

Commentary

Data Generated by Quantitative LC-MS Proteomics Are Only the Start and Not the Endpoint: Optimization of QconCAT-Based Measurement of Hepatic UDP- Glucuronosyltransferase Enzymes with Reference to Catalytic Activity

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Running Title: Data Analysis Optimization for QconCAT-Based UGT Proteomics

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Abbreviations:

AZT, Zidovudine; BCA, bichinchoninic acid assay; CDCA, chenodeoxycholic acid; HLM, human liver microsomes; IVIVE, in vitro-in vivo extrapolation; LC, liquid chromatography; MRM, multi-reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PBPK, physiologically-based pharmacokinetics; QconCAT, quantification concatemer; QIS, QconCAT-based internal standard; SIL, stable isotope-labeled; UDP, uridine-5'-diphosphate; UGT, uridine-5'-diphosphoglucuronosyltransferase.

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Abstract

Quantitative proteomic methods require optimization at several stages, including sample preparation, LC-MS/MS and data analysis, with the final analysis stage being less widely appreciated by end-users. Achour et al. (2017b) previously reported measurement of eight uridine-5'-diphosphoglucuronosyltransferases (UGT) generated by two laboratories [using stable isotope-labeled (SIL) peptides or quantitative concatemer (QconCAT)], which reflected significant disparity between proteomic methods. Initial analysis of QconCAT data showed lack of correlation with catalytic activity for several UGTs (1A4, 1A6, 1A9, 2B15) and moderate correlations for UGTs 1A1, 1A3 and 2B7 ($R_s=0.40-0.79$, $p<0.05$; $R^2=0.30$); good correlations were demonstrated between cytochrome P450 activities and abundances measured in the same experiments. Consequently, a systematic review of data analysis, starting from unprocessed LC-MS/MS data, was undertaken, with the aim of improving accuracy, defined by correlation against activity. Three main criteria were found to be important: choice of monitored peptides and fragments, correction for isotope-label incorporation, and abundance normalization using fractional protein mass. Upon optimization, abundance-activity correlations improved significantly for six UGTs ($R_s=0.53-0.87$, $p<0.01$; $R^2=0.48-0.73$); UGT1A9 showed moderate correlation ($R_s=0.47$, $p=0.02$; $R^2=0.34$). No spurious abundance-activity relationships were identified. However, methods remained sub-optimal for UGT1A3 and UGT1A9; here hydrophobicity of standard peptides is believed to be limiting. This commentary provides a detailed data analysis strategy and indicates, using examples, the significance of systematic data processing following acquisition. The proposed strategy offers significant improvement on existing guidelines applicable to clinically-relevant proteins quantified using QconCAT.

Introduction

Robust quantification of proteins involved in drug pharmacokinetics is required for reliable *in vitro-in vivo* prediction (IVIVE) of drug-related outcomes (Al Feteisi et al., 2015b). Various quantitative proteomic strategies share several key steps: (1) selection of signature peptides that represent target proteins in biological samples, with stable isotope-labeled (SIL) versions used as standards; (2) isolation of tissue fractions that contain these proteins; (3) sample preparation for mass spectrometry, by solubilization and proteolysis of proteins into peptides; and (4) simultaneous analysis of standard and native peptides by LC-MS/MS (Wegler et al., 2017). Furthermore, using concatenated standards (QconCAT) requires additional steps to ensure that standard proteins are successfully expressed and sufficiently labeled, purified and digested (Russell et al., 2013).

Uridine-5'-diphospho-glucuronosyltransferases (UGT) have recently attracted more clinical attention (Guillemette et al., 2014), leading to increased interest in UGT abundance and activity data (Margaillan et al., 2015). Our laboratories previously reported comparability of two proteomic assays: *in-solution* sample preparation with quantification using SIL peptide standards (Fallon et al., 2013) and *gel-based* sample preparation with QconCAT proteomics (Achour et al., 2014b). Disparities between abundances generated by these methods pointed to the necessity of validating measurements using UGT-isoform specific activity. Reliable correlation was demonstrated for the SIL-based measurements; discrepancies remained for the QconCAT-based dataset (Achour et al., 2017b).

The QconCAT methodology has been validated in various contexts (Scott et al., 2016), including, most notably, cytochrome P450 quantification carried out with the UGT measurements (Achour et al., 2014b). Quantification of UGTs led to complications that had not been observed with bacterial (Al-Majdoub et al., 2014) and yeast samples (Brownridge et al., 2011). The proteomic strategy used in this study is inherently complex; however, many steps have been taken to validate the LC-MS/MS multi-reaction monitoring (MRM) assay, including assessment of precision and accuracy of measurements, as well as the associated analytical and technical errors (Achour et al., 2014b). Data acquisition constitutes only the first step of data processing, with several subsequent stages aimed at converting these data into abundance levels and then making sense of such levels. These tasks include

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deciding which elements of the raw data should be used, normalization processes, and quality control checks as applicable. Discrepancies arising at the data analysis stage are not widely appreciated by end-users of proteomic data, especially modelers, and therefore warrant more attention. The aim of this commentary is to highlight the impact of optimizing UGT-specific quantitative factors at the level of data analysis on QconCAT-measured abundances with reference to catalytic activity.

Re-assessment of the Methodological Workflow

Human liver microsomal (HLM) samples ($n=24$) and methods used to measure UGT abundance and activity were previously described (Achour et al., 2017b). Briefly, eight UGTs (1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7 and 2B15) were independently quantified using QconCAT (Achour et al., 2014b) and SIL-peptide standards (Fallon et al., 2013). Activities of seven enzymes were measured by monitoring the glucuronides of substrates: β -estradiol (UGT1A1), chenodeoxycholic acid (UGT1A3), trifluoperazine (UGT1A4), 5-hydroxytryptophol (UGT1A6), propofol (UGT1A9), zidovudine (UGT2B7), and *S*-oxazepam (UGT2B15).

