Global Proteomic Analysis of Human Liver Microsomes: Rapid Characterization and Quantification of Hepatic Drug-Metabolizing Enzymes

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Abbreviations
ADME, absorption, distribution, metabolism and excretion; BSA, bovine serum albumin; DDI, drug-drug interaction; DME, drug-metabolizing enzyme; GO, Gene Ontology project; HLM, human liver microsomes; HPLC, high performance liquid chromatography; IMS, ion-mobility spectrometry; IVIVE, in vitro-in vivo extrapolation; LC-MS, liquid chromatography in conjunction with mass spectrometry; LysC, lysyl endopeptidase; MS/MS, tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; MPPGL, mg protein per gram liver; P450, cytochrome P450; PBPK, physiologically-based pharmacokinetics; QconCAT, quantification concatemer; UGT, uridine 5´-diphosphoglucuronosyltransferase.
Abstract

Many genetic and environmental factors lead to inter-individual variations in metabolism and transport of drugs, profoundly affecting efficacy and toxicity. Precision dosing, targeting drug dose to a well-characterised sub-population, is dependent on quantitative models of the profiles of drug-metabolizing enzymes and transporters within that sub-population, informed by quantitative proteomics. We report the first use of ion mobility-mass spectrometry for this purpose, allowing rapid, robust, label-free quantification of human liver microsomal (HLM) proteins from distinct individuals. Approximately 1000 proteins were quantified in four samples, including an average of 75 drug-metabolizing enzymes. Technical and biological variability were distinguishable, technical variability accounting for about 10% of total variability. The biological variation between patients was clearly identified, with samples showing a range of expression profiles for cytochrome P450 and uridine 5’-diphosphoglucuronosyltransferase enzymes. Our results showed excellent agreement with previous data from targeted methods. The label-free methodology, however, allowed a fuller characterization of the in vitro system, showing, for the first time, that HLMs are significantly heterogeneous. Further, the traditional units of measurement of drug-metabolizing enzymes (pmol mg\(^{-1}\) HLM protein) are shown to introduce error arising from variability in unrelated, highly abundant proteins. Simulations of this variability suggest that up to 1.7-fold variation in apparent CYP3A4 abundance is artefactual, as are background positive correlations of up to 0.2 (Spearman correlation coefficient) between the abundances of drug-metabolizing enzymes. We suggest that protein concentrations used in pharmacokinetic predictions and scaling to in vivo clinical situations (PBPK-IVIVE) should be referenced instead to tissue mass.
Introduction

Designing patient-specific dosage regimens within the framework of precision medicine has recently been emphasized as a key future direction in biomedical and pharmaceutical research, with physiologically-based pharmacokinetics and in vitro-in vivo extrapolation (PBPK-IVIVE) expected to play an important role in this application (Jamei, 2016). In pharmacogenomics, one of the pillars of personalized medicine, a recent survey of 517 submissions assessed by the European Medicines Agency between 1995 and 2014 showed that approximately 15% of approved medications have on-label pharmacogenomic information that directly affects therapy, indicating the recent move into tailoring drug use for specific patient sub-populations (Ehmann et al., 2015). Within this framework of targeted therapy evaluation, IVIVE-PBPK is expected to shift its focus to sub-populations with specific therapeutic needs, with increasing demand to populate these new models with expression and functional data of proteins involved in absorption, distribution, metabolism and excretion (ADME) (Turner et al., 2015; Jamei, 2016). This is supported by the substantial number of novel drug submissions (136 between 2008 and 2014) to the Food and Drug Administration for approval where PBPK has beneficially informed drug development, especially in the areas of drug-drug interactions and paediatrics (Huang et al., 2013; Jamei, 2016). Comprehensive and detailed information about the abundance and activity of ADME proteins, which play a central role in drug metabolism and disposition, is therefore required, and crucially needs to be generated with clear inter-relations with genetic, demographic, environmental and clinical information (Schadt and Björkegren, 2012; Turner et al., 2015).

Proteomics is expected to play a more prominent role in the qualitative and quantitative characterization of proteins involved in disease development and progression and modulating drug therapy, with applications ranging from biomarker discovery and disease monitoring to
dosage regimen design (Auffray et al., 2016; Masys et al., 2012). Biomolecular data acquisition and analysis should be guided by the intended clinical application with particular emphasis on disease prevention and therapy based on inter-individual variability in genetic, lifestyle and environmental factors (McGrath and Gheresi, 2016).

With recent advances in tandem mass spectrometry, many laboratories have started to contribute to the wealth of ADME protein abundance literature (Ohtsuki et al., 2012; Prasad et al., 2014; Achour et al., 2014a; Harwood et al., 2015; Vildhede et al., 2015; Fallon et al., 2016). Protein abundance values from these experiments are used in several drug pharmacokinetic prediction exercises including scaling parameters from in vitro models to in vivo clinical situations using computational PBPK models (Knights et al., 2016; Rostami-Hodjegan, 2012). However, cross-laboratory and inter-study heterogeneity highlighted recently (Achour et al., 2014b; Badée et al., 2015) have led to ongoing efforts to investigate variability originating from using different methodological workflows, taking into consideration their advantages and limitations in relation to their intended applications (Harwood et al., 2016; Al Feteisi et al., 2015).

There is little consistency in proteomic protocols used for protein quantification in a wide variety of samples, including heterogeneous membrane fractions: crude total membrane, plasma membrane and microsomal fractions (Schaefer et al., 2012; Russell et al., 2013; Groer et al., 2013; Fallon et al., 2013), and whole tissue lysates (Weiss et al., 2015; Wiśniewski et al., 2014; Wiśniewski et al., 2016a). The effects of different methodological processes on determining protein abundance were previously investigated with different levels of evidence, sometimes of conflicting nature; however, the general idea emphasized by these studies is that differences in sample preparation and in proteomic methods can contribute to considerable overall variability in end-point measurements (Balogh et al., 2013; Qiu et al., 2013; Chiva et al., 2014; Harwood et al., 2016), which makes assessment of true biological inter-individual
variability a difficult challenge. Different mass spectrometry platforms can also have an effect on the quality and robustness of analysis, with promising improvements in instrumentation making proteomic analysis more reliable. Particularly, liquid chromatography in conjunction with ion mobility spectrometry and tandem mass spectrometry (LC-IMS-MS/MS) is a relatively new approach that allows robust global analysis of entire proteomes, and has recently been applied to proteomic analysis of HeLa cell lines (Distler et al., 2014) and breast tumor xenografts (Burnum-Johnson et al., 2016).

