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Multiplex and Label-Free Relative Quantification Approach for Studying Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS

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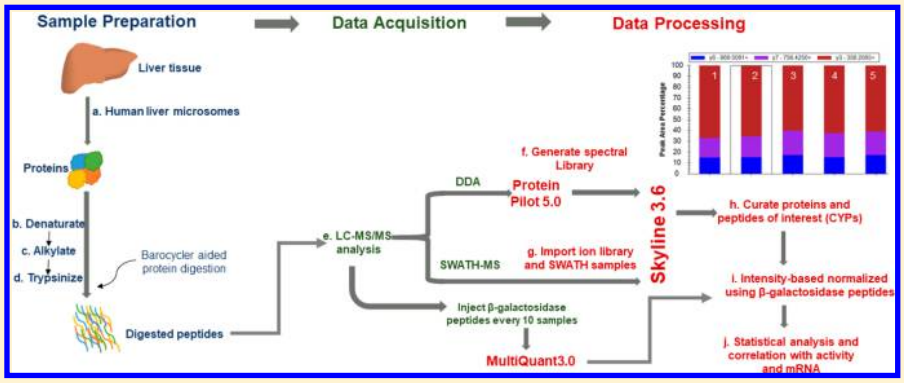
Supporting Information



ABSTRACT: We describe a sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS) based method for label-free, simultaneous, relative quantification of drug metabolism enzymes in human liver microsomes (HLM; n = 78). In-solution tryptic digestion was aided by a pressure cycling method, which allowed a 90 min incubation time, a significant reduction over classical protocols (12−18 h). Digested peptides were separated on an Acquity UHPLC Peptide BEH C18 column using a 60 min gradient method at a flow rate of 0.100 mL/min. The quadrupole-time-of-flight mass spectrometer (ESI-QTOFMS) was operated in positive electrospray ionization mode, and data were acquired by data-dependent acquisition (DDA) and SWATH-MS^{ALL} mode. A pooled HLM sample was used as a quality control to evaluate variability in digestion and quantification among different batches, and inter-batch %CV for various proteins was between 3.1 and 7.8%. Spectral library generated from the DDA data identified 1855 distinct proteins and 25 681 distinct peptides at a 1% global false discovery rate (FDR). SWATH data were queried and analyzed for 10 major cytochrome P450 (CYP) enzymes using Skyline, a targeted data extraction software. Further, correlation analysis was performed between functional activity, protein, and mRNA expression for ten CYP enzymes. Pearson correlation coefficient (r) between protein and activity for CYPs ranged from 0.314 (CYP2C19) to 0.767 (CYP2A6). A strong correlation was found between CYP3A4 and CYP3A5 abundance and activity determined using midazolam and testosterone ($r > 0.600$, $p < 0.001$). Protein-to-activity correlation was moderate ($r > 0.400$−0.600, $p < 0.001$) for CYP1A2, CYP2A6, CYP2B6, CYP2C9, and CYP2E1 and significant but poor ($r < 0.400$, $p < 0.05$) for CYP2C8, CYP2C19, and CYP2D6. The findings suggest the suitability of SWATH-MS based method as a valuable and relatively fast analytical technique for relative quantification of proteins in complex biological samples. We also show that protein abundance is a better surrogate than mRNA to predict the activity of CYP activity.

KEYWORDS: *proteomics, SWATH-MS, label-free quantification, human liver, cytochrome-P450, CYP3A4*

INTRODUCTION

Hepatic drug metabolizing enzymes (DMEs) are responsible for the clearance of pharmacological agents and xenobiotics. The abundance of these enzymes in liver tissue determines the rate and extent to which drugs are metabolized and cleared from systemic circulation. Cytochrome P450s (CYPs) and uridine diphosphate glucuronyltransferases (UGTs) constitute the majority of phase I and phase II DMEs, respectively.[1] CYPs are primarily involved in the oxidation of endogenous steroids, xenobiotics, and drugs.[2,3] Expression of CYPs thus impacts drug disposition, pharmacokinetics, and adverse drug reactions.[1] Characterization of expression and activity of DMEs in human liver microsomes and hepatocytes is a pivotal part of

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drug development. Moreover, induction potential of new chemical entities is typically screened using primary or cryopreserved hepatocyte culture.[4] It is important to understand how the level of different DMEs changes from one individual to another or how different disease states influence the abundance of these enzymes.

Classical methods for protein quantification include Western blotting and ELISA.[5] Western blotting is semiquantitative, low throughput, labor intensive, and requires the use of expensive antibodies.[6] ELISA methods can be higher throughput and more quantitative than Western analysis but are labor intensive, suffer from limited concentration range, and can lack specificity.[6] In the recent years, protein mass spectrometry has proved to be a powerful technique in different areas of biomedical research including drug development.[7] "Targeted" or "absolute" protein quantification methods use liquid chromatography coupled with a triple quadrupole mass spectrometer (LC−MS/MS) and consist of quantification of one or more signature peptides per protein.[8] These peptides are custom synthesized and are often paired with an isotope-labeled peptide as an internal standard. Traditionally, in the drug metabolism field, multiple-reaction monitoring (MRM) method has been used for absolute quantification of clinically relevant CYPs and UGTs in human liver tissue.[9−11] Others have measured the concentration of xenobiotic transporters using quantitative targeted proteomics.[12] However, significant upfront assay development with 1−2 synthetic peptides for each protein is often required for targeted MRM methods.