Initial QconCAT-based quantification did not show considerable correlation with catalytic activity, and therefore required systematic assessment of several data analysis steps. Figure 1 shows a schematic of the data assessment strategy, with a practical example shown in Figure 2 (for UGT2B15 in sample HH06). Re-analysis of elution profiles and fragment-based quantitative ratios was done using Skyline 3.7 (MacCoss Lab Software, WA). Measured abundances were re-assessed against activity data. An outline of the re-assessment strategy is described below.

A. Choice of Peptide Standards. The peptides that constitute the QconCAT were previously selected based on experimental design followed by theoretical assessment. This approach was limited by options in a data-dependent experiment, yielding 1-2 peptides per UGT (Russell et al., 2013). Extensive sequence homology between UGTs also contributed to this limitation. Initially, peptides that provided higher abundance were used in line with widely accepted literature (Brownridge et al., 2011; Lawless et al., 2016). Instead, we propose that better appraisal of quantification based on several peptides for each UGT should be adopted. In this report, assessment of suitability of peptides was carried out based on theoretical appraisal by two independent analysts and *in silico* evaluation

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using CONSeQuence algorithm (Eyers et al., 2011). Quantification was subsequently considered based on the more favorable peptide choice (Table 1). Theoretical criteria for peptide assessment are included in Supplemental Methods. Briefly, selected peptides should have unique sequences and mass-to-charge ratios (of parent and fragment), should not be mapped to membrane-associated domains or subject to polymorphisms or biological (post-translational) modifications, should be readily cleavable, of suitable length (6–20 amino acids), with favorable stability (to chemical modification due to handling/storage) and moderate hydrophobicity (Kamiie et al., 2008; Carroll et al., 2011). Choice of monitored peptide charge state ($z=+2$ or $+3$) was also considered.

B. Choice of Peptide Fragments. Fragment selection was initially conducted *in silico* using Skyline 1.4, with fragment ratios expected to return consistent quantification. Initially, 2-3 transitions per peptide were monitored with mean ratios being used for quantification. In this report, the uniqueness of fragment sequences and consistency between estimates based on monitored transitions were assessed, especially for low abundance proteins; less-specific fragments returning inconsistent ratios were excluded from analysis. In addition, the chromatographic trace of different fragment ions was assessed and poor quality signals were excluded. Since retention time and m/z values monitored had relatively large filters in the LC-MS/MS assay, the m/z values for the selected fragments were assessed, with isobaric and isomeric fragments being excluded (Table 1).

C. Correction for Efficiency of Label Incorporation. Assessment of efficiency of ^{13}C -label incorporation into QconCAT protein synthesis was previously reported as an in-house quality control step in QconCAT expression; constructs of $\geq 95\%$ purity are accepted as quantitative standards (Achour et al., 2015). The level of incorporation can vary batch-to-batch, and impurity is expected to affect quantification, especially of low abundance proteins (Carr et al., 2014). Uncorrected and corrected quantitative ratios were generated using Eqs. 1 and 2, respectively (Figure 2A and B):

$$R_{\text{peptide}} \frac{L}{H} = \frac{I_{L\text{peptide}}}{I_{H\text{QconCAT}}} \quad (1) \quad R'_{\text{peptide}} \frac{L}{H} = \frac{I_{L\text{peptide}} - I_{L\text{QconCAT}}}{I_{H\text{QconCAT}}} \quad (2)$$

where $R_{\text{peptide}} \frac{L}{H}$ and $R'_{\text{peptide}} \frac{L}{H}$ are uncorrected and corrected ratios, respectively, used to quantify a peptide representing a UGT enzyme or the QconCAT (using a QconCAT-based internal standard,

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QIS); $I_{L_{Peptide}}$ is the intensity of the light peptide signal measured in the quantitative experiment; $I_{L_{QconCAT}}$ is the peak intensity of the light peptide originating from the QconCAT measured in quality control experiments; and $I_{H_{QconCAT}}$ is the signal intensity of the heavy QconCAT peptide measured in the quantitative experiment.

D. Normalization of Abundance Measurements. Normalization is commonly applied relative to protein mass, leading to abundance levels expressed in units of pmol mg⁻¹ fractional protein. Protein mass measurement is normally done by a colorimetric assay, generating data that may not be reproducible. Commonly used assays include: Bradford assay (Bradford, 1976), BCA assay (Smith et al., 1985) and tryptophan fluorescence assay (Wiśniewski and Gaugaz, 2015). Abundance and activity were measured by independent laboratories and different protein assays were used (Bradford and BCA assays), demonstrating differences in reported contents for matched samples (Figure 2C). In this study, a proposed approach to resolving this issue was to normalize abundance and activity data using protein levels measured by the same assay (BCA assay), using Eq. 3.

$$[Enzyme] = [QIS] \cdot \frac{R'_{Peptide \frac{L}{H}}}{R'_{QIS \frac{L}{H}}} \cdot \frac{F_v}{PROTEIN \ MASS} \quad (3)$$

where $[Enzyme]$ is target enzyme abundance (expressed in pmol mg⁻¹ HLM protein); $[QIS]$ represents the concentration of the unlabeled internal standard used to quantify the QconCAT (a [Glu¹]-fibrinopeptide B analog, modified to reduce the incidence of missed cleavage due to the glutamate at the N-terminus (Lawless and Hubbard, 2012)); F_v is a volume correction factor relating the analyzed volume to the volume of HLM sample; and PROTEIN MASS is the protein content determined for each sample (BCA assay). The terms assessed in this report: target peptide and QIS ratios (parent-fragment selection and correction for label incorporation) and protein mass used for normalization (Figure 2D).