This report describes a proof-of-concept study that aims to apply a LC-IMS-MS/MS proteomic approach to the analysis of the human liver microsomal proteome, with specific focus on quantification of the expression of drug-metabolizing enzymes. Implications of this quantitative assessment for enzyme abundance measurements and expression correlations are subsequently considered.
Materials and Methods

Materials and chemicals

All reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise indicated. Lysyl endopeptidase (LysC) was purchased from Wako (Osaka, Japan) and recombinant proteomic-grade trypsin was supplied by Roche Applied Sciences (Mannheim, Germany). Label-free protein standards at 95% purity (bovine serum albumin (BSA), bovine cytochrome c, equine myoglobin) were purchased from Sigma-Aldrich. Solvents were of HPLC grade.

Human liver microsomal samples

Four individual human liver microsomal samples (HLM; nominally labeled as HLM01, HLM02, HLM03 and HLM04) provided by Pfizer (Groton, CT, USA), along with demographic, medication and genotype details of donors, were used in this study. Table 1 shows demographic and clinical information of the donors; suppliers of these samples were Vitron (Tucson, AZ, USA) and BD Gentest (San Jose, CA, USA). The same microsomal samples were used in the quantitative experiments using the label-free approach (the present study) and the QconCAT targeted approach (Achour et al., 2014a), which was used to analyze samples HLM01, HLM02, and HLM04. Microsomal fractions were prepared from liver tissue by the two suppliers, both using fractionation methodology based on differential centrifugation of hepatic tissue homogenates. Low speed centrifugation (10,000 g) was used to separate the S9 fraction (supernatant), which was followed by an ultracentrifugation step (100,000 g) to isolate the microsomal fraction (pellet). Ethics were covered by the suppliers.

Methodological workflows

Supplemental Figure 1 shows a summary of the label-free global proteomic workflow followed in this study. The targeted QconCAT methodology is described elsewhere (Achour et al.,
2014a). Differences between the methodological steps in these approaches are shown in Supplemental Table 1.

**Proteolytic digestion of HLM samples and estimation of protein loss**

Protein content in microsomal samples was determined using a colorimetric protein assay (Bradford, 1976). Proteolytic digestion and gravimetric estimation of peptide loss were carried out in triplicate using methods previously reported by Harwood et al. (2015) with slight modifications. Briefly, HLM samples (50 μg total protein mass) were suspended in ammonium bicarbonate buffer (25 mM, pH 8.0) and combined with a standard mixture of unlabeled BSA, equine myoglobin and bovine cytochrome c (6 μL, at 0.1, 0.02 and 0.01 mg mL⁻¹, respectively) to a final volume of 50 μL. The rationale behind using non-human standard proteins is that species-specific peptides can be found in reference proteins that should not be found in the target human proteome to allow quantification without interference due to homology in protein sequences. Mixtures were then denatured with sodium deoxycholate (acid-labile detergent) at a final concentration of 10% (w/v) for 10 min at room temperature. Disulfide bonds were reduced (dithiothreitol, 60 mM final concentration) at 56°C for 20 min and subsequently alkylated (iodoacetamide, 15 mM final concentration) in the dark at room temperature for 30 min.

Sequential enzyme proteolysis was used to increase the scope and depth of analysis and reduce the number of missed cleavages (Achour and Barber, 2013; Wiśniewski and Mann, 2012; Al-Majdoub et al 2014). Samples were diluted 1:10 with ammonium bicarbonate (25 mM) and 1 μL of LysC (1 μg μL⁻¹) was added, followed by incubation at 30°C for 4 h. Trypsin (2.5 μL, 1 μg μL⁻¹) was then added followed by incubation at 37°C for 18 h. After removal of detergent by acidification with trifluoroacetic acid (~pH 3.0) and centrifugation, the supernatant containing the peptides was retained and evaporated by vacuum centrifugation. Peptide loss was estimated gravimetrically as described previously (Harwood et al., 2015). Supplemental
Figure 2 shows the measured protein concentration in the HLM samples and the mass of recovered peptides following sample preparation.

**MALDI-TOF mass spectrometric analysis**

To confirm the quality of sample protein digests prior to LC-IMS-MS/MS analysis, digested samples were analyzed using MALDI-TOF mass spectrometry performed on an Ultraflex II instrument (Bruker, Bremen, Germany). 20 mg mL\(^{-1}\) MALDI matrix was prepared by dissolving \(\alpha\)-cyano-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) in 0.1% trifluoroacetic acid in 50% acetonitrile in HPLC water. Samples (0.5 μL) were applied onto a MALDI target plate in triplicate. Once dry, matrix solution (0.5 μL) was added then the mixture was allowed to dry. Spectra were acquired in two \(m/z\) ranges: 700 to 2500 and 700 to 5000, to check for mis cleaved peptides. Laser frequency of 100 Hz and intensity of 30-35% were used. Spectra of 2000 laser shots were acquired per spot. Analysis of MALD-TOF MS data was performed using FlexAnalysis version 2.2 (Bruker). Quality of spectra was checked for peptide peak intensities and \(m/z\) range before proceeding to LC-MS experiments.