Label-free quantification (LFQ) techniques are now becoming common for analysis of proteins using mass spectrometry. Neilson et al. published a comprehensive review on LFQ approaches and compared it with other labeling based techniques.[13] Sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS) is a cost efficient, LFQ method that combines data-independent acquisition (DIA) and multiple reaction monitoring-like data processing for accurate peptide quantitation. It provides an advantage when compared to traditional mass spectrometry-based proteomics methods like shotgun (high throughput) and SRM (high reproducibility and consistency). The technique enables a complete and permanent recording of all fragment ions of the detectable peptide precursors present in a digested biological sample that can be interrogated retrospectively.[14] Data are acquired on the high-resolution time-of-flight mass spectrometer (TOF/MS) in consecutive continuous cycles through precursor isolation windows, which collect fragment ion spectra for all analytes in a sample. The collected data contain the spectra of fragment ions for all precursor ions that appear within the defined precursor retention time and m/z space. The combination of all the high-resolution spectra collected at $\geq$ 25 000 resolution (fwhm) generates a fragment ion map, which generates extracted ion chromatogram (XIC) measurements with high specificity derived by low mass error.[14] This provides an unbiased quantification method, which is reproducibly collected across all samples for relative quantitation and does not require a protein or sample specific method development. A peptide spectral library can be used for extraction of peptides of interest from the SWATH data and further statistical analysis.

The purpose of this work was to develop a SWATH-MS method for relative quantification of proteins in the microsomal fraction of 78 human liver tissue samples. Enzyme activity of DMEs as provided by the vendor were correlated with protein level determined in the human liver microsomes by using the current method. Additionally, we performed correlation analysis of protein and functional activity with mRNA expression for 10 major CYPs.

MATERIALS AND METHODS

Chemical and Reagents

Trypsin digested β-galactosidase (*E. coli*), protein preparation kit, and TPCK-treated trypsin were procured from Sciex, Framingham, MA. Mass spectrometry grade acetonitrile and formic acid were from ThermoFisher Scientific, Waltham, MA. Acquity UHPLC Peptide BEH C18 analytical column and VanGuard precolumn were from Waters Corp., Milford, MA. RNeasy Mini Kit was purchased from Qiagen Inc., Valencia, CA.

Human Liver Microsomes

Human liver tissues retrieved from brain dead individuals by Sekisui XenoTech, LLC, Kansas City, KS. were used in this study. A brief overview of the donor demographics is given in Table 1. Human liver microsomes (HLM) were prepared as described previously, with modifications.[15]

Table 1. Brief Demographic Summary of Donors

total number of donors (male, female)	78 (41, 37)
ethnicity (C, AA, H)[a]	66, 9, 3
	mean ± SD
age (years)	51.5 ± 12.9
weight (kg)	90.6 ± 27.2
height (cm)	169.6 ± 10.9

[a]C, Caucasian; AA, African-American; H, Hispanic.

Briefly, human liver samples were carefully thawed on ice and weighed. Tissue was immediately transferred to a precooled 7 mL homogenization bead-mill tube containing 50 mM Tris-HCl (pH 7.4) buffer having 0.5 mM EDTA, 0.25 M sucrose, and 20 μM BHT (3 mL/g wet liver weight) and homogenized using a bead homogenizer (Bead Ruptor 24, Omni International, Kennesaw, GA). Homogenate was centrifuged at 10 000g for 20 min at 4 °C (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany). The pellet containing cell debris was discarded, and supernatant (S9 fraction) was ultracentrifuged at 100 000g for 1 h at 4 °C (Beckman Coulter, Brea, CA). Further, the supernatant was separated and stored as a cytosolic fraction for future use. The pellet on the walls of the tubes was washed (50 nM sodium pyrophosphate, pH 7.4) and resuspended in homogenization buffer containing 20% glycerol (pH 7.4, 0.66 mL/g of tissue). The contents were carefully transferred to a 1 mL Dounce homogenizer for fine homogenization. The resulting microsomal fraction was stored at −80 °C until analysis. Microsomal protein concentration was estimated using a bicinchoninic acid method (Pierce-Fisher, Rockford, IL) with bovine serum albumin as a standard. The samples were diluted to 2.5 mg/mL in phosphate-buffered saline (pH 7.4) before protein digestion as described below. XTreme 200 pool HLM sample was purchased from Sekisui XenoTech.

Pressure Cycling Technology (PCT) Based Protein Digestion

In-solution trypsin digestion was performed on each biological sample in duplicates according to a published method with

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modifications.[16] Denaturation, reduction, and alkylation were performed in centrifuge tubes, while digestion was carried out in MicroTubes (Pressure BioSciences Inc., South Easton, MA) under oscillating high-pressure cycles in a Barocyler NEP2320–45k (Pressure BioSciences Inc.). Briefly, 150 μg of microsomal protein was denatured and reduced for 1 h at 60 °C in a shaking water bath (75 rpm). Reduced samples were alkylated for 10 min at room temperature to prevent free cysteine residues from reforming of peptide bonds. Subsequently, samples were diluted with equal volume of 100 mM Tris buffer (pH 8) containing 4 mM $MgCl_2$ and digested with TPCK-treated trypsin (protease/protein, 1:20) in the barocyler. PCT-aided digestion was performed at 50 °C for 90 cycles, 50 s at 35 kpsi and 10 s at ambient pressure for every cycle. Further, samples were transferred to a centrifuge tube, and digestion was stopped by addition of formic acid at a final concentration of 0.1%. The mixture was vortex-mixed for 10 s before centrifugation at 5000 rpm for 1 min at 10 °C. The supernatant was collected and transferred to a clean microinsert for further analysis. Two technical replicates for each HLM sample were digested and analyzed by mass spectrometry. XTreme 200 pool sample was used as digestion control to monitor the batch-to-batch variation of protein digestion carried out in six batches. Approximately 12 samples and one digestion control sample were digested and run in every batch.

