (Figure 6A). In contrast to adult HLM, FMO3 was barely above lower limit of quantification (0.33 pmol/mg HLM protein) in fetal HLM, averaging 0.7 pmol/mg HLM protein with a highest concentration of 2.2 pmol/mg HLM protein. In addition, cimetidine sulfoxidation activity also was measured in the fetal HLM panel and compared to FMO protein concentrations. Neither FMO1 ($r^2 = 0.41$, $P = 0.12$) nor FMO5 ($r^2 = 0.01$, $P = 0.83$) protein concentration correlated with the marker substrate activity (data not shown). Since FMO5 was reported to lack appreciable cimetidine sulfoxidation activity (Overby et al., 1997; Hai et al., 2009), correlation using a relative activity factor-adjusted FMO expression was not attempted.

FMO5 Expression in Fetal, Pediatric and Adult HLM. In addition to fetal and adult HLM described above, a panel of pediatric HLM ($n = 16$ individual donors; Supplemental Table 1) was analyzed for FMO1, FMO3 and FMO5 expression. The FMO1 and FMO3 expression in the pediatric HLM has been reported (Zane et al., the companion paper). The final FMO5 average protein concentration (range and 95% CI) in the pediatric HLM panel was 36.2 (2.9 – 110 and

DMD # 67538

20.1 – 52.3) pmol/mg HLM protein (Figure 6B). There was no statistically significant difference among the three age groups ($P = 0.317$; Figure 6C).

Comparison of Recombinant Proteins vs. Synthetic Peptides as Calibration Standards for

Absolute Quantification. Previously, our laboratory and others have reported signature peptide-dependent absolute quantification of CYPs and drug transporters using synthetic peptides as calibration standards (Wang et al., 2008; Balogh et al., 2013; Michaels and Wang, 2014; Prasad et al., 2014; Peng et al., 2015) . To assess such a scenario for the absolute quantification of FMOs, two signature peptides were selected for each FMO isozyme (Table 1) and quantification coherence between the two peptides was evaluated. When recombinant FMO Supersomes of known concentration were used to generate signature peptide standards, good coherence was observed, as described above (Figure 4). However, when synthetic peptides of known concentrations were used to generate signature peptide standards, good coherence was observed for FMO1, but not for FMO3 or FMO5 (Figure 7).

Absolute FMO concentrations measured using synthetic peptide standards were substantially greater than those determined using recombinant protein standards (*i.e.*, Supersomes) (Figure 7 vs. Figure 4). For example, the average FMO1 concentration in fetal HLM was 7.0 pmol/mg HLM protein with recombinant protein standards. In contrast, it was 29 or 32 pmol/mg HLM protein (4- to 5-fold higher) with synthetic FMO1_pep1_L or FMO1_pep2_L standards, respectively. Similarly, the average FMO3 and FMO5 concentrations in adult HLMs were 46 and 27 pmol/mg HLM protein, respectively, with recombinant protein standards. In contrast, they were 259 or 412 pmol/mg HLM protein (5.6- to 9-fold higher) for FMO3 with synthetic

DMD # 67538

FMO3_pep1_L or FMO3_pep4_L standards and 21 or 32 pmol/mg HLM protein (0.8- to 1.2-fold higher) for FMO5 with synthetic FMO5_pep1_L or FMO5_pep6_L standards.

Absolute Quantification of FMOs in Recombinant FMO Supersomes using Synthetic Peptide-generated Calibration Standards. To further investigate discrepancies in the absolute quantification of FMOs when recombinant proteins *vs.* synthetic peptides were used as standards, and determine the ratios of holoprotein *vs.* total protein, total FMO protein was quantified in recombinant FMO Supersomes of different concentrations using synthetic peptides as calibration standards. The measured total FMO protein amount was plotted against the nominal FMO protein amount based on FAD content, which represents the FMO holoprotein (Figure 8). Similar to the previously described signature peptide-dependent quantification, the ratio of total protein *vs.* holoprotein (slopes in Figure 8) for each recombinant FMO Supersomes also was dependent upon the signature peptide used. The ratio ranged from 5.0 to 5.6 for FMO1, 6.0 to 8.4 for FMO3, and 0.9 to 1.5 for FMO5.