**Liquid chromatography-ion mobility spectrometry-mass spectrometry (LC-IMS-MS/MS)**

Prepared HLM peptide samples were diluted 1:10, of which 2 μL were analyzed from each diluted sample. The mean HLM peptide mass analyzed in each run was 44.53 ± 5.19 ng (range: 39.59–49.66 ng). Analysis was carried out on a nanoACQUITY™ UPLC® system (Waters, Manchester, UK) connected to a SYNAPT™ G2-Si mass spectrometer (Waters). For 1D reversed-phase liquid chromatography, peptides were injected onto a Symmetry C\(_{18}\) trap column (5 μm, 180 μm × 20 mm), and then eluted onto a HSS T3 analytical column (1.8 μm, 75 μm × 250 mm), maintained at 35°C. The LC program consisted of a gradient of 3 to 60% acetonitrile in HPLC water (acidified with 0.1% v/v formic acid) over 40 minutes with a flow rate of 300 nL min\(^{-1}\), followed by a ramp to 95% acetonitrile for 5 min, then a return to the initial conditions over 10 min.
Mass spectrometry was performed based on data-independent acquisition using high-definition MS\textsuperscript{E} methodology (Distler et al., 2014). The following acquisition parameters were used on the SYNAPT G2-Si: HDMS\textsuperscript{E}, positive electrospray (ESI+) mode, V optics, scan time 0.5 seconds, cone voltage 25 V, \textit{m/z} range 50-2000, and lock mass [Glu\textsuperscript{1}-Fibrinopeptide B \([M+2H]\)^{+2} 785.8426 \textit{m/z}. Collision energy (CE) was ramped based on the mobility of ions for optimal collision-induced dissociation (CID). T-Wave ion mobility (IMS) parameters were as follows: IMS T-Wave height 40 V, wave velocity 400-800 m second\textsuperscript{-1}, helium cell gas flow 180 mL min\textsuperscript{-1}, IMS gas flow 90 mL min\textsuperscript{-1}, mobility trapping release time 450 microseconds, and trap height 15 V.

\textit{Analysis of MS\textsuperscript{E} data and database searching}

Analysis and searching of the LC-IMS-MS/MS data was performed using the ProteinLynx Global Server (PLGS) version 3.0.2 and Identity\textsuperscript{E} (Waters) search engine, whereby the precursor ions were aligned based on retention time (RT) and drift time. Once the fragment and parent data were matched, identification was carried out by searching against a customized database containing protein sequences from human UniProt database (154,434 sequences; January 2015) and the three reference proteins. Quantification was performed using the summed intensity of the top 3 peptide ions based on the acquired label-free data for the proteins of interest and the standard proteins. The following quantification equation was applied:

\[
[\text{Protein}] = [\text{Standard}] \cdot \left( \sum_{i=1}^{3} \frac{\text{Ion Intensity}_{\text{protein}}}{\text{Ion Intensity}_{\text{standard}}} \right)
\]

Where \([\text{Protein}]\) represents the abundance of a target protein, \([\text{Standard}]\) represents the abundance of the spiked standard in the sample (expressed in units of pmol mg\textsuperscript{-1} HLM protein), and the fraction refers to the ratio of the sum of the intensities of the three highest ion peaks for the target protein relative to the standard as described previously (Silva et al., 2006). The
integrated peak intensities of eluted peptides were used for quantification and calculations of the summed peak intensities were performed by PLGS software.

This ‘top 3 CID’ approach is an empirical label-free quantification method, which was previously shown to produce accurate quantification of mixtures of protein standards (Sliva et al., 2006) and to correlate with data from targeted proteomic analysis (Carroll et al., 2011). Other label-free approaches include the total protein approach, based on all quantifiable peptides from each target protein, an approach which was also previously applied to quantifying hepatic ADME proteins (Vildehede et al., 2015).

Any quantitative data below the limit of quantification were not considered reliable. The limit of quantification was nominally set using two criteria: the peptides had to be reliability identified in all three technical replicates and the replicate intensities of the peptides had to be within 20% CV of each other (i.e. consistent identification and reproducible quantification). Further appraisal of the protein standards used in this analysis is included in Supplemental Information.

Protein data annotation for function and sub-cellular localization

Proteins were classified based on their subcellular localization and function according to GO annotations (http://geneontology.org/) and database searching (http://www.uniprot.org/).

Meta-analysis of hepatic microsomal protein abundance

In order to assess the effects of variability in the most abundant ten proteins on the end-point abundance of cytochrome P450 enzymes and their expression correlations, a Matlab model was used. To inform the model with abundance values for these proteins, Medline/Pubmed (http://www.nlm.nih.gov/bsd/pmresources.html) and Web of Knowledge (http://wok.mimas.ac.uk/) electronic databases (between the years 1980 and 2016) were searched for relevant literature on the protein expression of abundant liver microsomal proteins.
(see Table 2 for a list of these proteins) using suitable keywords including: the protein name / gene name (e.g., carboxylesterase 1 / CES1), human liver / human hepatic, protein quantification / expression / abundance, microsomes / HLM. Searches were combined and articles inspected for relevant data. Inclusion criteria were: studies that quantified primarily microsomal proteins / enzymes identified in the present analysis in adult human livers in units of, or convertible to, pmol mg\(^{-1}\) HLM protein. This analysis was used to select the ranges of the ten most highly expressed proteins in HLM samples. For the two target enzyme families (cytochrome P450 and uridine 5\(^{\prime}\)-diphosphoglucuronosyltransferase enzymes), previously published meta-analyses on cytochrome P450 (Achour et al., 2014b) and UGT abundance data (Achour et al., 2014c) were used, assuming ranges and mean abundances have not changed significantly in the last two years.

**Statistical data analysis and modeling**

Microsoft Excel 2010 and GraphPad Prism\(^{\circledR}\) version 7.01 (GraphPad Software, San Diego, CA) were used for data analysis and generating graphs. Venn diagrams were generated using Venny version 2.1 (BioinfoGP, [http://bioinfogp.cnb.csic.es/tools/venny/](http://bioinfogp.cnb.csic.es/tools/venny/)). To obtain data from graphs in publications in the meta-analysis step, GetData Graph Digitizer version 2.26 ([http://www.getdata-graph-digitizer.com/](http://www.getdata-graph-digitizer.com/)) was used. The heat map was generated using QCana version 1.2.1 (Kim et al., 2012). Matlab R2015a (MathWorks Inc., Natick, MA, USA) was used for modeling effects of variability of the most abundant HLM proteins on abundance and correlation of P450 enzymes. Simulation was repeated ten times for n=2,000 livers in each simulation step.
Results

In this study, we set out to obtain a snapshot of the drug-metabolizing sub-proteome of four human livers, with a focus on rapid and robust sample preparation and measurement. The methodology used in this work consisted of in-solution preparation of samples followed by nanoLC-Q-IMS-TOF MS/MS, i.e. nanoflow-liquid chromatography, mass spectrometry and ion mobility both at the peptide level, then mass spectrometry at the fragment level (Supplemental Figure 1). The main aim was to identify and comprehensively quantify a complex hepatic sub-proteome in a relatively short time (<1 hour), with particular focus on drug-metabolizing enzymes.

