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Comparison of Proteomic Quantification Approaches for Hepatic Drug Transporters: Multiplexed Global Quantitation Correlates with Targeted Proteomic Quantitation[S]

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ABSTRACT

Targeted protein quantification using liquid chromatography–tandem mass spectrometry with stable isotope-labeled standards is recognized as the gold standard of practice for protein quantification. Such assays, however, can only cover a limited number of proteins, and developing targeted methods for larger numbers of proteins requires substantial investment. Alternatively, large-scale global proteomic experiments along with computational methods such as the "total protein approach" (TPA) have the potential to provide extensive protein quantification. In this study, we compared the TPA-based quantitation of seven major hepatic uptake transporters in four human liver tissue samples using global proteomic data obtained from two multiplexed tandem mass tag experiments (performed in two independent laboratories) to the quantitative data from targeted proteomic assays. The TPA-based quantitation of these hepatic transporters [sodium-taurocholate cotransporting polypeptide (NTCP/SLC10A1), organic anion transporter 2 (OAT2/SLC22A7), OAT7/SLC22A9, organic anion-transporting polypeptide 1B1 (OATP1B1/SLCO1B1), OATP1B3/SLCO1B3, OATP2B1/SLCO2B1, and organic cation transporter (OCT1/SLC22A1)] showed good-to-excellent correlations (Pearson r = 0.74–1.00) to the targeted data. In addition, the values were similar to those measured by targeted proteomics with 71% and 86% of the data sets falling within 3-fold of the targeted data. A comparison of the TPA-based quantifications of enzyme abundances to available literature data showed that the majority of the enzyme quantifications fell within the reference data intervals. In conclusion, these results demonstrate the capability of multiplexed global proteomic experiments to detect differences in protein expression between samples and provide reasonable estimations of protein expression levels.

Introduction

Quantification of transport proteins is an important component to facilitate in vitro–in vivo extrapolation (IVIVE), and thus the mechanistic modeling of drug disposition (Prasad and Unadkat, 2014; Vildhede et al., 2016). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is generally considered the method of choice for the identification and quantification of proteins (Han et al., 2008). Typically, the proteins are enzymatically digested into peptides, which are then separated and analyzed by LC-MS/MS (Walther and Mann, 2010). Peptide concentrations are thus measured as surrogates of protein abundance.

There are essentially two main strategies for mass spectrometry-based proteomic analysis. In targeted proteomics, a predefined set of peptide ions, uniquely identifying proteins of interest, and one or more of their fragment ions are selected and monitored for accurate quantification of each peptide using an external calibration curve. Because of the reproducibility and high selectivity and sensitivity of the method, targeted proteomics is considered the gold standard for protein quantification (Peterson et al., 2012). It does, however, suffer from limited throughput, as there are practical restrictions to the number of peptides that can be targeted in a single run with reliable quantification (Lange et al., 2008).

In contrast to targeted proteomics, global or shotgun proteomics aims to identify as many proteins as possible in a sample. This is achieved by operating the mass spectrometer in data-dependent acquisition mode (Zhang et al., 2013). Precursor ions are detected in a survey scan followed by fragmentation of a subset, typically selected by highest signal abundances. From the fragment spectra, the identities of the peptides can be determined by sequence database searching, and the peptides are then assigned to proteins. To convert the spectral intensities to protein concentrations, a computational method termed the "total protein approach" (TPA) was recently proposed and validated (Wiśniewski et al., 2012). The method does not require standards and is applicable to any large-scale proteomic data set. It thus offers an efficient way to quantify all identified proteins in a sample. This approach is, however, expected to be less reproducible than targeted proteomic quantifications owing to the stochastic sampling of precursor ions for fragmentation, thus affecting run-to-run reproducibility (Tabb et al., 2010).

To reduce the impact of run-to-run variability when comparing protein abundances between different samples, multiplexing at the sample level is possible. For example, tandem mass tags (TMT) can be used to introduce isotope-based differences to the samples via chemical

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ABBREVIATIONS: CYP, cytochrome P450; LC, liquid chromatography; MS/MS (MS2), tandem mass spectrometry; MS/MS/MS (MS3); triple-stage mass spectrometry; NTCP, sodium taurocholate cotransporting polypeptide; OAT2, organic anion transporter 2; OAT7, organic anion transporter 7; OATP1B1, organic anion-transporting polypeptide 1B1; OATP1B3, organic anion-transporting polypeptide 1B3; OATP2B1, organic anion-transporting polypeptide 2B1; OCT1, organic cation transporter 1; TMT, tandem mass tag; TPA, total protein approach; UGT, UDP-glucuronosyltransferase.

labeling (Thompson et al., 2003). This approach was initially reported to suffer from interference, resulting in ratio distortions, a problem that was solved by an additional fragmentation step, i.e., triple-stage mass spectrometry (MS3) (Ting et al., 2011).