E. Statistical Assessment of the Optimization Process. Correlations were assessed at each stage using Spearman correlation test (R_s) and scatter of data was assessed with linear regression (R^2). The following criteria were used: α -value of 0.05 (Bonferroni-corrected for correlation matrices), strong correlation ($R_s > 0.50$) and limited scatter ($R^2 > 0.30$), taking into account the effect of abundance/activity units, as previously advocated (Achour et al., 2017a).

Effects of Systematic Appraisal on Endpoint Measurements

To generate reliable proteomic data for IVIVE-PBPK, best practice must be ensured throughout the entire quantitative workflow, including data processing. It is important to note that sample preparation and LC-MS/MS methods have previously undergone quality evaluation and returned precise and accurate quantification of cytochromes P450 in relation to genotype and activity (Achour et al., 2014b). Therefore, only factors specifically affecting UGT measurements were considered.

Effects of the Choice of Monitored Peptides/Fragments. MS-based proteomic strategies rely on using peptides as surrogates for proteins, and the limitations of this approach are still being uncovered. In eukaryotes, protein truncation and splice variants can result in misleading measurements, while technical issues include signal overlap and variable peptide responses between runs. QconCAT design normally follows a pragmatic approach, with two or more peptides included for each protein (Pratt et al., 2006); however, only one peptide is ultimately used for quantification. Two peptides representing each UGT were therefore included in the QconCAT whenever possible. Table 1 shows sequences used in data acquisition, scored based on theoretical and *in silico* criteria; Supplemental Figure 1 shows peptide elution profiles. Theoretical assessment of peptide suitability involves consideration of several parameters, which tend to be prioritized somewhat subjectively, and was therefore conducted by two independent analysts; the analysts' scores were in agreement and compared well with *in silico* assessment. Three pairs of peptides (representing UGTs 1A1, 1A4, and 2B7) returned consistent quantification (Supplemental Figure 2), whereas three pairs (UGTs 1A6, 2B4 and 2B15) showed significant differences.

When two or more peptides are used to quantify a protein, preference has conventionally been given to peptides that return higher concentrations (Brownridge et al., 2011; Lawless et al., 2016); the assumption is that underestimation can occur due to differences in efficiency of release of peptides. It has recently become clear that several peptide-related factors can affect measurements and should therefore be considered when peptide choice is made, ideally in the design stage, but this is not always possible, especially in global proteomics. The strategy of monitoring the "highest reporter" is likely to

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work well when soluble proteins are quantified; however, for membrane-bound proteins, especially those with unknown structures (e.g. UGTs), a more secure strategy is to monitor “best performer” peptides. Discerning “best performers” will almost always involve visual examination of MS and MS/MS spectra. For example, isobaric sequences representing UGT1A6 (WIYGVSK) and UGT2B7 (ADVWLIR) overlapped on the chromatogram, leading to overestimation of abundance, especially for UGT1A6. Discrepancies in abundance in relation to peptide choice have previously been reported to reach up to 30-fold in the case of P-glycoprotein in hepatocytes (Chen et al., 2017), and therefore applying suitable criteria is essential to ensure quality of surrogate peptides (Bhatt and Prasad, 2017). Such criteria were previously used in a label-free experiment on matched liver samples, which resulted in good agreement with QconCAT measurements of several UGT enzymes (Achour et al., 2017a).

Because targeted quantification is based on MS/MS data, the properties of fragments are as important as those of parent peptides. Carr et al. (2014) recommended monitoring 3-5 fragments, allowing inconsistencies in measurements to be reconciled. Fragments are typically selected *in silico* based on predicted peak intensities rather than sequence properties (Carr et al., 2014). There are several potential pitfalls with this approach. Firstly, MS/MS spectra may have several low intensity peaks (especially with proline-containing peptides) so that a lower number of consistent measurements can be made. Two peptides, YLSIPTVFFLR (UGT1A3) and ESSFDAVFLDPFDNCGLIVAK (UGT1A9), were subject to this error, where peaks of lower quality returned inconsistent ratios. Although optimizing transition selection resulted in improved correlation with activity, quantitative methods for these two enzymes still require improvement. Additionally, erroneous quantification can occur when uniqueness of peptide-to-fragment m/z values cannot be ensured, most often in complex biological mixtures, with short sequences being most affected (Carr et al., 2014). The MRM filters (retention time, parent ion m/z and fragment ion m/z) are normally sufficient to ensure exclusive selection of fragments, but not always, especially in non-scheduled experiments, where retention times are not specified. Peptide sequences WIYGVSK (UGT1A6), ADVWLIR (UGT2B7) and WIYGVSK (UGT2B15) were eventually excluded from analysis for isobaric interference despite returning higher levels than their alternatives. The combined effect of

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peptide and fragment selection was shown to be substantial (Supplemental Table 1), with abundance values changing 0.5 to 3-fold upon re-assessment. This led to improved abundance-activity correlation for most UGT enzymes. The initially weak correlation for UGTs 1A4, 1A6 and 2B15 ($R_s=0.19-0.35$, $p>0.05$; $R^2=0.01-0.14$) became moderate ($R_s=0.52-0.56$, $p<0.01$; $R^2=0.24-0.31$). For other UGTs (1A1, 1A3, 2B7), this improved mainly in terms of data scatter (from $R^2=0.30$ to $R^2=0.47-0.57$), while correlation for UGT1A9 remained weak ($R_s=0.20$, $p=0.33$, $R^2=0.07$).