Assessment of protein abundance measurements

The starting total protein mass for all samples was 50 µg, out of which 35.50±2.22 µg (range 33.78-38.74 µg) was recovered (Supplemental Figure 2), indicating an overall recovery of 71% as estimated gravimetrically (Harwood et al., 2015). The number of identified proteins was 901-1,018 proteins, of which 706-816 were quantifiable (Figure 1A) with abundances above the lower limit of quantification, estimated at ~0.03 fmol peptide (translating to protein abundance of ~0.6 pmol mg⁻¹).

To assess the reproducibility and precision of the methodology, the overlap of the number of quantified proteins between samples was estimated and the coefficients of variation related to technical replicates were calculated. In addition, relative error of measurements was estimated for drug-metabolizing P450 and UGT enzymes, which were quantified previously in three of the four HLM samples using QconCAT methodology (Achour et al., 2014a) (Supplemental Table 3) to allow cross-methodology comparison. The number of quantified drug-metabolizing enzymes ranged from 63 to 76, containing 10-14 drug-metabolizing P450 enzymes and 9-11 drug-metabolizing UGT enzymes (Figure 1B). Overlap of the quantified enzymes, including P450 and UGT enzymes, between the four samples is shown in Supplemental Figure 4.
Figure 1C shows significant linear correlation between label-free and QconCAT measurements in three samples ($R^2=0.70$; $R_s=0.84$, $p<0.0001$) that were analyzed previously (Achour et al., 2014a), with measurements within 2.5-fold across the two methodologies (Figure 1D). In the data of the present study, variability in cytochrome P450 and UGT enzyme abundances between samples was estimated at up to 20 fold (total inter-individual variability). Abundance values showed technical variability of less than 20% (CV) for all protein measurements. Therefore, the expected variability related to technical error; i.e., fold difference between the 5th and 95th centiles of measurements, calculated as $(1 + 2 \text{CV})(1− 2 \text{CV})$, was 2 fold. This means technical variability constituted up to 10% of total variability (2 fold out of a total of 20 fold). The variation due to the inherent reproducibility of mass spectrometry based experiments was therefore very small compared with the biological variability found in these samples.

Protein expression profiles of drug-metabolizing enzymes

Assessment of the protein expression levels of drug-metabolizing enzymes is summarized in Figure 2. The assessed abundances were within reported values where literature was available (Figure 2A and B). The overlap between mid-to-high abundance drug-metabolizing P450 and UGT enzymes was approximately 80%, with the most abundant enzymes being CYP3A4, CYP2E1, CYP2C9, UGT2B4 and UGT2B7. The expression profiles of the quantified drug-metabolizing enzymes in the samples under study are shown in Figure 2C, showing a distinct visual difference in the expression of enzymes in sample HLM03, which is confirmed by the heat map and rank order cluster analysis shown in Figure 2D.

Components of human liver microsomal fractions

In the liver, hepatocytes are the primary site of drug metabolism. Along with hepatocytes, liver tissue contains other non-parenchymal cell types including Kupffer, stellate, and biliary endothelial cells. Human liver microsomes are used as an in vitro model of drug metabolism,
in early studies of drug development, but to date, their composition has not been systematically investigated.

A specific cell surface marker for hepatocytes, asialoglycoprotein receptor 1, ASGR1 (Peters et al., 2016), was abundant in the microsomal fraction, whereas specific markers for other types of cells were not detected in any of the samples analyzed. Within hepatocytes, the main site of metabolism is the endoplasmic reticulum; however, other sub-cellular compartments, such as the cytosol and mitochondria, also contain drug-metabolizing enzymes. Figure 3A shows identified and quantified specific membrane marker proteins that reside in the membranes of different organelles within hepatocytes (Vildhede et al., 2015). The most abundant markers were those of the endoplasmic reticulum membrane (calnexin), mitochondrial membrane (cytochrome c oxidase subunit 4, COX4), and plasma membrane (CD81, ATP1A1), with little difference in their abundances between analyzed samples. These specific markers suggest the presence of membranes from these compartments in the microsomal fraction and their contribution to drug metabolism in HLM preparations, although the extent of such contribution has yet to be systematically investigated.

The ten most abundant proteins in HLM samples were shown to be localized mainly in the endoplasmic reticulum (Table 2); however, when the list is expanded to include all identified proteins (1,276), the distribution of the HLM proteins was shown to be balanced between the endoplasmic reticulum (429 proteins), plasma membrane (406), cytosol/cytoplasm (411) and mitochondria (243), with overlap in a number proteins between different compartments (Figure 3B and Supplemental Figure 5A). The localization of drug-metabolizing enzymes in different cellular compartments and the corresponding overlap are also shown in Figure 3C and Supplemental Figure 5B, with most enzymes shown to be localized within the endoplasmic reticulum (50 enzymes).
Thus, although HLMs exhibit no detectable contamination from other hepatic cell types, these findings suggest HLMs are far from pure in terms of sub-cellular composition, with many sub-cellular compartments other than endoplasmic reticulum being represented.

**The use of total HLM protein mass for enzyme abundance normalization**

Although drug-metabolizing cytochrome P450 and UGT enzymes are mainly present in the endoplasmic reticulum, normalization of abundance values has historically been done using total HLM protein mass, routinely measured using a colorimetric assay. However, HLM samples represent a mixture of proteins from different compartments as shown above, and therefore, the effect of the most highly expressed proteins in this system, which are not directly related to drug metabolism, was investigated in this study. The top part of Table 2 shows the ten most abundant proteins in the microsomal samples, and for comparison, the bottom part shows the ranks and abundances of drug-metabolizing P450 enzymes. Figure 4A shows that although 600 proteins in the HLM fractions make up the bulk of sample mass (>99%), these top ten proteins constitute approximately 15-20% of protein mass in this fraction.