LC-QTOF/MS Analysis

All experiments were performed on a SCIEX 5600 TripleTOF mass spectrometer equipped with a DuoSpray ion source (SCIEX, Concord, Canada) coupled to Acquity UHPLC HClass system (Waters Corp., Milford, MA, USA). The mass spectrometer was operated in positive electrospray ionization mode for the analysis. The peptides were separated on Acquity UHPLC Peptide BEH C18 (2.1 × 150 mm^2, 300 Å, 1.7 μm) equipped with Acquity VanGuard precolumn (2.1 × 5 mm^2, 300 Å, 1.7 μm). Digested samples were maintained at 10 °C in the autosampler, and the analytical column temperature was kept at 40 °C. The amount of protein per injection on the column was 10 μg. The chromatographic separation was achieved with a runtime of 60 min at 100 μL/min with a gradient method using mobile phase A (98% water, 2% acetonitrile, 0.1% formic acid) and mobile phase B (98% acetonitrile, 2% water, 0.1% formic acid). A linear gradient scheme was used with solvent composition as follows: 98% A from 0 to 3 min, 60% to 90% A from 3 to 48 min, 20% A held from 49 to 52 min to flush the column, 98% A at 53 min. The column was allowed to equilibrate at 98% A from 53 to 60 min before the start of next run. In each batch, trypsin-digested β-galactosidase peptides were injected (~30 pmol/injection) every 10 samples during the analysis to monitor mass calibration of the TOF detector and normalization of intensity during relative quantification (described below). The average intensity of the β-galactosidase peptide (APLDNDIGVSEATR) in a batch was used for data normalization of the respective batch of samples.

Standard DDA and SWATH-MS Data Acquisition

Mass spectrometry analysis was performed according to a previously described method with modifications.[17] Analyst TF 1.7 was used to acquire data during the study (SCIEX, Framingham, MA). DDA was used to acquire data for generation of peptide ion library, and SWATH-MS^{ALL} mode for relative quantification of the proteins. Positive ionization monitoring was utilized for all the experiments during the study. DDA experiments were performed over a mass range of m/z 350–950, and all ions exceeding 350 cps, with a charge state 2 to 4, and quadrupole resolution of 0.7 AMU were used for automated MS/MS analysis. The mass tolerance was set at 50 mDa during the initial 250 ms (ms) survey scan, and eight ions were selected for product scan per cycle (total cycle time: 900 ms). A DuoSpray ion source was used for all the experiments. Source specific parameters settings for the analysis were ion source gas 1 (GS1), 55 psi; ion source gas 2 (GS2), 60 psi; curtain gas (CUR), 25 psi; source temperature (TEM), 500 °C; and ionspray voltage floating (ISVF), 5500 V. Compound-specific parameters for acquisition were declustering potential (DP), 120 and collision energy (CE), 10 (product ion experiments were carried out using rolling collision energy). SWATH-MS based spectra were acquired for mass range m/z 400–900 Da with SWATH window width of 25 m/z resulting in 20 overlapping mass windows per cycle. Accumulation time of 109 ms per window was used, which resulted in a total cycle time of 2.29 s. Rolling collision energy for +2 and +3 charges with collision energy spread of 15 V was applied to each SWATH window upon automatic calculation of the collision energy center value, dependent on the m/z range according to this rolling collision energy equation, $CE = 0.044 \times (m/z) + 9$.[18]

Generation of Spectral Library

Protein database searching was performed against reference UniProt human proteome library (July 2015) by ProteinPilot 5.0 (SCIEX; Framingham, MA, USA) using Paragon algorithm (5.0). A comprehensive spectral library of proteins and peptides from DDA runs of the HLM samples was prepared. Data were uploaded to ProteinPilot Software to carry out protein identification against a Human Uniprot FASTA database. Search parameters in ProteinPilot were as follows: Cys alkylation-MMTS; digestion-Trypsin; instrument, TripleTOF 5600; ID focus-Biological modifications, search effort-Thorough ID, detected protein threshold-0.05 (10%), and false discovery rate analysis, yes. The resulting library file (.group) was uploaded to Skyline (http://proteome.gs.washington.edu/software/skyline), and label-free analysis of data was performed as described below. The spectral library is available on PeptideAtlas (Identifier number PASS01078).

Data Processing Using Skyline

Skyline is an open source, Windows-based software for curating and analyzing data from proteomic experiments.[19] Reviewed protein sequences of DMEs of interest was retrieved from Uniprot and uploaded onto Skyline. Detailed Skyline and data processing settings are given in Supporting Information I.

Briefly, spectral library generated from DDA files was uploaded in Skyline, and SWATH-MS data files were processed using the full scan MS/MS filtering at a resolution of 10 000. Unique, nonrepetitive peptides were refined and curated for reproducible fragment ions, and peak boundaries for each selected peptide were manually supervised and when necessary, adjusted. The reproducibility and reliability of selected peptides and transitions were verified visually by looking at the peak area ratio of the ion across the samples. We used two peptides per protein and three fragment ions per peptide for every protein. Selected peptides for each protein for the relative quantification of the CYPs described in this study were also correlated (protein specific) to validate the selection of peptides (Supplementary Figure 1).

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The total area of representative peptides for a protein was summed, and resulting intensity was normalized by total intensity of tryptic peptide of β-galactosidase. MultiQuant v 3.0 (SCIEX, Framingham, MA) was used to retrieve intensity for APLDNDIGVSEATR peptide [$(M+2H)^{2+}$: 729.365] and was subsequently used for normalization among different batches as described above (Supporting Information II).[20] Percent coefficient of variation (CV%) of the proteins of interest (CYPs) between six batches was calculated and plotted using Prism 6.0 (GraphPad Inc., La Jolla, CA). Peptides used for relative quantification and the transitions for precursor and product ions are given in Supporting Information II.

Quantification of Hepatic mRNA Expression

Total cellular RNA was isolated from the samples using RNeasy Mini Kit (QIAGEN inc., Valencia, CA). The total RNA was reverse-transcribed, and the single-stranded DNA was used for real-time PCR. The mRNA expression of hepatic CYP was quantified in duplicates by real-time PCR using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. 18S rRNA (rRNA) was also quantified as an internal control. The primers used for CYPs are given in Supporting Information III.