Effects of Correction for the Quality of Isotopically-Labeled Standards. QconCATs are artificial proteins expressed in-house, and the extent of labeling varies depending upon the construct and culture conditions from ~95% to 99% (Achour et al., 2015). This means that QconCATs can contribute unlabeled peptide which may affect quantification when the analyte is expressed at low levels (Carr et al., 2014). Table 1 shows the extent of label incorporation into each peptide, reflecting more efficient labeling of arginines than lysines (Russell et al., 2013). The outcome of correction for purity was that levels of UGTs had a variable artefactual component of up to 10%, with little effect on correlation with activity. A similar trend was observed with efflux transporters, BCRP and MRP2, quantified in human jejunum using QconCAT methodology (Harwood et al., 2015), which were overestimated by on average 10% and 7%, respectively, when corrections for purity were not considered (Harwood et al., 2016a). However, these errors do not always lead to meaningful differences in pharmacokinetic outcomes (Harwood et al., 2016b).

Effects of Normalization of Abundances. Normalization relies on protein content determination using colorimetric assays, which are prone to interference from reagents commonly used in routine sample processing, including detergents, chaotropes and reducing agents (Wiśniewski and Gaugaz, 2015). In particular, BCA assay is incompatible with commonly used concentrations of urea and dithiothreitol (Smith et al., 1985), while Bradford reagent tends to interact with sodium dodecyl sulfate (Bradford, 1976), a detergent used for gel-based sample preparation. A limitation of the cross-laboratory study (Achour et al., 2017b) was the use of Bradford assay with QconCAT measurements, whereas both activity and SIL-based measurements were normalized using BCA assay. Comparing the two protein measurements (Figure 2C) indicates that, while average protein content in the samples was similar, there was no correlation between individual values. Normalization

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against the same protein content resulted in changes in enzyme levels reaching up to 50%, with improved correlation with activity, mainly in terms of scatter for six UGTs (from $R^2=0.24-0.57$ to $R^2=0.48-0.73$), while UGT1A9 showed substantial improvement to moderate correlation ($R_s=0.47$, $p=0.02$; $R^2=0.34$). It is not clear, however, whether BCA-normalization leads to better results; it may just lead to more consistent error. We have recently illustrated that normalization relative to tissue mass instead of fractional protein introduces less artefactual variability to end-point measurements (Achour et al., 2017a).

Overall Effects of Systematic Re-assessment on UGT Measurements. Specific effects of the assessed factors on abundance of each enzyme are described in Supplemental Results. Collectively, the proposed strategy led to 0.5 to 3.3-fold change in UGT levels, with substantial improvement in correlation with activity (Figure 3) and tighter levels of inter-individual variability in abundance (26%-86%), matching variability in activity (27%-67%), in line with recent literature (Margaillan et al., 2015). In addition, cross-laboratory comparison of UGT abundances seemed to indicate overall agreement, returning generally interchangeable abundance values (Supplemental Figures 3 and 4). Based on these considerations, a list of established UGT-specific methods was generated (Supplemental Table 4).

The correlation matrix (Figure 4) confirms specificity of protein and activity data, with no evidence of spurious abundance-activity relationships. A similar complementary approach was used previously to discern tissue-specific glycolytic and gluconeogenic pathways (Wiśniewski et al., 2015). Expression inter-correlations uncovered in the UGT dataset (Supplemental Table 3) were also in line with literature (Achour et al., 2014a; Margaillan et al., 2015). Correlations of enzyme expression have recently been adopted for more realistic model-based predictions of drug clearance and drug-drug interactions (Barter et al., 2010; Doki et al., 2018), with additional established correlations making their way into commonly used platforms, such as Simcyp.

Conclusions. The QconCAT approach offers several advantages (Al Feteisi et al., 2015a), and therefore it has recently been adopted for various clinical and biological applications (Dzieciatkowska et al., 2015; Wang et al., 2015; Kito et al., 2016). While initial applications were primarily aimed to quantify soluble proteins, often in simple organisms, it is clear that QconCAT can

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be applied to membrane-bound mammalian proteins. However, monitored peptides and fragments need to be chosen carefully, preferably using *a priori* selection, and corrections are required for relatively low purity standard peptides targeted at low abundance proteins. We continue to advocate using tissue mass for abundance normalization and activity data for quality control. We propose optimized QconCAT methods for the quantification of several UGTs (1A1, 1A6, 2B4, 2B7 and 2B15) and a robust data analysis strategy for targeted proteomic quantification, particularly applicable for QconCAT-based measurements.

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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Achour, Dantoni, Niosi, Novak, Al-Majdoub, Goosen, Rostami-Hodjegan, Barber.

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Figure Legends

Figure 1 Schematic of the methodological approach used to assess abundance levels of UGT enzymes based on raw data acquired using LC-MS/MS. Representative peptides are selected using criteria outlined in Supplemental Methods. This selection process applies to targeted (MRM) and untargeted/global studies. Selection from peptides that are detected consistently in a LC-MS/MS experiment should take into account the uniqueness and the stability of the peptides. Selected fragments should be stable and representative of the peptide (of sufficient length) in order to return consistent quantification. Correction factors should be applied for label incorporation, especially when low abundance proteins are analyzed. The spike ratio should be consistent with the dynamic range of expression of the target proteins. Normalization should be consistent across all samples and measured parameters. An example of this process is shown in Figure 2

Figure 2 An example of the assessment process applied to UGT2B15 in sample HH06: choice of peptide standard and correction for label incorporation efficiency (A and B), featuring elution profiles of QconCAT alone (dashed lines), and QconCAT and analyte sample (continuous lines) for heavy (blue) and light (red) peptides (Peptide 1: WIYGVSK; Peptide 2: SVINDPVYK). Differences between total protein mass measurements using Bradford and BCA assays (C). Calculation of UGT2B15 abundance in sample HH06 using the outlined correction factors and their contributions to the change in reported abundance (D). In C, the arrow shows sample HH06, and data points in red reflect a difference in content higher than a cut-off relative error ($\%RE = 100 \cdot (x_{BA,j} - x_{BCA,j}) / x_{BCA,j}$) of 15% for each sample j between the two protein content assays. Overall differences in mean and distribution between data from the two assays were non-significant according to Mann-Whitney U -test; however, individual values were poorly correlated. In D, the overall shift in abundance was -19.8% ($\%RE = 100 \cdot (x_{2,i} - x_{1,i}) / x_{1,i}$) for enzyme $i=UGT2B15$ before and after optimization, with the main contributing factor being the selection of peptide/fragment transitions ($\%RE=-29.8\%$), followed by total HLM protein content (+18.3%) and correction for label incorporation (+6.1%). Text in purple font reflects corrected values. BA, Bradford assay; BCA, bicinchoninic acid assay