In order to assess the effect of expression variability in these ten abundant proteins on end-point measurement of drug-metabolizing cytochrome P450 enzymes, in terms of their abundance and correlation of expression, two simulations were performed. This was done based on data from literature studies, collated using meta-analysis, and our experimental data. The first simulation was intended to describe the effect of variation in the set of 10 abundant proteins on CYP3A4 abundance (Figure 4B) and the second was intended to investigate the effects of variability in these proteins on correlation between CYP3A4 and CYP2C8 (reported in the literature to be strongly correlated, Rs=0.68, p<0.0001 (Achour et al., 2014b)). This latter simulation was intended to probe how much of the strong correlation could be attributed simply to the units of measurement.
Ten simulations of 2,000 livers each with variable CYP3A4 amounts (in pmol expressed in one mg of tissue) showed that there is an overall significant decreasing trend in CYP3A4 abundance in units of pmol per mg HLM protein (Rs=-0.25 to -0.20, p<0.0001, n=2,000), assuming independent regulation of expression. When the amount of CYP3A4 in simulated livers was kept constant in tissue at the median, the apparent abundance of CYP3A4 changed 1.4-1.7-fold as a function of overall random variability in the most abundant HLM proteins (Figure 4B). When CYP2C8 and CYP3A4 were simulated independently (with variable amounts of these two enzymes in tissue), the level of correlation increased from Rs=0.0 (with no statistical significance) for random abundance values (decoy simulation) to correlation coefficients of approximately +0.1 to +0.2 (p<0.0001, n=2,000) in ten repeated simulations as a function of variability in the ten most abundant proteins. These preliminary simulations suggest that the variation in levels of proteins unrelated to drug metabolism can significantly influence the apparent levels of target enzymes if correction factors are not applied, such as MPPGL (mg protein per gram liver), to relate protein abundance levels to tissue mass instead of protein mass.
Discussion

Qualitative and quantitative protein characterization can afford substantial insight into the biochemical state of cells (Collins et al., 2016), and proteomics is therefore becoming increasingly important in clinical and biomedical research. Scientists and clinicians are required to make important decisions as to whether to employ a targeted approach to robustly analyze a limited set of proteins or to apply a non-targeted discovery-like methodology, which is more comprehensive but generally produces data of lower quality (Auffray et al., 2016; Collins et al., 2016). The present study involved the application of both approaches to human liver microsomal samples from the same patients to generate quantitative data for a set of drug-metabolizing enzymes, demonstrating the wide scope of analysis offered by the global (label-free) approach. It was particularly gratifying that the results showed good agreement with targeted quantification using QconCAT as a standard (Achour et al., 2015). Our results show that it is possible to obtain robust global proteomics measurements when quality control steps are taken to ensure successful implementation of quantitative analysis. In these experiments, there was rigorous quality control of sample preparation, standards, LC-IMS-MS/MS measurement and data analysis. A similar assessment of label-free quantification of a set of yeast glycolytic enzymes also demonstrated agreement with quantification using QconCAT standards (Carroll et al., 2011), further supporting previous reports of consistency in measurements carried out within the same laboratory setting (Qiu et al., 2013; Prasad and Unadkat, 2014).

The global proteomic experiment was designed to be both robust and relatively quick. The time of the experiment was intended to be less than 1 hour to demonstrate the possibility of using this technique in screening processes. For this purpose, liquid chromatography, ion mobility and mass spectrometry were used to provide three layers of separation including the physical size of analyzed peptides (Supplementary Figure 6) in order to analyze as many proteins as
possible with high reliability (Distler et al., 2014). With this snapshot type of analysis, a set of a few hundred proteins (706-816) were successfully quantified, out of which a subset of 63-76 drug and xenobiotic-metabolizing enzymes were characterized. The abundances of measured cytochrome P450 (12) and UGT (9) enzymes were within previously published literature ranges (Achour et al., 2014b; Achour et al., 2014c). The phenotypic fingerprint generated using the expression profiles and the heat map of drug-metabolizing enzymes revealed a range of abundance levels exhibiting differences between the four individual samples, with rank order cluster analysis showing sample HLM03 to have the most distinct expression profile.

The expression fingerprint of sample HLM03 showed overall lower abundances of a set of ADME proteins, exemplified by cytochrome P450 enzymes, including CYP1A2, CYP2A6, CYP2C9/19 and CYP3A4/5. Differences in the characteristics of the corresponding donor included exposure to medications, including an opioid analgesic (morphine) and a non-steroidal anti-inflammatory agent (ibuprofen), as well as certain genetic differences, including polymorphic CYP2C9 (*1/*2), CYP2C19 (*1/*2) and CYP3A5 (*3/*3). Inflammatory conditions and polymorphism were previously reported to reduce the catalytic activity of CYP1A2, CYP2C9/19 and CYP3A4/5 (Zanger and Schwab, 2013; Zanger et al., 2014). Notably, severe reduction in the expression levels of CYP3A5*3/*3 compared to the wild type and CYP3A5*1/*3 variant is well-documented in the literature (Lin et al., 2002; Achour et al., 2014a). In addition, murine hepatic expression of P450 enzymes after exposure to a derivative of morphine showed significantly lower abundances of CYP2C and CYP2E enzymes determined using immunoblotting (Sheweita, 2003). However, due to the small sample size in the present study, the effects of these differences may require further investigation in order to confirm and elucidate them.

Human liver microsomes are routinely used in the metabolic characterization of new and existing compounds, with the idea that most of the metabolic activity in these systems is
attributed to enzymes localized in the endoplasmic reticulum, which is believed to be preferentially enriched using differential centrifugation (Zhang et al., 2015). However, there is little evidence in the literature that defines the biomolecular composition of these fractions with suggestions that centrifugation can lead to either enrichment or loss of different membrane components (Harwood et al., 2014). For the purpose of addressing this gap, annotation related to subcellular localization was performed for all identified proteins in the analyzed HLM samples (1,276 proteins). This revealed information about the composition of this in vitro system, with the main components being the endoplasmic reticulum (34% of all proteins), the plasma membrane (32%) and the cytosol/cytoplasm (32%). Mitochondrial proteins also constituted a large proportion of proteins identified in HLM samples (19%). This finding is supported by the identification and quantification of specific membrane markers for the endoplasmic reticulum, mitochondria and plasma membrane in this fraction, indicating that HLM samples represent a crude, heterogeneous mixture of proteins from different cell compartments (i.e., a crude total membrane fraction), including but not limited to the endoplasmic reticulum. Technical differences in the microsomal preparation method can theoretically lead to differences in the composition of the final microsomal fraction. However, the fractionation methods used by the suppliers of these samples were very similar and the abundances of marker proteins from different cell compartments were not significantly different. Importantly, the presence of proteins from the nucleus (11%) and Golgi body (12%) shows that the initial centrifugation step may require further optimization to achieve better enrichment of endoplasmic proteins. A useful approach to eliminate the effect of fractionation on measuring protein expression profiles may be to examine the expression levels in liver tissue homogenates instead.

