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Sample matrix has a major impact on accuracy of peptide quantification: Assessment of calibrator and internal standard selection and method validation

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Abstract

Protein quantification based on peptides using LC-MS/MS has emerged as a promising method to measure biomarkers, protein drugs and endogenous proteins. However, the best practices for selection, optimization and validation of the quantification peptides are not well established, and the influence of different matrices on protein digestion, peptide stability and MS detection has not been systematically addressed. The aim of this study was to determine how biological matrices affect digestion, detection and stability of peptides. The microsomal retinol dehydrogenase (RDH11) and cytosolic soluble aldehyde dehydrogenases (ALDH1As) involved in the synthesis of retinoic acid (RA) were chosen as model proteins. Considerable differences in the digestion efficiency, sensitivity and matrix effects between peptides were observed regardless of the target protein's subcellular localization. The precision and accuracy of the quantification of RDH11 and ALDH1A were affected by the choice of calibration and internal standards. The final method using recombinant protein calibrators and stable isotope labeled (SIL) peptide internal standards was validated for human liver. The results demonstrate that different sample matrices have peptide, time and matrix specific effects on protein digestion and absolute quantification.

Relative and absolute quantification of protein expression by LC-MS/MS provides critical information of the biological importance of specific proteins.¹⁻³ While methods for relative quantification of proteins of interest have been well established, targeted absolute quantification of proteins by LCMS/MS still has many challenges due to lack of methods to predict the analytical performance of specific peptides,⁴⁻⁶ the poor understanding of matrix effects on protein solubilization, digestion and analytical behavior and the lack of blank matrices for endogenous proteins. The sequence and MS/MS transitions of the peptides generated by protease digestion can be easily predicted by in silico methods,⁷ but no systematic way has been established to select the most robust, selective and sensitive quantification peptides for different matrices. With therapeutic proteins or when

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Supporting Information

Supporting information includes Supplemental tables 1-3 and Supplemental Figures 1-6. This material is available free of charge via the Internet at <http://pubs.acs.org>

Author Contributions

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recombinant protein is available, digestion and analysis of purified protein in different matrices offers a direct approach for peptide selection and validation.^{2,8} However, with endogenous proteins, especially membrane proteins that are challenging to purify and express, identification of the optimal quantification peptides for the matrix of interest is non-trivial. For endogenous proteins an additional challenge is introduced by the general lack of blank matrix for method development. Different matrices may result in variable degrees of ion suppression, endogenous matrix interference, and variability in sample recovery and protein digestion. In fact, inter-individual variability in protein digestion efficiency has been observed in human serum samples.⁹ Yet, the influence of different matrices on protein digestion efficiency has not been thoroughly evaluated.

Appropriate calibrator and internal standard selection is another crucial step in protein quantification by LC-MS/MS, and the selection could be affected by sample matrix and sample preparation protocols. Common choices for internal standard include stable isotope labeled (SIL) peptides,^{8,10,11} SIL-protein,^{8,12-14} SIL extended peptide or concatenated (QconCat) peptides.^{8,15} The unlabeled analogs can all be used as quantification standards. SIL peptide internal standards are ideal for addressing ion suppression by matrix components,¹⁶⁻¹⁸ but simple peptide standards and internal standards suffer from the fact that they do not require digestion or sample preparation for detection. This may lead to considerable quantification error^{4,8} as digestion may be incomplete or lack specificity.¹⁹ On the other hand, peptide formation from an extended peptide can occur at a different rate than from whole protein resulting in low quantification accuracy.^{15,20} As such, recombinant protein standards have been established as the most accurate calibrators for protein quantification^{8,21,22} and SIL-protein is considered as the ideal internal standard.²³ However, since SIL-proteins are not native in the sample matrix, they may not address variability in extraction of endogenous proteins from a membrane. This is critical as protein extraction from tissue has been shown to contribute to 72% of the quantification variability,²⁴ highlighting the significance of sample preparation in absolute quantification of proteins.