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Figure 3 Correlation between individual UGT enzyme abundances and activity rates ($n=24$) using the original dataset (A) and the re-assessed data based on the proposed strategy (B). Moderate to strong, statistically significant correlations are shown in blue and weak correlations in gray. Units of abundance measurement are pmol mg^{-1} HLM protein, and units of catalytic activity are $\text{nmol (glucuronide) min}^{-1} \text{mg}^{-1}$ HLM protein. Substrates used for activity measurement are: β -estradiol (UGT1A1), chenodeoxycholic acid (UGT1A3), trifluoperazine (UGT1A4), 5-hydroxytryptophol (UGT1A6), propofol (UGT1A9), zidovudine (UGT2B7), *S*-oxazepam (UGT2B15). R_s , Spearman correlation coefficient. Dashed lines represent lines of regression

Figure 4 Correlation matrix of QconCAT-derived individual UGT enzyme abundances ($n=24$) and activity rates (abundance vs activity). Strong, statistically significant correlations are shown in blue. Units of abundance measurement are pmol mg^{-1} HLM protein, and units of catalytic activity are $\text{nmol (glucuronide) min}^{-1} \text{mg}^{-1}$ HLM protein; EST, β -estradiol; CDCA, chenodeoxycholic acid; TFP, trifluoperazine; 5HTOL, 5-hydroxytryptophol; PRO, propofol; AZT, zidovudine; OXAZ, *S*-oxazepam. Supplemental Table 2 shows the statistical analysis used to generate the abundance-activity correlation matrix

Table 1 Assessment of peptides and fragments used to quantify each of the eight UGT enzymes

Protein	Peptide sequence ^a	In silico score (0-1) ^d	Theoretical assessment ^e	Incorporation correction factor (%) ^f	MRM transitions monitored ^g		
					$[(m/z)^z]_{peptide} \rightarrow [(m/z)^z]_{fragment}$		
UGT1A1	D ₇₀ GAFYTLK ₇₇ ^b	0.354	++	4.0	457.73 ²⁺ /524.31 ⁺¹ (y ₄)	457.73 ²⁺ /671.38 ⁺¹ (y ₅)	457.73 ²⁺ /742.41 ⁺¹ (y ₆)
	T ₇₈ YPVPFQR ₈₅	0.363	++	2.0	504.27 ²⁺ /547.30 ⁺¹ (y ₄)	504.27 ²⁺ /646.37 ⁺¹ (y ₅)	504.27 ²⁺ /743.42 ⁺¹ (y ₆)
UGT1A3	Y ₁₆₄ LSIPTVFFLR ₁₇₄ ^b	0.514	+++	1.0	678.39 ²⁺ /681.41 ⁺¹ (y ₅)	678.39 ²⁺ /782.46 ⁺¹ (y ₆)	678.39 ²⁺ /879.51 ⁺¹ (y ₇)
UGT1A4	Y ₁₇₅ IPCDLDFK ₁₈₃ ^{b,c}	0.432	+++	5.0	585.78 ²⁺ /637.32 ⁺¹ (y ₅)	585.78 ²⁺ /797.35 ⁺¹ (y ₆)	585.78 ²⁺ /894.40 ⁺¹ (y ₇)
	G ₁₈₄ TQCPNPSSYIPK ₁₉₆ ^c	0.522	++	5.0	724.85 ²⁺ /791.43 ⁺¹ (y ₇)	724.85 ²⁺ /905.47 ⁺¹ (y ₈)	724.85 ²⁺ /1002.53 ⁺¹ (y ₉)
UGT1A6	S ₁₀₃ FLTAPQTEYR ₁₁₃ ^b	0.554	+++	2.0	656.83 ²⁺ /793.38 ⁺¹ (y ₆)	656.83 ²⁺ /864.42 ⁺¹ (y ₇)	656.83 ²⁺ /965.47 ⁺¹ (y ₈)
	V ₂₅₀ SVWLLR ₂₅₆ ^{b,i}	0.354	+	1.5	436.77 ²⁺ /587.37 ⁺¹ (y ₄)	436.77 ²⁺ /686.43 ⁺¹ (y ₅)	
UGT1A9	E ₁₃₉ SSFDAVFLDPFDNCGLIVAK ₁₅₉ ^{b,c}	0.553	++	4.0	1172.56 ²⁺ /1233.63 ⁺¹ (y ₁₁)	1172.56 ²⁺ /1348.66 ⁺¹ (y ₁₂)	1172.56 ²⁺ /1461.74 ⁺¹ (y ₁₃)
UGT2B4	F ₁₇₄ SPGYAIEK ₁₈₂	0.391	+++	5.0	506.26 ²⁺ /623.34 ⁺¹ (y ₅)	506.26 ²⁺ /680.36 ⁺¹ (y ₆)	506.26 ²⁺ /777.41 ⁺¹ (y ₇)
	A ₃₂₁ NVIASALAK ₃₃₀ ^b	0.412	+++	4.0	479.29 ²⁺ /560.34 ⁺¹ (y ₆)	479.29 ²⁺ /673.42 ⁺¹ (y ₇)	479.29 ²⁺ /772.49 ⁺¹ (y ₈)
UGT2B7	T ₄₁ ILDELIQR ₄₉ ^b	0.426	++	2.0	550.82 ²⁺ /658.3 ⁺¹ (y ₅)	550.82 ²⁺ /773.42 ⁺¹ (y ₆)	550.82 ²⁺ /886.50 ⁺¹ (y ₇)
	A ₂₅₃ DVWLIR ₂₅₉ ⁱ	0.300	+	1.5	436.75 ²⁺ /587.37 ⁺¹ (y ₄)	436.75 ²⁺ /686.43 ⁺¹ (y ₅)	436.75 ²⁺ /801.46 ⁺¹ (y ₆)
UGT2B15	W ₉₇ IYGVSK ₁₀₃ ^h	0.325	+	3.0	426.73 ²⁺ /553.30 ⁺¹ (y ₅)	426.73 ²⁺ /666.38 ⁺¹ (y ₆)	
	S ₄₃₂ VINDPVYK ₄₄₀ ^b	0.406	++	5.0	517.78 ²⁺ /506.30 ⁺¹ (y ₄)	517.78 ²⁺ /735.37 ⁺¹ (y ₆)	517.78 ²⁺ /848.45 ⁺¹ (y ₇)
MetCAT ^j	GVNDNEEGFFSAR ^k	0.561	++++	2.5	721.32 ²⁺ /813.39 ⁺¹ (y ₇)	721.32 ²⁺ /942.43 ⁺¹ (y ₈)	721.32 ²⁺ /1056.47 ⁺¹ (y ₉)