DMD # 67538

Discussion

In this study, a UPLC-MRM-based targeted quantitative proteomic method has been developed for the multiplexed absolute quantification of FMOs 1, 3 and 5 in HLM. This method has a lower limit of quantification of 0.33 pmol/mg HLM protein for each FMO when 30 μ g of HLM is used. Using the developed quantitative proteomic method, protein concentrations of the three FMOs were determined in three panels of HLM, one derived from adult livers ($n = 10$), one from pediatric livers ($n = 16$), and one from fetal livers ($n = 7$). In the adult HLM, FMO3 was more abundant than FMO5 (46 *vs.* 27 pmol/mg HLM protein), which supports earlier reports (Cashman, 1995; Cashman, 2000) but not the most recent report (Cashman and Zhang, 2006), both of which were based on mRNA expression. FMO1 was below the lower limit of quantification (0.33 pmol/mg HLM protein). In the fetal HLM (14-20 weeks gestation), FMO1 was expressed at relatively high levels (7.0 pmol/mg HLM protein), similar to what was reported previously (7.8 pmol/mg HLM protein; 8-15 weeks gestation) (Koukouritaki et al., 2002). FMO3 was barely above the lower limit of quantification, averaging 0.7 pmol/mg HLM protein. Interestingly, FMO5 was the predominant FMO isozyme in fetal HLM, averaging 3-fold greater protein expression than FMO1 (21 *vs.* 7.0 pmol/mg HLM protein). In the pediatric HLM, FMO5 also appeared to be the predominant FMO isozyme (36.2 *vs.* 20.0 pmol/mg HLM protein for FMO3), whereas FMO1 was barely detected (Zane et al., the companion paper). Although FMO5 expression was not significantly different among the three age groups, larger interindividual variability was observed in the pediatric HLM (38- fold *vs.* 4.3- and 2.3-fold in adult and fetal HLM, respectively) (Figure 6). These targeted quantitative proteomic results confirm previous reports that FMO1 and FMO3 expression undergo a developmental transition and also discovered that FMO5 has relatively stable expression throughout development.

DMD # 67538

However, these results should be interpreted with caution, as our study only included a small number of HLM from each age group, fetal samples only represented the second trimester, and neonatal samples (birth to first month) were absent (Supplemental Table 1). As such, future studies employing larger panels of HLM are warranted.

LC-MRM-based targeted quantitative proteomic methods for the absolute quantification of CYPs, UGTs and drug transporters were first reported in the late 2000s (Fallon et al., 2008; Kamiie et al., 2008; Li et al., 2008; Wang et al., 2008). These methods rely on the identification and detection of signature peptides for each target protein. The selection and verification of suitable signature peptides can be time-consuming and costly, mainly due to peptide synthesis after *in silico* selection. The ability to verify LC-MRM detection of the selected signature peptides in a protein digest prior to committing to peptide synthesis is therefore desirable. As such, we implemented a post-*in silico* product ion screening step to verify the detection of selected signature peptides (Figure 1) prior to their synthesis in order to reduce unnecessary peptide synthesis and costs. For example, only two FMO3 signature peptides (FMO3_pep1_L and FMO3_pep4_L) were synthesized in this study, rather than all eight candidate signature peptides (Supplemental Table 2).

To achieve absolute quantification using an LC-MRM-based targeted proteomic approach, two types of standards are typically employed, recombinant proteins of known concentration or synthetic signature peptides of known concentration. Due to the poor coherence (*i.e.*, signature peptide-dependent quantification) when synthetic peptides were employed as standards (Wang et al., 2008; Balogh et al., 2013; Michaels and Wang, 2014; Prasad et al., 2014; Peng et al., 2015),

DMD # 67538

we prefer to use recombinant proteins when available (e.g., CYPs) to generate standards and employ at least two signature peptides for each protein to ensure quantification coherence. In the current study, poor quantification coherence was observed for FMO3 and FMO5 when synthetic peptides were used as standards (Figures 7B and 7C). Presumably, the presence of multiple acidic amino acid residues (D or E) close to the tryptic cleavage sites (e.g., FMO1_pep1, FMO3_pep1 and FMO5_pep1; Table 1) could cause missed cleavage (Yen et al., 2006), resulting in lower recovery of signature peptides and underestimation of protein concentration. In contrast, quantification was coherent between signature peptides for all three FMOs when recombinant FMO Supersomes were used to generate standards (Figure 4). Therefore, we recommend the use of recombinant proteins, when available, to generate standards for LC-MRM-based targeted protein quantification. Moreover, we call for a coordinated effort to produce reference protein standards, especially in the case of drug transporters, for use as calibration standards for targeted quantitative proteomics. It is not completely understood yet what may cause the lack of coherence in signature peptide-dependent quantification when synthetic peptides are used as standards. We have proposed that different digestion efficiencies (e.g., missed cleavage) and/or unexpected post-translational modifications of signature peptides were the underlying causes (Peng et al., 2015), and warrant future investigation.