In this study, we aimed to evaluate the quantitative ability of the total protein approach using MS3 spectral data obtained from two independently performed TMT experiments. To assess quantification accuracy, calculated protein levels were compared with the abundances determined by targeted proteomics for a set of hepatic uptake transporters that are of relevance in drug disposition.

Materials and Methods

Human Liver Tissue. Normal human liver tissue samples from four different donors were obtained from the Pfizer Tissue Bank (Groton, CT). Collection of these samples was conducted in a manner compliant with Pfizer policies, including ethical approval.

Targeted Proteomic Quantification of Hepatic Uptake Transporters. Surrogate peptide levels for sodium taurocholate cotransporting polypeptide (NTCP), organic anion transporter 2 (OAT2), OAT7, organic anion transporting polypeptide 1B1 (OATP1B1), OATP1B3, OATP2B1, and organic cation transporter 1 (OCT1) were measured by modification of previously published quantitative targeted proteomic methods (Kimoto et al., 2012; Qiu et al., 2013), and with guidance on the choice of proteotypic surrogate peptides from other publications (Sakamoto et al., 2011; Groer et al., 2013). Details of the targeted proteomic methodology are provided in Supplemental Methods. In brief, membrane proteins were extracted from the human liver samples and digested with trypsin (Supplemental Fig. 1). The resulting mixture of peptides was spiked with known quantities of stable isotope-labeled peptides as internal standards. Peptides were then separated and analyzed by LC-MS/MS in multiple-reaction monitoring mode. Chromatographic peak areas of target fragment ions in relation to their respective stable isotope-labeled internal standard were used to calculate peptide concentrations (as surrogate of protein levels) with an external calibration curve prepared in digested human serum albumin matrix.

Multiplexed Global Proteomic Analysis of Liver Tissue Samples and Quantification of Protein Abundances. Liver tissue samples from the same donors included in the targeted proteomic quantification were analyzed by multiplexed global proteomics in two independent laboratories: MS Bioworks (Ann Arbor, MI) and IQ Proteomics (Cambridge, MA) using their in-house protocols. For the global proteomic experiments, proteins (both soluble and membrane-bound) were extracted by homogenization and lysis of the liver tissue samples (Supplemental Fig. 1). Proteins were digested with trypsin (laboratory 1) or Lys-C and trypsin in tandem (laboratory 2), and the resulting peptide samples were labeled with isobaric TMT reagents in technical duplicates. After labeling, samples were mixed and peptides were subjected to high pH reverse-phase fractionation, followed by LC-MS/MS/MS analysis on Orbitrap Fusion Lumos instruments (Thermo Fisher Scientific, Waltham, MA). MS3 spectral intensities of the reporter ions, formed upon fragmentation, were used to calculate protein abundances under the assumption that the MS signal for a given protein in relation to the summed MS signals for all identified proteins reflects its partial abundance in the sample (Wiśniewski, 2017). A more detailed description of the TMT experiments is provided in Supplementary Methods.

Statistical Analysis. Correlations of protein abundance measurements across laboratories were assessed by Pearson's correlation coefficients (r). Protein abundance values calculated from the TMT data were compared with those determined in the targeted proteomic experiment by Dunnett's multiple comparison tests and by calculating fold errors.

Results and Discussion

To evaluate the quantitative merits of the multiplexed global proteomics approach, and to justify its utility in quantitative translation needed for system pharmacokinetics, we compared transporter protein levels in human liver samples acquired from TMT data to the abundances determined by validated targeted proteomic methods. For this comparison, TMT data obtained from two independent laboratories with different sample processing protocols were used.

Surrogate peptide abundance levels of the major hepatic uptake transporters involved in drug disposition, namely NTCP, OAT2, OAT7, OATP1B1, OATP1B3, OATP2B1, and OCT1, were measured in four human liver tissue samples. All these transporters were detected in the liver tissue samples analyzed with measured abundances within the ranges observed in previous studies (Kimoto et al., 2012; Qiu et al., 2013; Peng et al., 2015; Wang et al., 2015). For the four samples included in this study, NTCP showed ~6-fold intersubject variability, whereas the variability of the other transporters was ~2- to 3-fold (Table 1).