A similar trend was seen with annotated drug and xenobiotic-metabolizing enzymes, with most enzymes coming from the endoplasmic reticulum (nearly 60%), the cytosol and the
plasma/exosomal membrane. However, the contribution of these non-endoplasmic reticulum enzymes to drug metabolism is only hypothesized at this stage. This observation of heterogeneity is in line with the findings of a recent global proteomic analysis that showed that the distribution of drug-metabolizing enzymes in fractions of liver tissue homogenate is complex (Wiśniewski et al., 2016b). Both the current work and that of Wiśniewski et al. point to caution in applying scaling factors when enzyme abundances are measured in membrane fractions.

Implications of this level of heterogeneity in HLMs are relevant to both the way abundance levels of ADME proteins are reported and the assessment of their correlations of expression. Abundance levels of enzymes and transporters have traditionally been measured in units of pmol per mg of total microsomal protein mass. We highlight two problems with this tradition. Firstly, the total protein mass of microsomal samples represents proteins from different compartments of the cell, and the relative contribution of each compartment can, presumably, vary. In addition, the apparent expression of enzyme/transporter abundances can vary based on the total amount of protein in this system even in the cases where the level of the target enzyme/transporter is constant in tissue. In this study, the ten most abundant proteins in HLM samples were shown to constitute 15-20% of protein mass in these samples, and their expression can vary, leading to apparent variation in abundance of CYP3A4 by up to 1.7-fold (p<0.0001). Further, enzymes enriched in this system can achieve a level of background correlation based on variability of unrelated but highly expressed proteins, a hypothesis proposed in our earlier reports (Achour et al., 2014b; Achour et al., 2014c). This effect was simulated by randomly varying the amount of CYP2C8 and CYP3A4 in tissue and then normalizing by total protein mass with variations in the abundance of these ten unrelated proteins. This simulation revealed a level of positive background correlation (Spearman correlation coefficient, Rs = +0.1 to +0.2) with statistical significance (p < 0.0001) for all
assessed enzymes, further supporting the use of tissue mass, instead of total HLM protein mass, as the normalization factor, as previously advocated by Milne et al. (2011). The units of protein abundance would then be pmol mg\(^{-1}\) tissue. Although strong correlations between enzymes with common genetic regulatory mechanisms are highly expected (Wortham et al., 2007; Jover et al., 2009), the reported level of background correlation encourages exercising caution when interpreting and using weak to moderate expression relationships reported in the literature when abundance values are expressed in the traditional units even if the correlation exhibits statistical significance.

In conclusion, this report constitutes a proof-of-principle study that demonstrates the utility of snapshot global profiling of enzymes in biological systems as a screening method and raises cautionary arguments about using abundance levels of ADME proteins reported in the literature and their correlations of expression. The report also provides preliminary qualitative and quantitative details about the protein composition of HLM samples. Limitations of the current work consist of mainly the low sample size (4 HLM samples), which renders comprehensive elucidation of inter-individual variability in a population using the data in this report highly unlikely.
Acknowledgments

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Authorship Contributions

Participated in research design: Achour, Rostami-Hodjegan, Barber

Conducted experiments: Achour, Al-Feteisi, Lanucara

Performed data analysis: Achour, Lanucara

Wrote or contributed to the writing of the manuscript: Achour, Rostami-Hodjegan, Barber
References


Footnotes

Financial support from the Division of Pharmacy and Optometry, School of Health Sciences, University of Manchester, is acknowledged.
**Figure legends**

**Figure 1** Proteomic analysis of microsomal sub-proteome in the HLM samples showing the total number of identified and quantified proteins in the fraction (A); the number of all drug-metabolizing enzymes, DME, drug-metabolizing cytochrome P450 enzymes, CYP450, and drug-metabolizing uridine 5'-diphosphoglucuronosyltransferase enzymes, UGT (B); Spearman correlation with linear regression of measurements of drug-metabolizing enzymes using the label-free methodology described in this report and measurements of the same enzymes in three of the analyzed samples using QconCAT targeted methodology (C); fold difference in abundance of enzymes of label-free measurements (method 1) in each sample relative to QconCAT measurements (method 2) expressed as a ratio \( \left[ \frac{x_{1,i}}{x_{2,i}} \right] \) for enzyme \( i \), with all pairs of measurements within approximately 2.5-fold (gray box) (D). Average fold error (AFE) is a measure of bias in the data, whereas absolute average fold error (AAFE) is a measure of scatter or spread of measurements; the closer these two measures to 1, the lower the bias and scatter in measurement; there was limited bias in the two method and a level of spread in the data (see Supplemental Table 3). In panel (C), abundances are expressed in units of pmol mg\(^{-1}\) HLM protein.

**Figure 2** Patterns of expression of drug-metabolizing enzymes in liver samples: cytochrome P450 enzyme abundances compared to literature values (A), UGT abundances compared to literature values (B), patterns of expression of quantified drug-metabolizing enzymes in the HLM samples (C), and heat map of the expressed P450 and UGT enzymes with samples classed using rank order clustering (D). In panels (A) and (B), the gray highlights indicate literature derived ranges, the bars indicate literature means and the scatter points indicate experimentally derived values in this study. In panel (C), BLQ is assigned for values below the limit of quantification. In panel (D), the abundance values are normal log modified. Abundances are expressed in units of pmol mg\(^{-1}\) HLM protein.