FMOs, specifically the holoprotein, require an FAD prosthetic group for catalytic activity. Recombinant FMO Supersomes can be quantified based on their FAD content to give a holoprotein concentration. In contrast, the use of synthetic peptides as standards for targeted proteomic quantification provides a total protein concentration (*i.e.*, holoprotein + apoprotein) for a sample. Such a distinction was seen (Figure 8), as the total FMO protein amount exceeded

DMD # 67538

its nominal holoprotein amount 5- to 6.6-fold for FMO1 and 6- to 8.5-fold for FMO3, while only a small difference (0.9- to 1.5-fold) was seen for FMO5. These results suggest that a large portion of FMO1 and FMO3 proteins in Supersomes are present as apoprotein without the FAD prosthetic group, whereas most FMO5 proteins are holoproteins. This is consistent with a much greater FAD content in FMO5 Supersomes (2700 pmol/mg protein; lot#3154943) relative to those in FMO1 and FMO3 Supersomes (500 and 810 pmol/mg protein, respectively; lot#3098891 and lot#3130681, respectively) reported by the vendor, although differential expression efficiency also could contribute to FAD content differences in FMO Supersomes.

For both conventional immunoquantification and the targeted proteomic quantification described here, an assumption was made that the holoprotein:apoprotein ratio remains the same between a recombinant system (*e.g.*, Supersomes) and HLM. However, this assumption remains to be examined. Deviation from this assumption could result in either underestimation or overestimation of enzymatic activity in HLM, depending on how the ratio in HLM deviates relative to that in the recombinant system. For example, if the ratio deviates upward in HLM (*i.e.*, higher proportion of holoproteins), this will result in an underestimation of HLM holoprotein concentration and the measured HLM activity will exceed the predicted activity calculated as the product of recombinant enzyme activity and HLM protein expression. To test this, one could first determine the rate of a probe substrate reaction, which needs to be catalyzed exclusively by the enzyme of interest, in HLM and then compare the measured HLM activity with the predicted activity based on the measured activity of the recombinant enzyme and measured expression level of the enzyme in HLM. Using FMO3 and cimetidine sulfoxidation as an example, the average measured cimetidine sulfoxidation activity in the adult HLM panel was

DMD # 67538

1.25 nmol/min/mg HLM (Figure 5A), the measured cimetidine sulfoxidation activity of recombinant FMO3 was 6.0 nmol/min/nmol FMO3 (unpublished data), and the measured FMO3 expression in HLM was 0.046 nmol/mg HLM (Figure 4B). The predicted activity is 0.28 nmol/min/mg HLM, substantially less than the measured activity of 1.25 nmol/min/mg HLM. Thus, an upward deviation of the holoprotein:apoprotein ratio in HLMs could have contributed to the under-prediction, in addition to other possibilities proposed in the companion paper (Zane et al., the companion paper). The questionable assumption regarding the holoprotein:apoprotein ratio for FMOs, as well as for CYPs, is underappreciated and requires further investigation using newly available analytical tools (*e.g.*, targeted quantitative proteomics).

In summary, a UPLC-MRM-based targeted proteomic assay has been developed for the absolute protein quantification of FMOs 1, 3 and 5 in HLM. Our results corroborated the developmental transition in FMO1 and FMO3 expression and revealed relatively stable FMO5 expression throughout development. The developed FMO assay and other previously developed targeted quantitative proteomic assays are expected to assist in addressing previously unanswered questions in quantitative pharmacology.

DMD # 67538

Authorship Contributions

Participated in research design: YC, NRZ, DRT, and MZW

Conducted experiments: YC and NRZ

Performed data analysis: YC, NRZ, DRT and MZW

Wrote or contributed to the writing of the manuscript: YC, NRZ, DRT and MZW

DMD # 67538

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DMD # 67538

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DMD # 67538

Zane NR, Chen Y, Wang MZ, and Thakker DR (the companion paper) Age-Dependent

Differences in Expression and Functional Activity of CYP and FMO Families. *Drug Metab*

Dispos (currently under review).