Human liver tissue samples from the same donors included in the targeted proteomic quantification were analyzed in two separate TMT experiments, performed in two independent laboratories. The samples were analyzed in technical duplicates. Very high correlations (Pearson $r > 0.99$) in MS3 intensities for the replicate measurements were observed, indicating a good precision of the TMT method. More than 5000 proteins were identified in each TMT experiment, demonstrating a sufficient depth of analysis for the TPA-based quantification of protein abundances (Wiśniewski, 2017). Among the identified proteins were the seven uptake transporters of interest (Table 1). For laboratory 1, however, NTCP was not detected in MS3 mode and, thus, MS2 data were used instead. The lack of NTCP detection in MS3 mode was likely a result of the shorter MS acquisition time for this TMT experiment compared with the other one (24-hour compared with 36-hour).

TABLE 1

Comparison of the average protein abundance and range (interindividual variability) in protein abundance for four human liver tissue samples measured with two different proteomic methodologies, targeted proteomics, and multiplexed global proteomics using tandem mass tag (TMT) reagents

LC-MS/MS/MS run time was 24 hours for laboratory 1 and 48 hours for laboratory 2.

Transporter	Average Protein Abundance ± S.D. (pmol/mg membrane protein)			Range in Protein Abundance (max/min)		
	Targeted Data	TMT Data		Targeted Data	TMT Data	
		Laboratory 1	Laboratory 2		Laboratory 1	Laboratory 2
NTCP	1.28 ± 0.67	1.09 ± 0.39[a]	4.81 ± 1.52*	6.03	2.60[a]	2.32
OAT2	1.15 ± 0.45	0.74 ± 0.32	2.74 ± 0.94*	2.50	2.71	2.30
OAT7	5.42 ± 1.77	1.32 ± 0.55*	4.16 ± 1.38*	1.97	2.53	1.97
OATP1B1	8.08 ± 2.24	2.80 ± 0.85*	4.28 ± 1.47*	2.07	2.24	2.52
OATP1B3	4.39 ± 1.88	1.24 ± 0.24*	2.11 ± 0.98*	2.60	1.58	2.69
OATP2B1	1.86 ± 0.73	1.72 ± 0.50	2.29 ± 0.79	2.23	1.96	2.35
OCT1	2.92 ± 0.80	5.69 ± 2.31*	3.37 ± 1.00	1.79	2.32	1.86

*Statistically different from the targeted proteomic data (adjusted $P < 0.05$).
[a]NTCP values represent MS2 data for laboratory 1 because of lack of detection of NTCP in MS3 mode.