Statistical Analysis

Enzymatic activity for 10 CYPs provided by Xenotech was used for correlation with the relative protein abundance estimated in HLM using the current method. The correlation analysis was also performed with mRNA levels determined from liver samples using method as described above. The incubation conditions, probe substrates, and other details of the enzymatic assays performed by Xenotech on the livers are given in Supporting Information IV. Information on mRNA primers is provided in Supporting Information III. A three-way correlation analysis was conducted between enzyme activity, protein levels, and mRNA (described below).

Normality tests were performed before statistical analysis, and to address the skewness, the data were natural log transformed (ln) before correlation analysis. Pearson correlation coefficient was used to determine the relationship between activity, protein, and mRNA level. Correlation coefficient (r) > 0.600 was considered strong while between 0.400 and 0.600 was considered moderate. Additionally, any correlation with r < 0.400 was considered poor in this work. Statistical values (p < 0.05) were considered significant for the analysis. All statistical analysis was performed with SPSS 24 (IBM Corp., Armonk, NY) and graphs were plotted on Prism 6.0 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Targeted Data Extraction Using Skyline

At a critical FDR of 1.0%, we detected 1855 distinct proteins and 25 681 distinct peptides from global FDR fit (Figure 1). The in-house generated spectral library was imported into Skyline, and data extraction was performed. The list of peptides and their transitions along with charge state is provided in Supporting Information II. The correlation analysis of two peptides for a protein is given in Supplementary Figure 1. Out of the 15 hepatic CYPs reported for xenobiotic metabolism, we were able to find 12 CYPs in this study.[21] Interbatch %CV for all the DMEs evaluated from quality control sample ranged from 3.1−7.8% (Supplementary Figure 2).

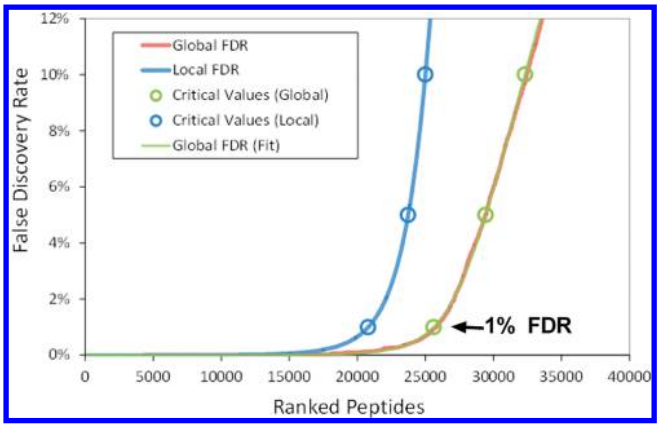

Figure 1. Identified proteins and peptides at 1% false discovery rate (FDR). The graph depicts identification of 1855 proteins and 25 681 peptides in the spectral library at 1% FDR.

Correlation between Protein Expression and Enzyme Activity

Pearson correlation coefficient (r) for CYPs ranged from 0.314 (CYP2C19) to 0.767 (CYP2A6). All the major CYP enzymes showed a significant (p < 0.05) correlation between enzyme activity and protein levels (Figure 2a−l). CYP3A4 and CYP3A5 abundance and activity determined using midazolam and testosterone showed a significant association (r > 0.650, p < 0.001). CYP3A4 and CYP3A5 protein expression correlated strongly but in a linear regression analysis, combination of the two protein levels did not improve prediction of CYP3A enzyme activity. A moderate protein-to-activity correlation (r = 0.400−0.600, p < 0.001) was also observed for CYP2A6, CYP1A2, CYP2C9, CYP2B6, and CYP2E1. The correlation for CYP2C8, CYP2D6, and CYP2C19 was significant but poor (r < 0.400, p < 0.05). A detailed correlation between activity and protein is provided in Table 2.

Correlation between mRNA Expression and Enzyme Activity

The correlation coefficient for mRNA and activity ranged from −0.067 to 0.729 (Figure 3a−l). CYP2C19 and CYP2E1 showed a slightly negative correlation but were not significant. The correlation between mRNA expression and activity for CYP1A2, CYP2A6 and CYP2C8 was significant and moderate (r = 0.400−0.600, p < 0.01), and strong for CYP3A4 and CYP2B6 (r > 0.600, p<0.01). CYP3A5 mRNA correlated significantly only with midazolam hydroxylation activity. CYP2D6 exhibited a poor but significant correlation (r = 0.306, p < 0.05). The correlation was poor and insignificant for other CYP isoforms. A detailed correlation table is provided in Table 2.

Correlation between mRNA and Protein Expression

We found that only CYP2A6 (r = 0.395), CYP1A2 (r = 0.271), CYP3A4 (r = 0.577), and CYP2B6 mRNA (r = 0.431) levels showed a correlation that was statistically significant (Figure 4a−j). There was some correlation (r < 0.200) between CYP2C9 and CYP2C19 mRNA and protein level but did not reach statistical significance. All other isoforms showed a poor correlation between mRNA and protein, which was again not significant (p > 0.10). A detailed correlation table is provided in Table 2.