**Figure 3** The abundance of specific membrane markers of hepatocytes (ASGR1), endoplasmic reticulum (calnexin), plasma membrane (ATP1A1, CD81), mitochondria (COX4) and peroxisomes (PEX14) (A), sub-cellular localization of all identified proteins (B) and drug-metabolizing enzymes (C) in analyzed samples, providing indication of the presence of membrane fractions from these organelles in human
liver microsomes. In panel (A), abundances are expressed in units of pmol mg\(^{-1}\) HLM protein. In panels (B) and (C), percentages represent the proportions of proteins identified in each sub-cellular location to the total identified number of proteins, the sum of which adds up to more than 100% due to overlap in localization of protein expression as shown in Supplemental Information.

**Figure 4** The contribution of the ten most abundant proteins to total HLM protein mass from 4 human livers (A) and simulated effect of variability of the top ten proteins in HLM samples on CYP3A4 abundance in 2,000 human livers (B). When the amount of CYP3A4 within tissue in simulated livers is kept constant, the abundance of CYP3A4 changes on average 1.4-1.7 fold, representing the effect of overall random variability in the most abundant proteins. Simulations were based on data obtained from this experimental study and a meta-analysis of available literature. In panel (B), abundances are expressed in units of pmol mg\(^{-1}\) HLM protein.
Tables

**Table 1** Demographic and clinical details of the individual liver donors of samples used in this study. The final column shows the suppliers of samples.

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>Age (years)</th>
<th>Ethnicity</th>
<th>Gender</th>
<th>Cause of death</th>
<th>Smoking</th>
<th>Alcohol use</th>
<th>Medical history</th>
<th>Medication</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM01</td>
<td>31</td>
<td>C</td>
<td>F</td>
<td>Motor vehicle accident</td>
<td>Yes</td>
<td>No</td>
<td>None</td>
<td>None</td>
<td>BD Gentest</td>
</tr>
<tr>
<td>HLM02</td>
<td>62</td>
<td>C</td>
<td>F</td>
<td>Head trauma</td>
<td>No</td>
<td>No</td>
<td>Hypertension</td>
<td>Hypertension medications</td>
<td>BD Gentest</td>
</tr>
<tr>
<td>HLM03</td>
<td>41</td>
<td>H</td>
<td>F</td>
<td>CVA</td>
<td>No</td>
<td>Occasional</td>
<td>Hypertension, mild stroke</td>
<td>Atenolol, Dobutamine, Morphine, Nuprin</td>
<td>BD Gentest</td>
</tr>
<tr>
<td>HLM04</td>
<td>50</td>
<td>C</td>
<td>M</td>
<td>CVA</td>
<td>No</td>
<td>No</td>
<td>Healthy</td>
<td>None</td>
<td>Vitron</td>
</tr>
</tbody>
</table>

C, Caucasian; H, Hispanic; F, Female; M, Male; CVA, Cerebrovascular aneurysm

Human liver microsomal (HLM) samples were prepared by the suppliers using differential centrifugation of hepatic tissue homogenates.
Table 2  Rank orders, abundance levels and primary subcellular localization of the ten most abundant proteins in the analyzed HLM samples and those of drug-metabolizing cytochrome P450 enzymes