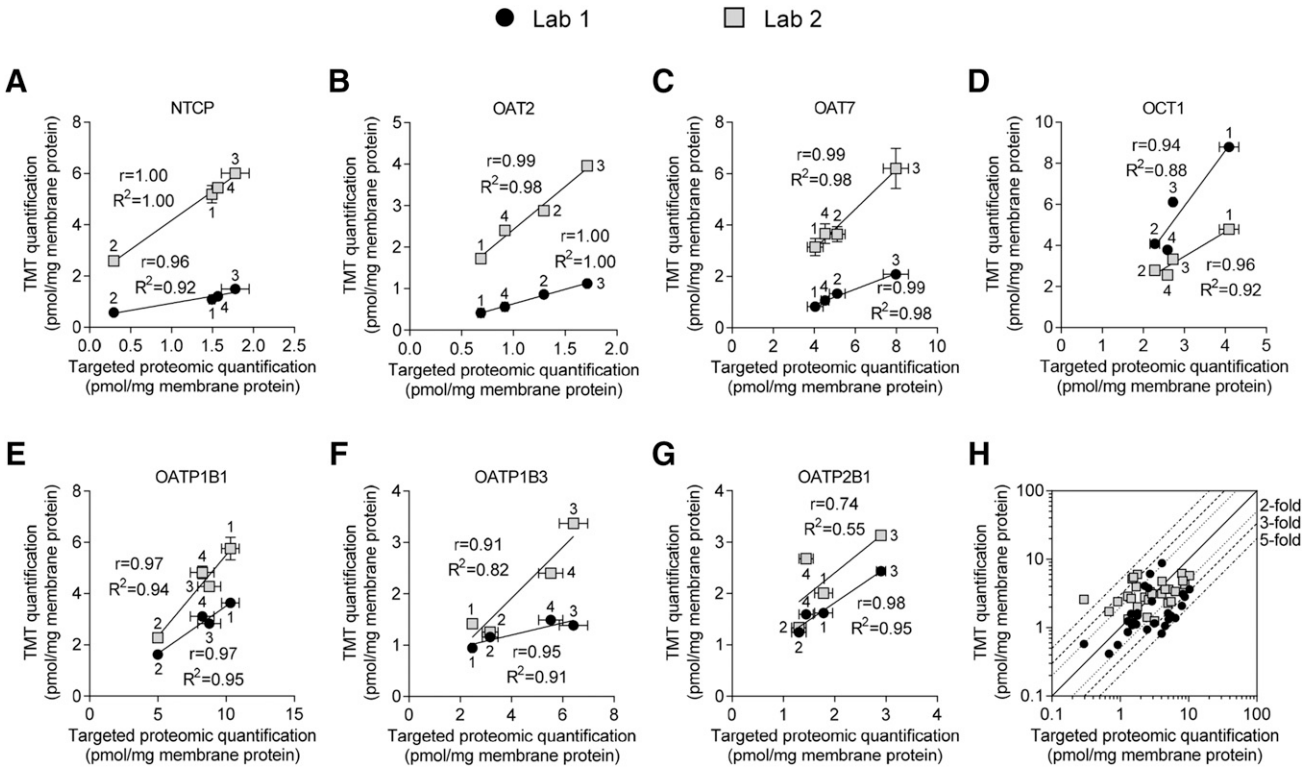

Fig. 1. Comparison of protein quantifications by multiplexed global proteomics (*y*-axis) and targeted proteomics (*x*-axis). Cross-laboratory correlation analysis of protein abundance measurements for NTCP (A), OAT2 (B), OAT7 (C), OCT1 (D), OATP1B1 (E), OATP1B3 (F), and OATP2B1 (G). Data are presented as arithmetic means with standard deviations (n = 3 for the targeted proteomic data, n = 2 for the TMT data). r = Pearson correlation coefficient. Quantitative agreement of protein abundances calculated from the multiplexed global proteomic data to those measured by targeted proteomics (H). The solid line represents the line of unity, dotted lines represent 2-fold deviations, dashed lines represent 3-fold deviations, and the dot-dash lines represent 5-fold deviations. LC-MS/MS/MS run time was 24 hours for laboratory 1 and 48 hours for laboratory 2. NTCP was not detected in MS3 mode for laboratory 1, and hence, MS2 data were used instead.

Moreover, because of the stochastic element of precursor ion fragmentation in data-dependent acquisition mode, proteins of interest are not always identified in complex samples (Tabb et al., 2010). This is especially true for less abundant proteins, such as NTCP, when the selection criterion for fragmentation is chosen on the basis of peptides with the highest signal abundances. To increase the probability of identifying the proteins of interest, enrichment through subcellular fractionation is possible, although isolated fractions are reported to be associated with lower yields than expected (Wiśniewski et al., 2016; Wegler et al., 2017). Another option is to perform data-independent acquisition on the proteome-scale using a recently introduced technique known as SWATH-MS (Gillet et al., 2012).

The calculated protein abundances from the TMT experiments were compared with the targeted protein quantifications (Fig. 1). For this comparison, a membrane-to-total protein ratio of 0.233 (in-house