DMD # 67538

FOOTNOTES

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DMD # 67538

Figure Legends

Figure 1. Post-*in silico* product ion screening of FMO3 signature peptides (FMO3_pep1_L and FMO3_pep4_L). Extracted product ion chromatograms of predicted y ions (**A** and **B**) and MS/MS spectra (**C** and **D**) of the detected FMO3 signature peptides are shown following product ion screening analysis of a recombinant FMO3 Supersomes tryptic digest (40.5 pmol FMO or 50 µg total protein). FMO3_pep1_L and FMO3_pep4_L eluted at 2.5 and 5.5 min, respectively.

Figure 2. Representative MRM chromatograms of FMO signature peptides in tryptic digests of (A) adult and (B) fetal HLM. Digestion mixtures, containing 30 µg HLM and 1 µg trypsin, were incubated for 4 h at 37°C prior to UPLC-MRM analysis.

Figure 3. Effects of trypsin digestion time and HLM protein loading on the UPLC-MRM signals of FMO signature peptides derived from pooled HLM. The UPLC-MRM peak areas of FMO signature peptides were normalized by those of corresponding stable isotope-labeled signature peptides spiked in as IS. For the digestion time study (**A** and **B**), each reaction contained 30 µg of pooled HLM and 1 µg of trypsin. For the protein loading study (**C** and **D**), each reaction contained 1 µg of trypsin and varying amounts of HLM proteins. Symbols and error bars represent the mean and standard deviation of triplicate determinations. In many cases, error bars are too small to be seen. Dashed lines (**C** and **D**) represent the best-fit lines of least-square linear regression analysis.

Figure 4. Coherence analysis of FMO protein quantification by UPLC-MRM-based targeted proteomic approach using different signature peptides and recombinant FMO

DMD # 67538

Supersomes-generated calibration standards. Quantification of FMO1 was performed using the fetal HLM panel, whereas quantification FMO3 and FMO5 was performed using the adult HLM panel. Symbols and error bars represent the mean and standard deviation of triplicate determinations for an individual donor HLM. In many cases, error bars are too small to be seen. Dotted lines represent the best-fit lines of least-square linear regression analysis.

Figure 5. Correlation analysis of FMO protein content and measured marker activity in (A) adult and (B) fetal individual donor HLM panels. Symbols and error bars represent the mean and standard deviation of triplicate determinations for an individual donor HLM. In many cases, error bars for protein concentration are too small to be seen. Dotted lines represent the best-fit lines of least-square linear regression analysis.

Figure 6. Comparison of FMO5 protein expression in the fetal, pediatric and adult HLM. FMO5 protein concentration was determined by UPLC-MRM-based targeted proteomic approach using recombinant FMO5 Supersomes-generated calibration standards. Donor age (gestational and postnatal age) was plotted in logarithm scale. Symbols in the scatterplots represent the mean of triplicate determinations of an individual HLM sample. Lines and error bars represent the mean and standard deviation of all HLM samples in an age group. ANOVA was used to compare all three age groups ($P = 0.317$).

Figure 7. Comparison of FMO protein quantification by UPLC-MRM-based targeted proteomic approach using different signature peptides and synthetic signature peptide-generated calibration standards. Symbols represent the mean of triplicate determinations for

DMD # 67538

an individual donor HLM. Lines and error bars represent the mean and standard deviation for a panel of HLM. Student's *t* tests (two-tailed, unpaired) were used to compare the pairs of signature peptides.

Figure 8. Comparison between total (holoprotein + apoprotein) FMO protein concentration and nominal holoprotein concentration in FMO Supersomes. The total FMO protein concentration was determined using synthetic signature peptides as calibration standards, while the nominal holoprotein concentration was determined based on FAD content (provided by the vendor). Dotted lines represent the best-fit lines of least-square linear regression analysis.

Tables

Table 1. Signature peptides for human FMO1, FMO3, and FMO5.