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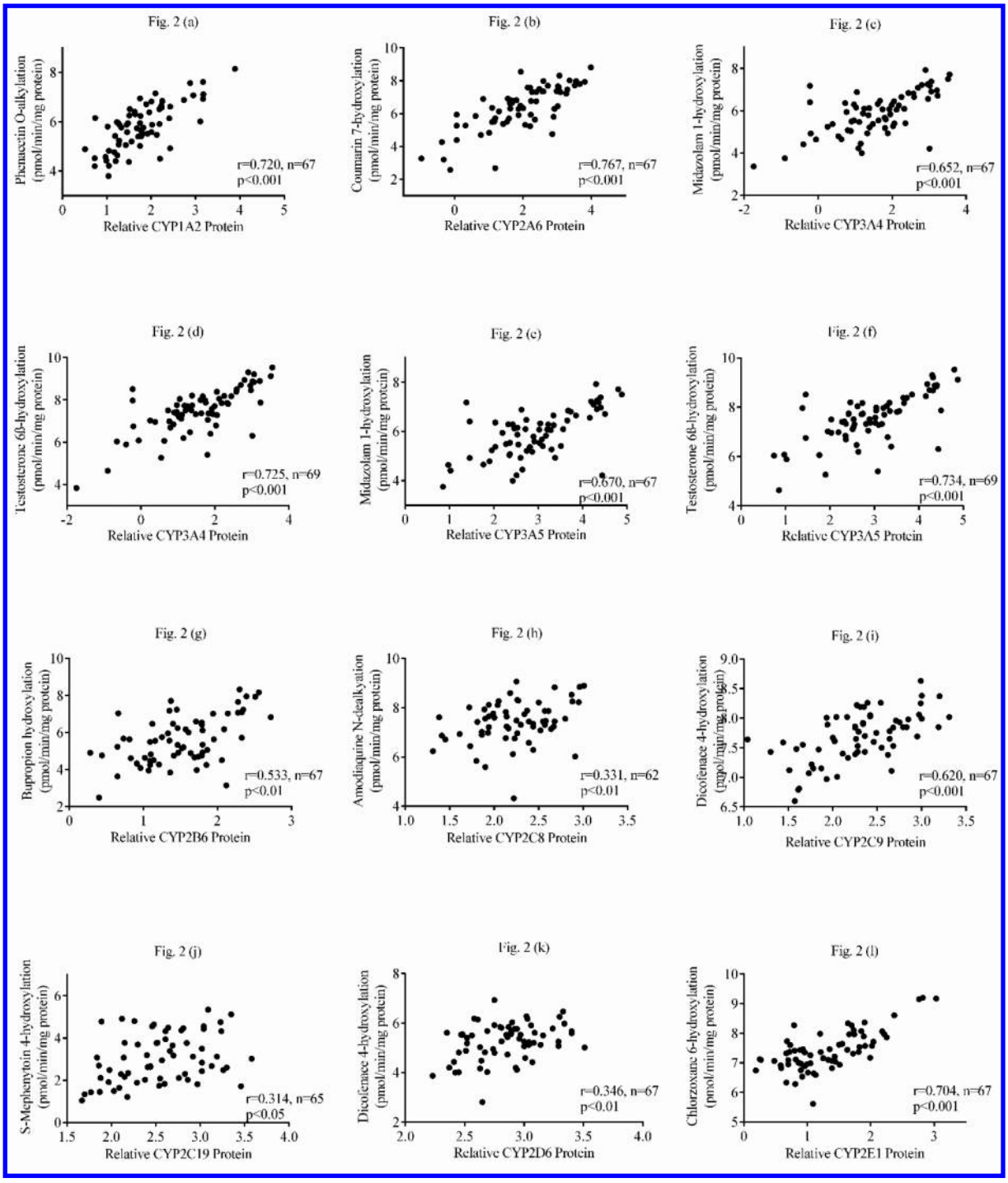

Figure 2. (a–l) Correlation plots for relative protein expression and enzyme activity. Protein abundance values were expressed relative to APLDNDIGVSEATR peptide from *E. coli* β-galactosidase. The enzyme activity was represented as CYP-specific product formation/min/mg protein. All values were natural logarithm (ln) transformed before Pearson correlation analysis.

DISCUSSION

Studies involving drug metabolizing enzymes (DMEs) are critical for evaluating drug efficacy and safety. Thus, an understanding of the biological variation of these DMEs could provide useful insight into pharmacokinetics or drug interaction potential of new chemical entities. Conventional targeted methods for protein quantification rely on the use of one or two unique peptides per protein. The data are further acquired using MRM, and a ratio of unlabeled (light) to labeled peptide (heavy) is used to determine the level of that peptide present in a digested sample.[22] However, a researcher would require a separate isotope-labeled peptide for each target peptide, and this leads to a significant cost. These high-purity isotope-labeled synthetic heavy peptides cost anywhere from $700–1000 for ∼1 mg of peptides with concentration certification by amino acid analysis. For the development of small numbers of assays, this is a reasonable investment. However, the cost can be prohibitive when the numbers of proteins of interest exceed a limited number or for experiments intended for biomarker discovery. In drug metabolism, usually

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Table 2. Correlation[a] between Protein Levels and Activity, mRNA and Activity, and mRNA and Protein

	protein and activity			mRNA and activity			mRNA and protein		
	sample size	Pearson correlation coefficient (r)	p-value	sample size	Pearson correlation coefficient (r)	p-value	sample size	Pearson correlation coefficient (r)	p-value
CYP1A2	69	0.720	<0.001	67	0.426	<0.001	76	0.271	<0.05
CYP2A6	67	0.767	<0.001	67	0.461	<0.001	78	0.395	<0.001
CYP3A4							78	0.577	<0.001
CYP3A4-M	67	0.652	<0.001	67	0.647	<0.001			
CYP3A4-T	69	0.725	<0.001	69	0.652	<0.001			
CYP3A5							78	0.058	0.616
CYP3A5-M	67	0.670	<0.001	67	0.330	0.006			
CYP3A5-T	69	0.734	<0.001	69	0.188	0.122			
CYP2B6	67	0.533	<0.001	63	0.729	<0.001	73	0.431	<0.001
CYP2C8	62	0.331	<0.01	66	0.524	<0.001	77	0.121	0.295
CYP2C9	67	0.620	<0.001	66	0.043	0.728	77	0.185	0.106
CYP2C19	65	0.314	<0.05	69	−0.067	0.584	78	0.197	0.084
CYP2D6	67	0.346	<0.01	67	0.306	0.012	78	0.110	0.341
CYP2E1	67	0.704	<0.001	67	−0.032	0.800	78	0.111	0.334

[a]All values were natural logarithm (ln) transformed before the correlation analysis. M, Midazolam 1-hydroxylation; T, testosterone 6β-hydroxylation.

only high abundance CYPs are measured using targeted approach and the low abundance CYPs or other microsomal enzymes are neglected.