- ^a Peptide sequences as defined by the human UniProtKB database (<http://www.uniprot.org>). Subscript number labels on the C- and N-terminal amino acids of peptide sequences denote their positions in the UGT protein sequences based on their database entries. The terminal lysine (K) and arginine (R) residues were labeled using [¹³C₆] stable isotopes in the QconCAT standard
- ^b Peptide selected for quantification of each UGT enzyme based on the selection criteria outlined in Supplemental Information and *in silico* appraisal
- ^c Cysteine residues were alkylated (by carbamidomethylation), necessitating an increment of +57.0215 Da in monoisotopic mass of peptides and certain fragments
- ^d *In silico* assessment was carried out using CONSeQuence algorithm based on charge, hydrophobicity and secondary structure (Eyers et al., 2011)
- ^e Theoretical assessment based on criteria outlined in Supplemental Information; arbitrarily, +, ++, +++ and ++++ scores were assigned to peptides under assessment (highest score, +++++) by two independent analysts
- ^f The proportion of light to heavy peptide due to inefficient incorporation of the ¹³C label needed to correct quantification ratios; this can be variable from batch to batch
- ^g Up to 3 transitions for each peptide were designed *in silico* using Skyline (superscript indicating charge states, z); selected fragments were then appraised based on unique sequences, *m/z*, quality of elution profiles and the CV of the returned quantitative ratios. In this table, only the light (native) peptide transitions are listed, where the y-ions (subscript indicates the length of the sequence) were used
- ^h Two transitions were designed and monitored for peptides VSVWLLR (UGT1A6) and WIYGVSK (UGT2B15), which returned low scores based on theoretical, *in silico* and fragment assessments. These peptides were excluded from analysis
- ⁱ The isobaric sequences VSVWLLR (UGT1A6) and ADVWLIR (UGT2B7) were overlapped on the chromatogram due to close retention times (with the same *m/z* of parents and fragments). These peptides were excluded from analysis
- ^j MetCAT: QconCAT used as a standard for the quantification of human liver P450 and UGT enzymes (Russell et al., 2013; Achour et al., 2015)
- ^k Sequence of QconCAT-based internal standard (QIS) used for quantification of the QconCAT