Protein	Signature Peptide ^a	Peptide Sequence ^b	Start-End ^c	Average Mass MH ⁺ (Da) ^d	MRM (m/z)		CE (V)
					Precursor Ion	Product Ion	
FMO1	FMO1_pep1_L	FTEHVEEGR	43-51	1104.2	552.5	589.3 (y5)	20
	FMO1_pep1_H	FTEHVEEG(R)		1114.2	557.5	599.3 (y5)	20
	FMO1_pep2_L	VEDGQASLYK	345-354	1110.2	555.5	881.5 (y8)	15
	FMO1_pep2_H	VEDGQASLY(K)		1118.2	559.6	889.5 (y8)	15
FMO3	FMO3_pep1_L	FSDHAEGR	43-51	1048.1	524.5	561.3 (y5)	20
	FMO3_pep1_H	FSDHAEGR(R)		1058.1	529.5	571.3 (y5)	20
	FMO3_pep4_L	SNDIGGLWK	34-42	990.1	495.4	560.3 (y5)	15
	FMO3_pep4_H	SNDIGGLW(K)		998.1	499.4	568.3 (y5)	15
FMO5	FMO5_pep1_L	FQENPEEGR	44-52	1106.1	553.4	587.3 (y5)	18
	FMO5_pep1_H	FQENPEEG(R)		1116.1	558.4	597.3 (y5)	18
	FMO5_pep6_L	WATQVFK	388-394	880.0	440.5	622.4 (y5)	10
	FMO5_pep6_H	WATQVF(K)		888.0	444.5	630.4 (y5)	10

^a L and H indicate unlabeled and stable isotope-labeled peptides, respectively.^b Stable isotope-labeled amino acid residues are included in parentheses.^c Start and end residue positions of peptides in the corresponding full-length protein.^d Theoretical average mass of mono-protonated molecular ion.

Figure 1.

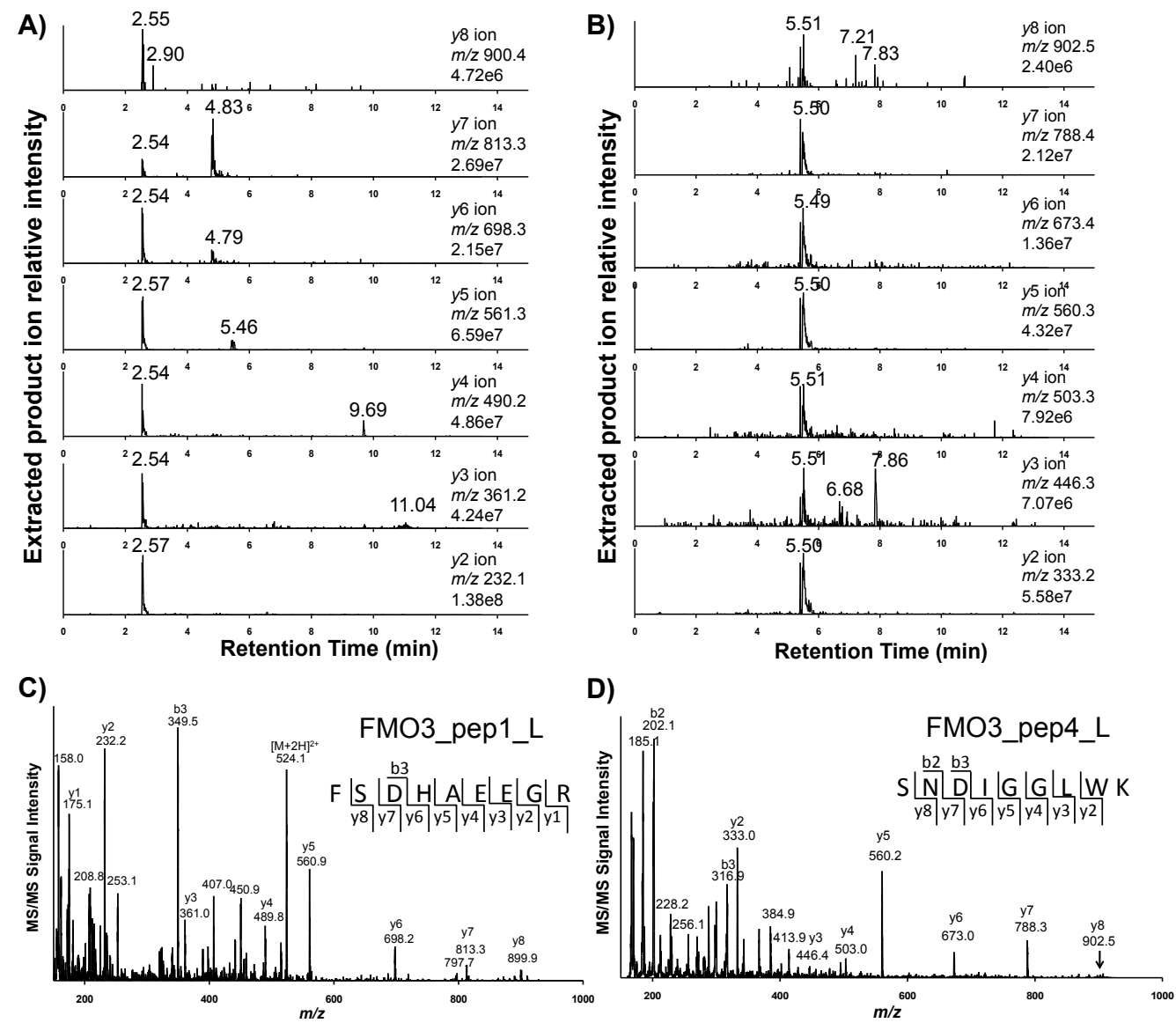
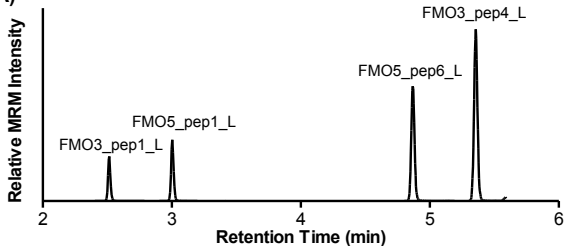