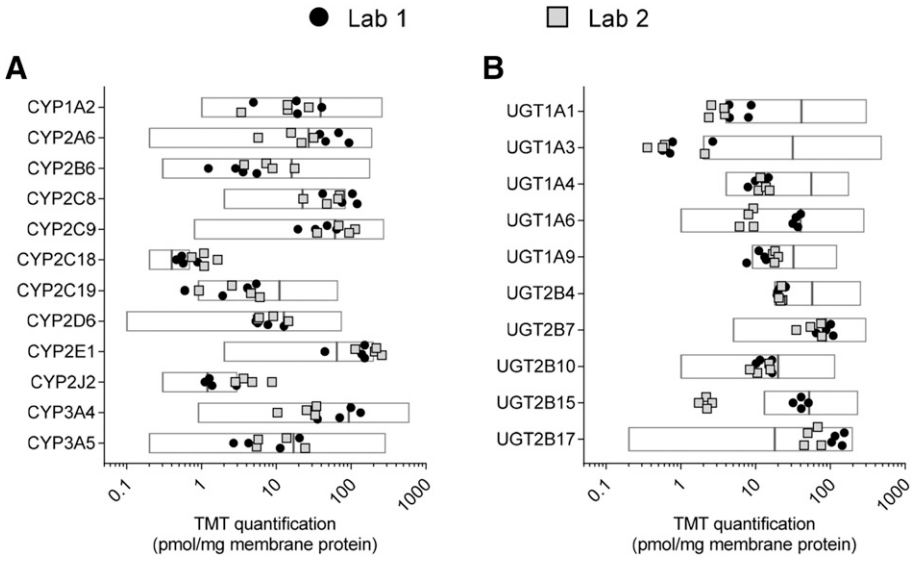

Fig. 2. Comparison of cytochrome P450 (A) and UGT (B) enzyme abundances, quantified by multiplexed global proteomics, to the range (white bars) and weighed mean (solid gray line) from two recent meta-analyses (Achour et al., 2014a,b).

data, $n = 42$) was used to convert the measured abundances to the same unit. The TMT data showed good-to-excellent correlations (Pearson r = 0.74–1.00) to the targeted proteomic data (Fig. 1, A–G). We attribute this cross-laboratory agreement to the confident identification of peptides in the TMT experiments and to the thorough analytical validation of the targeted proteomic assays to avoid false positive identifications caused by matrix interference (Balogh et al., 2012; Qiu et al., 2013; Carr et al., 2014). The good correlations observed in our study are in stark contrast to the predominantly poor correlations of transporter and enzyme protein levels across laboratories reported elsewhere (Achour et al., 2017; Wegler et al., 2017). In such cases, correlations with activity measurements are necessary to determine which proteomic methodology results in expression data that align with functional activity. Unfortunately, it is not possible to measure transport activities in frozen liver tissue samples.

In addition to the excellent correlations of measured protein concentrations across methods observed in our study, the transporter abundances calculated from the TMT data resulted in values comparable to those measured by targeted proteomics with absolute fold errors within 3-fold for 71% and 86% of the data set for the two TMT experiments, respectively (Fig. 1H). Furthermore, the range (interindividual variability) in protein expression was very similar across methods (Table 1), suggesting that the multiplexed global proteomic approach can be used to reliably detect differences in protein levels across samples.

The better quantitative agreement for laboratory 2 suggests that longer run-times are required for more reliable quantifications. This observation is in agreement with the finding that higher quantification accuracy is obtained when proteins are identified by a larger number of peptides (Wiśniewski and Rakus, 2014). Differences in the sample processing protocols, including the use of sequential enzymatic digestion with Lys-C and trypsin (laboratory 2) compared with digestion with trypsin alone (laboratory 1), can also add to the quantitative differences observed, when the former is expected to reduce missed cleavages and enhance protein sequence coverage (Guo et al., 2014).

To further assess the accuracy of the TPA-based quantifications, we compared the abundances of cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) drug-metabolizing enzymes with literature data (Fig. 2). Most of the enzyme quantifications fell within the ranges reported in literature. In addition, there was a good quantitative agreement between the two different TMT experiments with an average absolute fold error of 2.1 for the CYP enzymes and 3.5 for the UGT enzymes, respectively.

In summary, our results suggest that multiplexed global proteomic experiments with sufficient proteome coverage can provide reasonable estimates of protein concentrations, as demonstrated with a set of major hepatic uptake transporters, as well as drug-metabolizing enzymes. More importantly, such experiments accurately capture differences in protein levels across samples and may thus be used to compare in vitro and in vivo expression for IVIVE exercises. The global proteomic approach also supports the simultaneous quantification of thousands of other proteins, thus providing a much larger dataset than that obtained by targeted proteomics. Such data can, for example, be useful when studying the age-, gender-, ethnicity-, disease-, and drug-dependent expression of transporters and other drug disposition-related proteins in various tissues or when assessing the appropriateness of an in vitro model compared with the in vivo counterpart. Our study is the first to present evidence suggesting that MS3 intensities from TMT experiments can be applied to TPA-based protein quantifications. Additional validation employing the multiplexed global proteomic approach presented herein can further strengthen its application in quantitative proteomics.

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