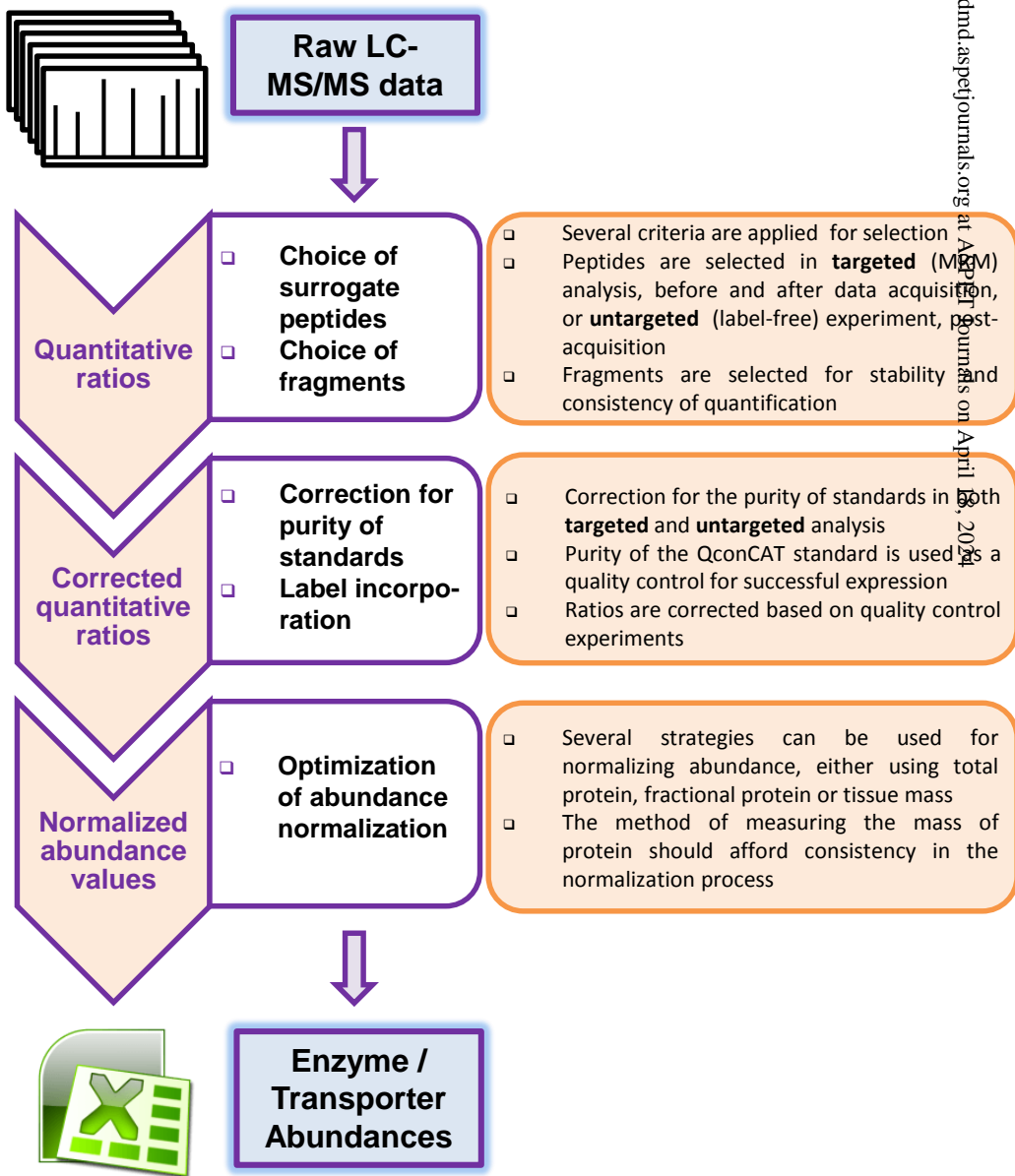


Figure 1

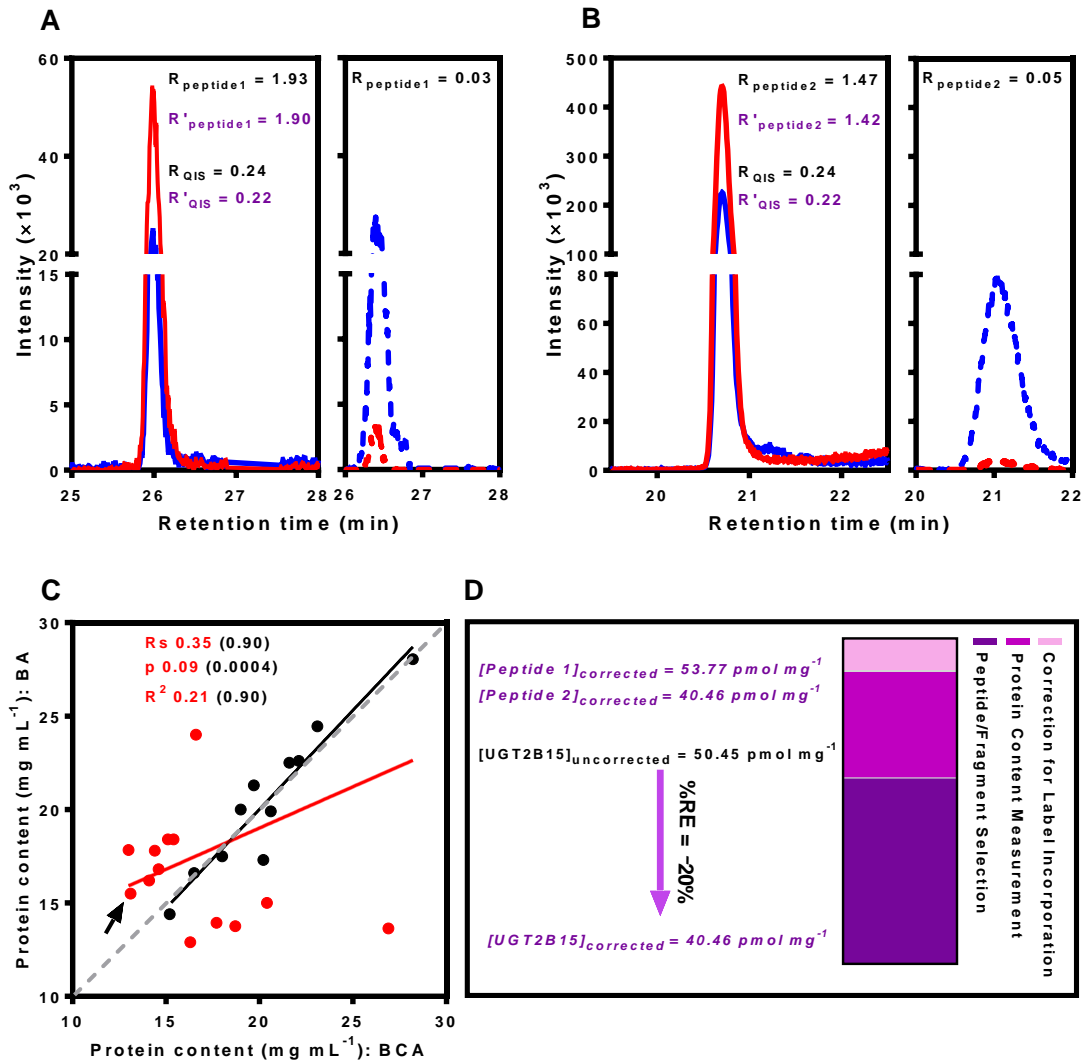


Figure 2