Figure 2.

A)



B)

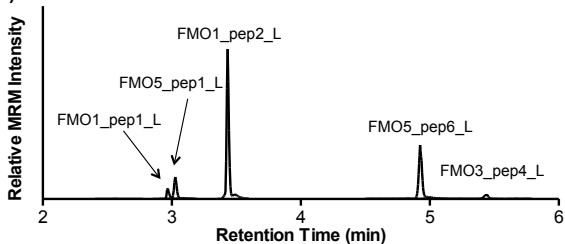


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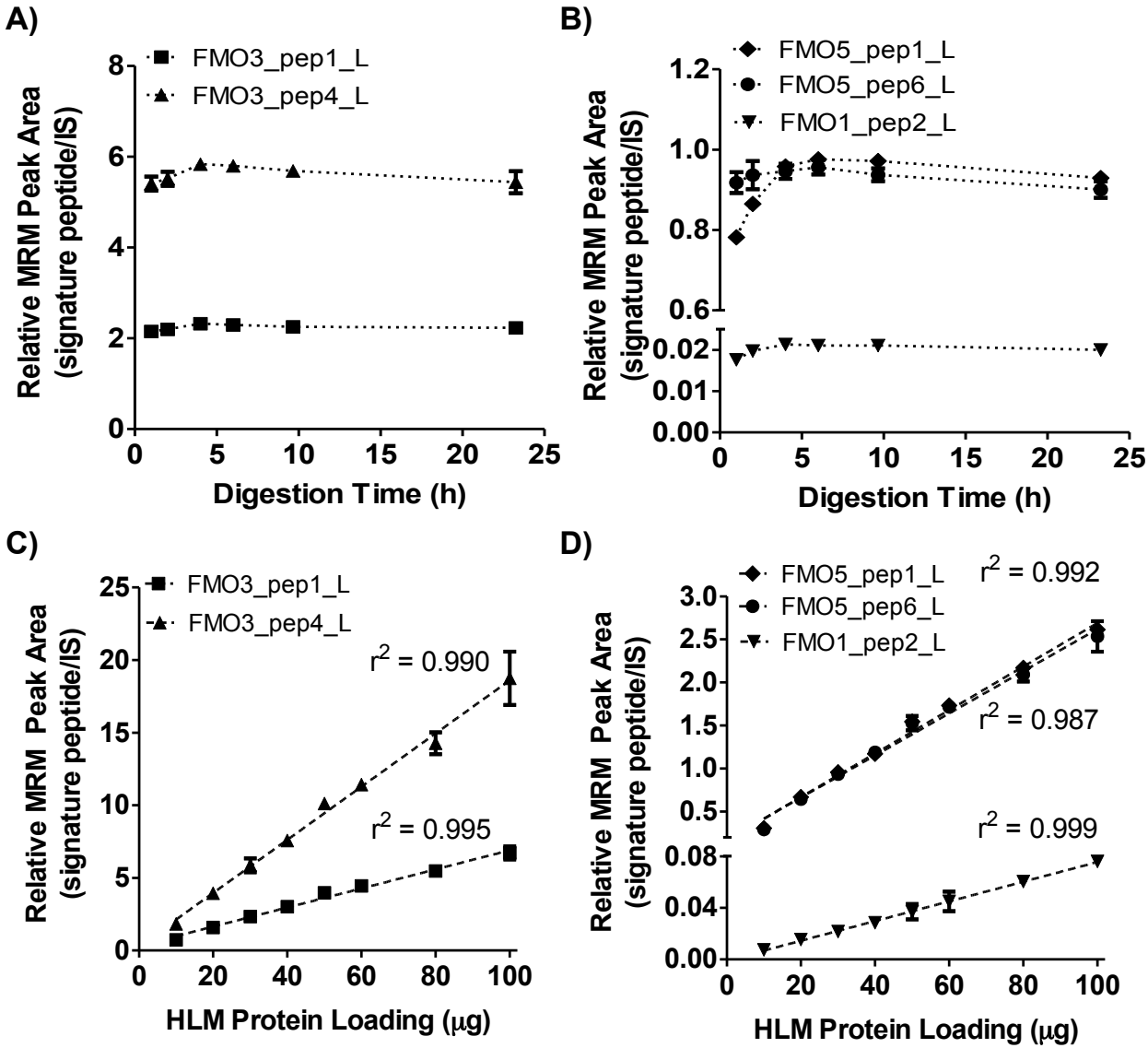