Several groups have used high-resolution mass spectrometry methods to analyze the expression of drug-metabolizing enzymes and transporters in human tissue.[3,23] SWATH-MS has found an important application is the discovery of novel biomarkers.[24,25] Drawbacks of traditional MRM based approaches of protein quantification make it unsuitable for the research area.[26] Ortea and colleagues used SWATH-MS for mining potential protein biomarkers of lung adenocarcinoma.[27] Quantitative mapping of ErbB2, a receptor tyrosine kinase biomarker was recently demonstrated using SWATH-MS approach and highlights the application of technique.[28]

Pressure cycling technology (PCT) based digestion along with SWATH-MS acquisition (PCT-SWATH) was used to reduce the sample preparation time.[16] The typical incubation time for digestion with trypsin is time-consuming and range from 12−18 h (usually overnight). PCT enhances proteolytic action by inducing denaturation of proteins, therefore allowing better access to trypsin for cleavage sites.[29] This also significantly reduces the digestion and overall sample preparation time.

Skyline is a popular tool used for targeted data analysis of mass spectrometry data. It supports spectral library generation as well as data analysis of SWATH-MS files.[19] Similar to other LFQ approaches, it relies on retention time (RT) alignment between the data files and the spectral library. A commonly used method for retention time normalization relies on the use of synthetic iRT peptides, which are spiked to ever sample before analysis, and has few drawbacks.[30] Complex and widely different matrices might significantly affect the ionization and retention time reproducibility of these peptides within their LC retention time space and would compromise the evaluation of peptide peak area and FDR calculation in the case of a low signal within background noise. After thorough literature review and taking into consideration the costs associated with insertion of standards for RT normalization, we used the method suggested by Parker et al. and Nakamura et al. in recent articles.[21,30] A retention time predictor was created from endogenous peptides present in our sample using Skyline allowing integration of fragment intensity over different batches.

Correlations between mRNA expression, protein abundance, and functional activity are not always tight due to complex regulation mechanisms involving pre- and post-transcriptional events, translational modifications, and subsequent protein localization events.[31] Among other mechanisms, stability and half-life of protein and mRNA in their in vitro conditions also contribute to poor correlation.[31,32] Moreover, as reviewed by Zanger and Schwab, post-translational modifications, as well as certain genetic polymorphisms and epigenetic variations can decrease or increase the activity of cytochrome P450 enzymes.[33] In the proteomics field, poor selection of peptides for quantification of protein expression can also affect protein versus activity association.

In this work, we found that protein levels are better surrogates for estimating the activity of major CYP isoforms than mRNA. Additionally, we observed that both protein, as well as mRNA, can be used to access the functional activity of CYP2A6, CYP1A2, CYP2B6, CYP2C8, and CYP3A4. Interestingly, apart from CYP3A4, CYP1A2, CYP2B6, and CYP2A6, protein expression of none of the other six CYP isoforms showed a significant correlation with the mRNA expression. Pretranslational regulation of CYP1A2, CYP3A4, and CYP2B6 expression has already been reported to be responsible for good correlation between mRNA expression and enzyme activity.[34]

Al Koudsi et al. found that CYP2A6 protein determined by Western blotting significantly correlate with nicotine C-oxidation activity in human livers.[35] We also observed a significant correlation between protein, activity, and mRNA for CYP2A6. The correlation was in general stronger between activity and protein expression, followed by activity and mRNA. Similar correlations have been published previously for microsomal CYP2A6.[35]

CYP3A activity for testosterone and midazolam hydroxylation correlated strongly with mRNA as well as protein. There was a good correlation between CYP3A4 mRNA, protein, and activity suggesting that both protein and mRNA could be used to estimate the functional activity of this enzyme. Similar results on the correlation of CYP3A4 activity with mRNA and protein have been previously published.[36] CYP3A5 protein expression correlated strongly with midazolam and as