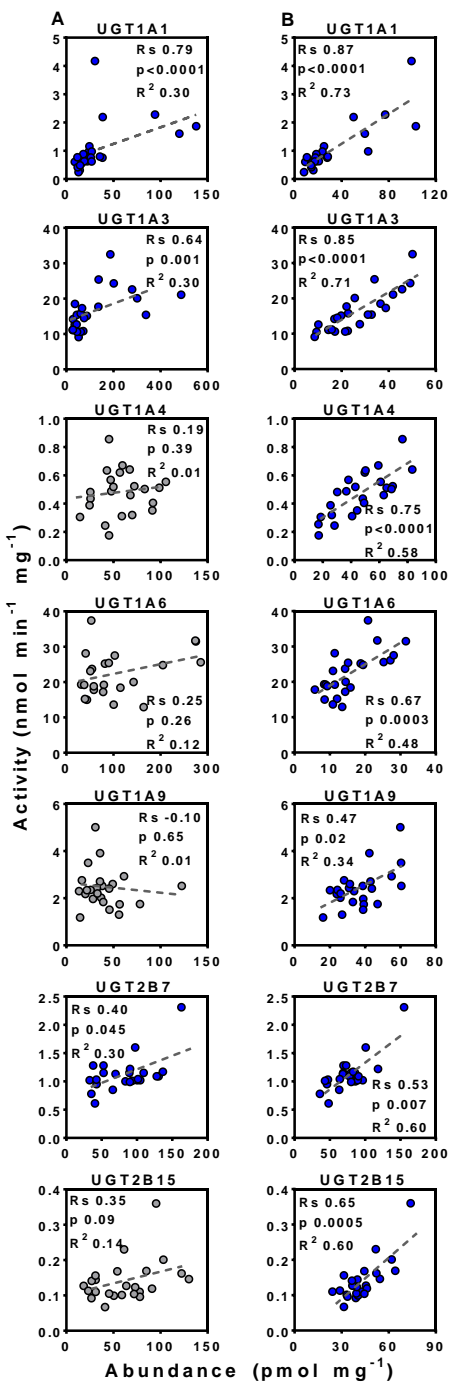


Figure 3

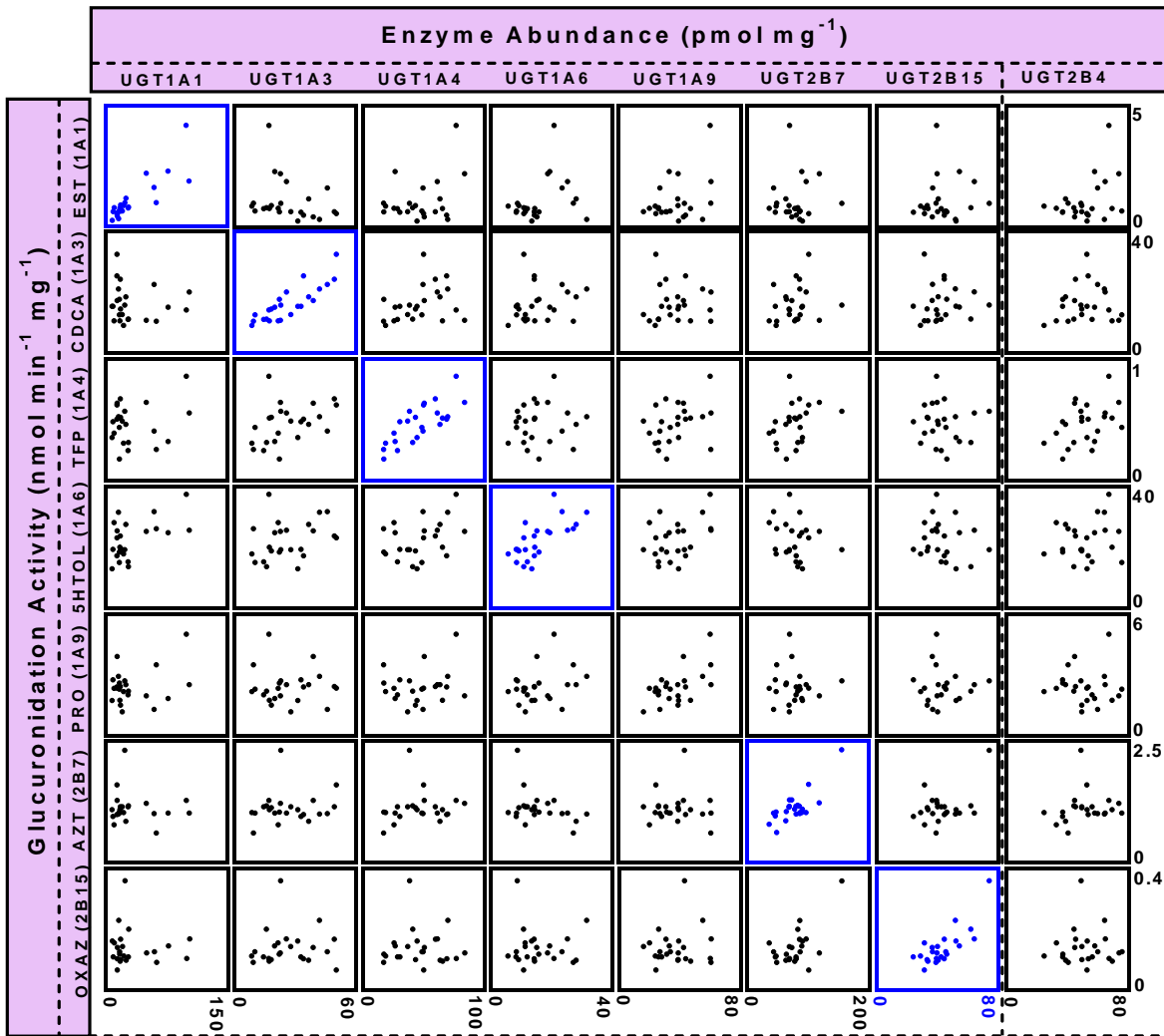


Figure 4