Figure 4.

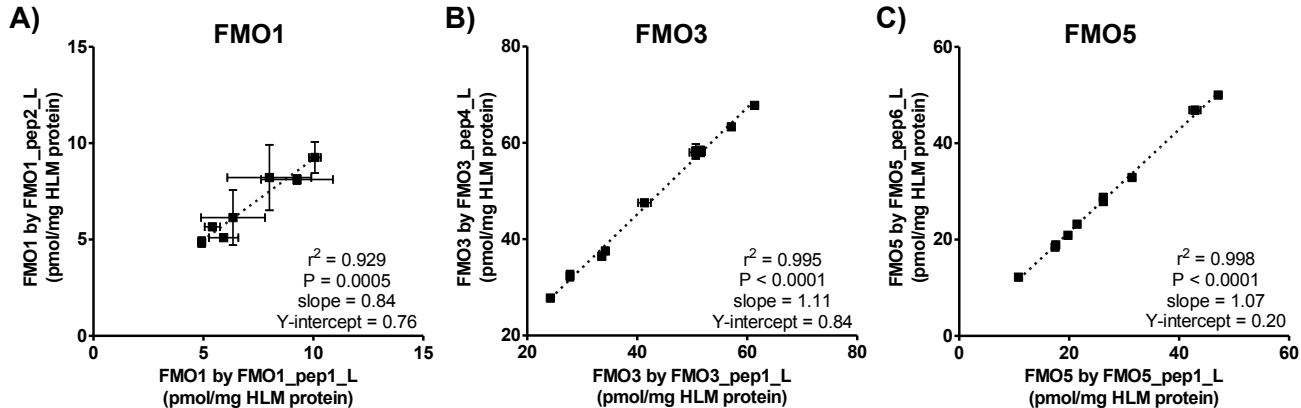
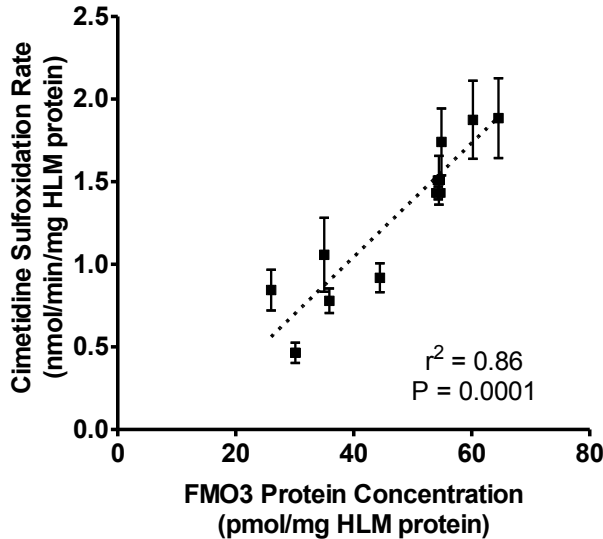


Figure 5.