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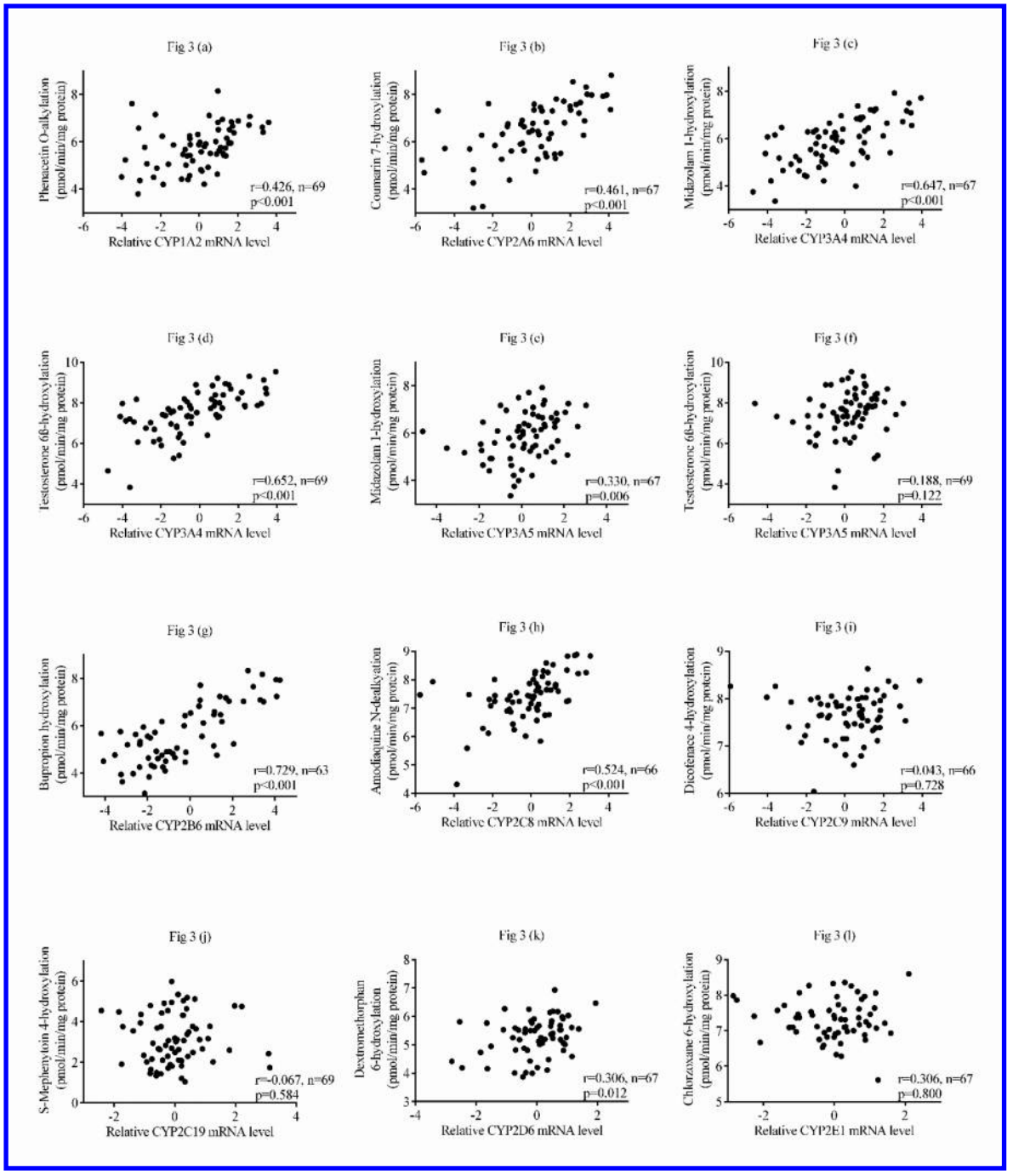

Figure 3. (a–l) Correlation plots for mRNA and enzyme activity. Messenger RNA (mRNA) was expressed relative to 18S rRNA. Protein abundance values were expressed relative to APLDNDIGVSEATR peptide from *E. coli* β-galactosidase. The enzyme activity was represented as CYP-specific product formation/min/mg protein. All values were natural logarithm (ln) transformed before Pearson correlation analysis.

well as testosterone hydroxylation ($r > 0.600$, $p < 0.05$). In contrast, the correlation between CYP3A5 mRNA and activity was poor for testosterone activity ($r < 0.188$, $p > 0.10$), while it was significant for midazolam activity ($r = 0.330$, $p < 0.01$). There was no relationship observed between CYP3A5 protein levels and mRNA ($r < 0.100$, $p > 0.10$).

As previously reported, CYP2E1 activity correlated strongly with protein level but not with the mRNA expression.[36,37] This again highlights the role of post-translational modifications of protein on the enzyme activity. Conversely, a pharmacogenomics study of CYP1A2 in human liver samples ($n = 150$) found that mRNA and protein correlated with the functional activity of this enzyme.[38] The results are in line with the significant correlation observed for CYP1A2 in the current study.

CYP2C9 metabolic activity was shown to have a higher correlation with protein than mRNA.[34] This agrees with our findings for strong CYP2C9 protein and activity correlation ($r = 0.620$, $p < 0.001$). However, we found an insignificant correlation for mRNA with CYP2C9 activity and protein levels. Interestingly, CYP2B6 and CYP2C8 activity showed a better correlation with mRNA than protein levels. It was not unexpected as Ohtsuki and colleagues have earlier reported that mRNA is a better surrogate than protein level for