<table>
<thead>
<tr>
<th>Rank</th>
<th>Overall rank</th>
<th>Protein (gene name)</th>
<th>Overall abundance</th>
<th>HLM01</th>
<th>HLM02</th>
<th>HLM03</th>
<th>HLM04</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>Liver carboxylesterase 1 (CES1)</td>
<td>403.14 ± 92.8</td>
<td>485.94 ± 24.31 (1)</td>
<td>396.81 ± 31.63 (1)</td>
<td>275.38 ± 13.45 (5)</td>
<td>454.42 ± 19.15 (1)</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>Cytoplasmic actin 1 (ACTB)</td>
<td>316.94 ± 74.63</td>
<td>325.51 ± 50.06 (8)</td>
<td>363.32 ± 21.94 (2)</td>
<td>370.02 ± 24.37 (2)</td>
<td>208.93 ± 23.54 (10)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Protein disulfide isomerase (P4HB)</td>
<td>304.25 ± 101.48</td>
<td>415.74 ± 12.23 (3)</td>
<td>324.86 ± 18.57 (3)</td>
<td>169.96 ± 4.02 (9)</td>
<td>306.44 ± 12.18 (2)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>78 kDa glucose-regulated protein (HSPA5)</td>
<td>284.23 ± 62.67</td>
<td>352.90 ± 10.52 (6)</td>
<td>284.86 ± 24.83 (5)</td>
<td>201.29 ± 2.06 (8)</td>
<td>297.86 ± 10.86 (3)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>ATP synthase subunit beta (ATP5B)</td>
<td>264.10 ± 96.66</td>
<td>140.23 ± 1.59 (24)</td>
<td>270.66 ± 25.26 (6)</td>
<td>376.41 ± 19.64 (1)</td>
<td>269.09 ± 2.31 (5)</td>
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<tr>
<td>6</td>
<td>6</td>
<td>Protein disulfide isomerase A3 (PDIA3)</td>
<td>262.73 ± 108.03</td>
<td>387.29 ± 12.61 (4)</td>
<td>252.14 ± 17.28 (7)</td>
<td>127.46 ± 2.23 (27)</td>
<td>286.05 ± 12.36 (4)</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>Calreticulin (CALR)</td>
<td>257.40 ± 86.90</td>
<td>372.06 ± 7.27 (5)</td>
<td>223.98 ± 36.44 (10)</td>
<td>166.32 ± 13.55 (11)</td>
<td>267.26 ± 4.77 (6)</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>Haptoglobin (HP)</td>
<td>253.47 ± 155.76</td>
<td>457.21 ± 34.92 (2)</td>
<td>292.00 ± 22.60 (4)</td>
<td>117.54 ± 10.34 (30)</td>
<td>147.11 ± 1.66 (21)</td>
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<tr>
<td>9</td>
<td>9</td>
<td>Endoplasmin (HSP90B1)</td>
<td>243.64 ± 78.95</td>
<td>335.09 ± 9.03 (7)</td>
<td>231.93 ± 17.82 (9)</td>
<td>144.52 ± 15.96 (14)</td>
<td>263.01 ± 6.13 (7)</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>Cytochrome b5 (CYB5A)</td>
<td>226.79 ± 18.36</td>
<td>251.61 ± 26.34 (11)</td>
<td>209.61 ± 15.40 (12)</td>
<td>217.01 ± 7.03 (6)</td>
<td>228.93 ± 36.13 (8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rank</th>
<th>Drug-metabolizing P450 enzymes</th>
<th>Overall rank</th>
<th>Overall abundance</th>
<th>HLM01</th>
<th>HLM02</th>
<th>HLM03</th>
<th>HLM04</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
<td>Mean ± SD [pmol mg⁻¹ (rank)]</td>
</tr>
<tr>
<td>44</td>
<td>CYP3A4</td>
<td>80.87 ± 58.48</td>
<td>126.83 ± 4.43 (26)</td>
<td>134.71 ± 13.65 (26)</td>
<td>19.32 ± 1.63 (231)</td>
<td>42.64 ± 1.02 (122)</td>
<td></td>
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<tr>
<td>53</td>
<td>CYP2E1</td>
<td>76.28 ± 14.78</td>
<td>73.50 ± 6.23 (57)</td>
<td>73.36 ± 8.65 (65)</td>
<td>60.84 ± 6.38 (78)</td>
<td>96.63 ± 13.80 (34)</td>
<td></td>
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<tr>
<td>101</td>
<td>CYP2C9</td>
<td>50.37 ± 30.63</td>
<td>94.26 ± 13.22 (43)</td>
<td>29.89 ± 0.73 (192)</td>
<td>28.82 ± 0.40 (171)</td>
<td>48.49 ± 2.83 (106)</td>
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<tr>
<td>109</td>
<td>CYP4F4</td>
<td>48.31 ± 19.96</td>
<td>28.37 ± 2.56 (165)</td>
<td>68.91 ± 8.19 (70)</td>
<td>34.32 ± 1.79 (142)</td>
<td>61.63 ± 2.33 (80)</td>
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<tr>
<td>180</td>
<td>CYP2A6</td>
<td>48.11 ± 43.44</td>
<td>108.60 ± 13.01 (36)</td>
<td>38.44 ± 3.53 (147)</td>
<td>5.11 ± 0.57 (597)</td>
<td>40.30 ± 1.37 (127)</td>
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<tr>
<td>186</td>
<td>CYP1A5</td>
<td>31.07 ± 9.68</td>
<td>37.92 ± 4.13 (117)</td>
<td>24.22 ± 1.54 (231)</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>201</td>
<td>CYP2C8</td>
<td>30.44 ± 7.19</td>
<td>24.25 ± 2.59 (184)</td>
<td>28.72 ± 2.81 (196)</td>
<td>–</td>
<td>–</td>
<td>38.33 ± 5.88 (134)</td>
</tr>
<tr>
<td>262</td>
<td>CYP2B6</td>
<td>27.57 ± 25.42</td>
<td>64.37 ± 3.03 (66)</td>
<td>23.22 ± 1.72 (244)</td>
<td>6.92 ± 0.77 (516)</td>
<td>15.78 ± 1.25 (296)</td>
<td></td>
</tr>
<tr>
<td>425</td>
<td>CYP2D6</td>
<td>20.98 ± 1.22</td>
<td>20.98 ± 1.22 (225)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>560</td>
<td>CYP3A7</td>
<td>12.45 ± 4.84</td>
<td>10.74 ± 1.07 (372)</td>
<td>8.70 ± 1.72 (538)</td>
<td>17.91 ± 1.79 (245)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>815</td>
<td>CYP2C19</td>
<td>9.21 ± 0.62</td>
<td>9.21 ± 0.62 (413)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4.98 ± 0.76 (604)</td>
</tr>
<tr>
<td>1066</td>
<td>CYP3A43</td>
<td>5.47 ± 0.69</td>
<td>5.96 ± 0.17 (542)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5.96 ± 0.17 (542)</td>
</tr>
<tr>
<td>51</td>
<td>NADPH cytochrome P450 reductase (POR)</td>
<td>77.95 ± 19.18</td>
<td>85.88 ± 3.27 (53)</td>
<td>100.12 ± 5.52 (35)</td>
<td>55.97 ± 8.61 (87)</td>
<td>69.85 ± 3.27 (62)</td>
<td></td>
</tr>
</tbody>
</table>

a SD representing combined biological and technical variability, b SD representing technical variability, c subcellular localization: endoplasmic reticulum, d subcellular localization: cytoplasm, e subcellular localization: mitochondria, f subcellular localization: secreted
Figure 1

A

[Bar chart showing the number of proteins identified and quantified across HLM01 to HLM04.]

B

[Bar chart showing the number of enzymes (DME, CYP450, UGT) across HLM01 to HLM04.]

C

[Scatter plot showing a linear relationship between QconCAT targeted measurements and label-free measurements. The equation is \( y = 0.84x \), with \( R^2 = 0.70 \), \( R_s = 0.84 \), and \( p < 0.0001 \).]

D

[Plot showing the fold difference between methods, with \( AFE = 0.99 \) and \( AAFE = 1.46 \).]
Figure 2

A

B

C

D

Enzyme abundance (pmol mg⁻¹)

Enzyme abundance (pmol mg⁻¹)

Enzyme abundance (pmol mg⁻¹)

Enzyme abundance (pmol mg⁻¹)

BLQ

HLM01

HLM02

HLM04

HLM03
Figure 3

A

B

C

Abundance (pmol mg^{-1})

Number of Proteins

Number of enzymes

Membrane marker proteins

Endoplasmic reticulum
Mitochondria
Plasma membrane/exosomes
Cytosol/cytoplasm
Secreted
Nucleus
Peroxisomes
Golgi body

Endoplasmic reticulum
Mitochondria
Plasma membrane/exosomes
Cytosol/cytoplasm
Secreted
Nucleus
Peroxisomes

58%
26%
29%
8%
6%
3%

19%
34%
12%
11%
32%
32%

34%
2%
12%
11%
32%
32%

58%
26%
29%
8%
6%
3%
Figure 4

A

Cumulative protein mass (%) vs. Ranked proteins

- HLM01
- HLM02
- HLM03
- HLM04

>99% total protein mass

15-20% total protein mass

B

CYP3A4 abundance (pmol mg⁻¹) vs. Total mass of the ten most abundant proteins in HLM samples (µg × 10⁻³)

- Variable CYP3A4 amount
- Median CYP3A4 amount