A)



B)

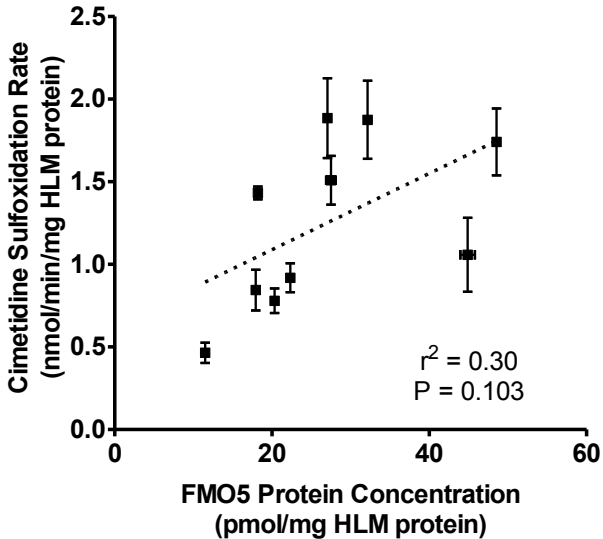


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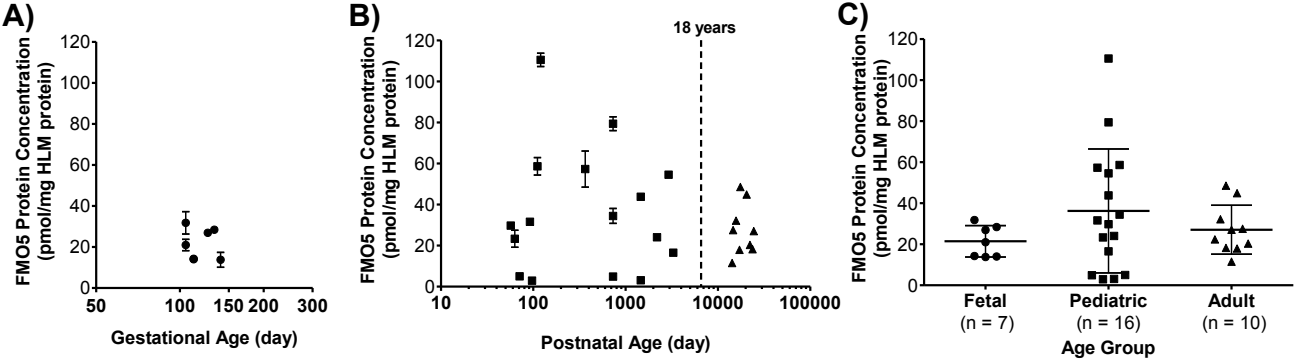


Figure 7.

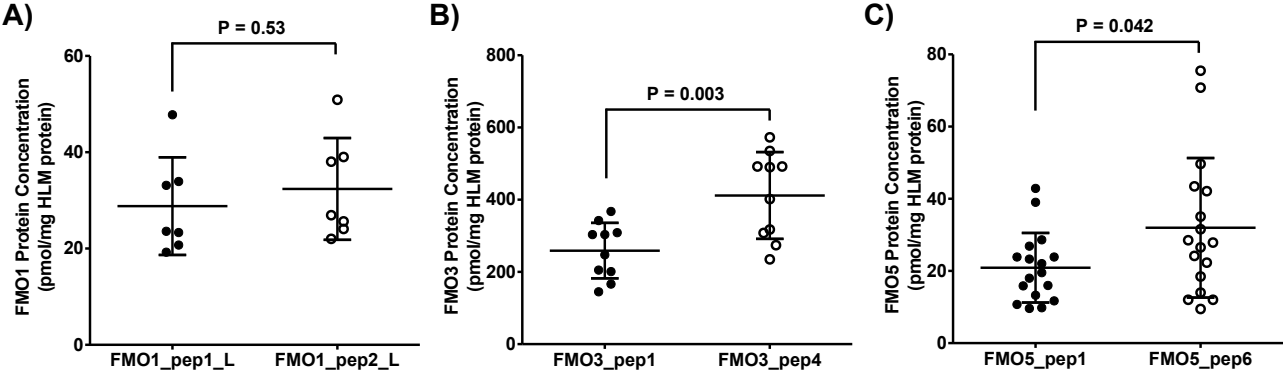


Figure 8.