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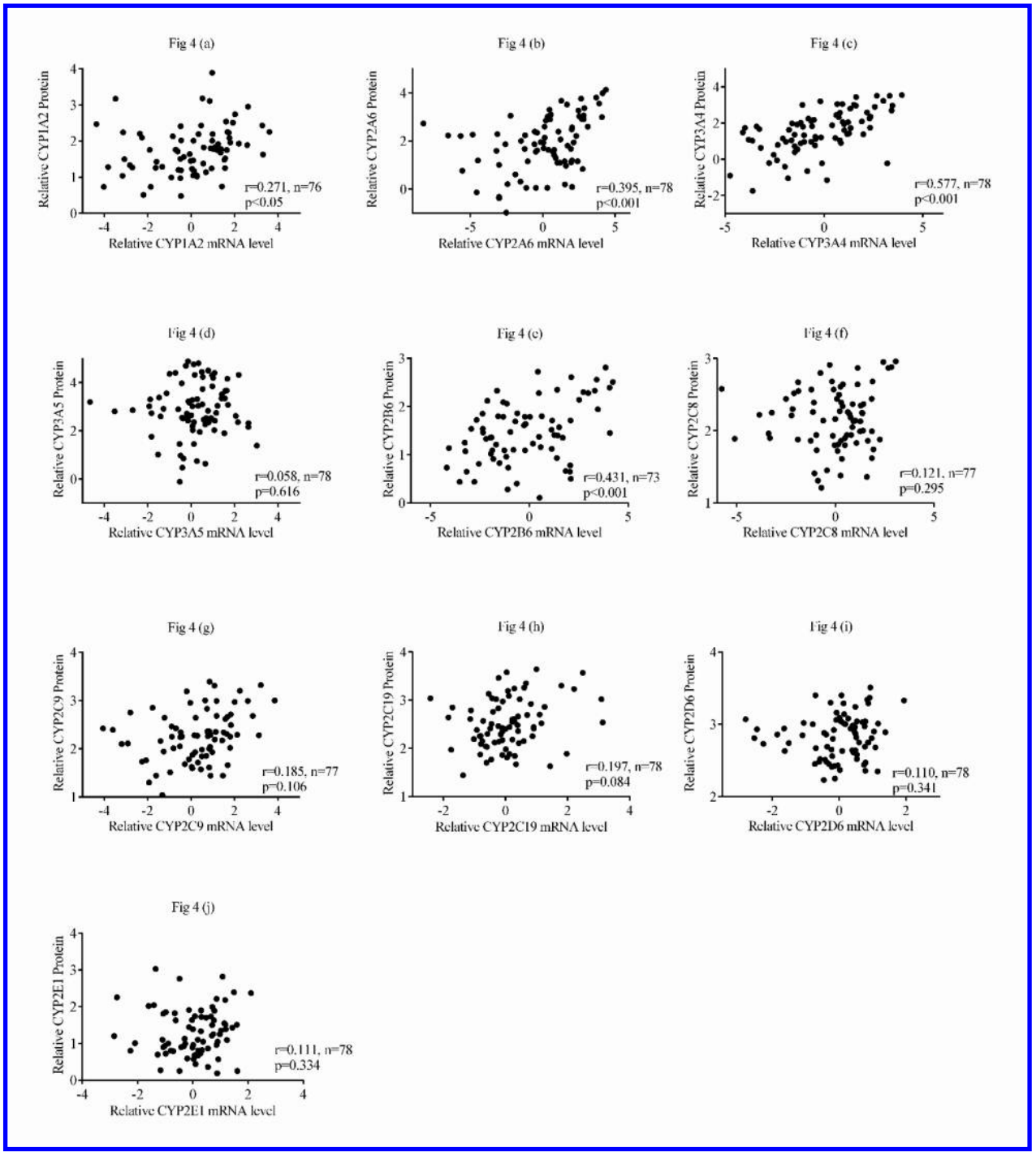

Figure 4. (a–j) Correlation plots for mRNA and protein expression. Messenger RNA (mRNA) was expressed relative to 18S rRNA. Protein abundance values were expressed relative to APLDNDIGVSEATR peptide from *E. coli* β-galactosidase. All values are natural logarithm (ln) transformed before Pearson correlation analysis.

prediction of CYP2B6 activity.[3] For CYP2B6, our data agree with finding as we also found a strong correlation (r = 0.729, p < 0.001) between mRNA and activity, while there was a moderate correlation (r = 0.533, p < 0.001) between activity and protein. The correlation between CYP2B6 protein and mRNA was also moderate (r = 0.431, p < 0.001). Genetic polymorphisms were previously suggested to be responsible for such correlation (Spearman r = 0.44) between CYP2B6 mRNA and protein expression.[39] Studies have also reported that the genetic polymorphism in CYP2C8, CYP2C19, and CYP2D6 account for major variability in the activity of these enzymes.[40] Protein stability in isolated microsomes could also be attributed to poor correlation with mRNA.

Rodríguez-Antona et al. observed a moderate correlation between CYP2C8 protein abundance and enzyme activity.[41] CYP2C8 activity correlated moderately with protein (r = 0.331, p < 0.01), but a strong correlation was observed with mRNA (r = 0.524, p < 0.01) suggesting the role of post-translational modifications. A significant correlation between mRNA and activity for CYP2C8 and CYP2D6 suggests the utility of mRNA

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for studying the functional activity of these CYP isoforms. Poor correlation of CYP2C19 mRNA and activity suggests toward major post-translational changes influencing its activity in human and makes a case for use of protein levels to predict the activity of this enzyme.

CONCLUSIONS

SWATH-MS exemplifies a powerful LFQ technique, which addresses the limitations of the shotgun and targeted proteomics to provide a permanent digital repository of all peptides present in a sample. This method can serve as a valuable post hoc tool for studying new hypothesis and ideas without the need to reacquire data. To the best of our knowledge, this is the first report demonstrating the use of SWATH-MS and pressure-cycling based digestion for relative quantification of drug metabolizing enzymes in human liver microsomes and the correlation with their functional activity and mRNA expression. The studies also highlight the importance of protein levels for prediction of the functional activity of CYP enzymes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00505.

Supplementary Figure 1. Correlation analysis of surrogate peptides used for relative quantification (TIF)

Supplementary Figure 2. Percent coefficient of variation for 10 CYPs between different batches (TIF)

Skyline peptide and transition settings, peak scoring model; list of precursor and product ions for major drug metabolism enzymes quantified using current method; quantification of hepatic CYP mRNA expression; enzyme assay conditions (PDF)

Extended list of precursor and product ions for major drug metabolism enzymes quantified using the current method (XLSX)

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Notes

The authors declare the following competing financial interest(s): Brigitte L. Simons was actively employed by SCIEX, Canada at the time when this work was conducted as well at the date of submission of this manuscript. The authors would also like to highlight that this research was presented at 47th Gordon Research Conference for Drug Metabolism at Holderness, New Hampshire in July 2017.

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ABBREVIATIONS

BHT, butylated hydroxytoluene; CYP, Cytochrome P450; DMEs, drug metabolizing enzymes; DDA, data-dependent analysis; FDR, false discovery rate; HLM, human liver microsomes; LFQ, label-free quantification; MRM, multiple reaction monitoring; PCT, pressure cycling technology; SRM, single-reaction monitoring; SWATH-MS, sequential windowed acquisition of all theoretical fragment ion mass spectra; TPCK, tosyl phenylalanyl chloromethyl ketone; UGT, UDP-glucuronosyltransferase; UHPLC, ultra high performance liquid chromatography

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