The goal of this study was to establish the extent to which digestion efficiency of protein in different sample matrices affect endogenous protein quantification and to determine the influence of calibration and internal standard selection on absolute protein quantification. Soluble cytosolic aldehyde dehydrogenases (ALDH1A) and membrane bound retinol dehydrogenase (RDH11) were used as model enzymes. In tissues that require retinoic acid signaling, ALDH1A and RDH enzymes are essential for maintaining tight control of retinoic acid synthesis and physiological processes.²⁵ Yet, very little is known about their tissue distribution and expression in adult humans. The sequence similarity between the members of the RDH and ALDH1A families has hindered the development of selective antibodies, and measurement of RDH or ALDH1A expression by western blot or ELISA has been challenging. Therefore LC-MS/MS quantification is particularly useful to characterize the expression patterns of these critical proteins in diverse matrices of interest.

EXPERIMENTAL PROCEDURE

Materials and Reagents

SIL-RDH11 and RDH11 were acquired from Origene (Rockville, MD). ALDH1A1 and ALDH1A2 were expressed and purified as previously described²⁶ and stored at 4°C. Mass spectrometry grade trypsin, dithiothreitol, iodoacetamide, optima grade water, optima grade acetonitrile, formic acid, and SIL peptides were purchased from Thermo-Fisher (Rockford, IL). Sodium deoxycholate was from Sigma (St. Louis, MO).

Microsomal and cytosolic tissue fractions

Mouse livers were from 3-5 month old BL/6-129 mice.²⁷ Human livers were obtained from the University of Washington, School of Pharmacy Human Tissue Bank. *Spodoptera frugiperda* (Sf9) insect cells (Invitrogen, Carlsbad, CA), commonly used for recombinant enzyme production following Baculovirus infection, were grown in Sf-900 II SFM liquid media (Invitrogen, Carlsbad, CA) with 2.5% fetal bovine serum according to manufacturer's protocol, mock infected with a baculovirus, harvested and stored at -80°C. Microsomal and cytosolic cell fractions were prepared by ultracentrifugation to separate cell organelles as previously described.²⁸ Fractions were prepared and stored in 50 mM potassium phosphate buffer (pH 7.4) with 250 mM sucrose and EDTA free protease inhibitor cocktail (Roche, San Francisco, CA). Protein concentrations were measured using BCA assay (Thermo Fisher, Waltham, MA).

In silico protein digestion and peptide screening

The protein sequences were downloaded from www.uniprot.org (RDH11- Q8TC12, Q9QYF1, ALDH1A1- P00352, P24549 and ALDH1A2- O94788-1, Q62148 for the human and mouse proteins, respectively) and tryptic signature peptide candidates were identified in silico using Skyline.⁷ Peptides with less than 7 residues or more than 20 residues and peptides with a predicted $m/z > 1100$ and < 100 were excluded from further analysis. Similarly, peptides conserved between human and mouse enzymes were excluded from further analysis. The predicted peptides for the target proteins were screened against in silico trypsin digested human and mouse proteomes obtained from www.uniprot.org, to ensure peptide selectivity. Nonspecific peptides were excluded. Peptides reported to contain sites of single nucleotide polymorphisms or posttranslational modifications or peptides that contained a proline residue on the carboxyl side of the lysine or arginine at the site of cleavage were excluded. Skyline was used to predict the precursor ions and fragments and corresponding declustering potentials and collision energies for the remaining peptides. A MS/MS method was generated using the predicted m/z transitions and mass spectrometric parameters (Supplemental Tables 1-3) to experimentally establish peptide sensitivity and stability.

Sample preparation, digestion and analysis by UHPLCMS/MS

20 μ L of purified protein standards or tissue fractions at concentrations indicated were added to 96- well plates followed by 4 μ L of 100 mM dithiothreitol and 10 μ L of 100 mM ammonium bicarbonate (pH 7.8). Based on previously reported protocols²⁹⁻³¹ after 20 min

incubation at room temperature, 5 μ L of 10% sodium deoxycholate was added, samples were incubated at 95°C for 5 minutes and cooled to room temperature. Then 4 μ L of 200 mM iodoacetamide was added before samples were incubated for 20 min at room temperature in the dark. Lyophilized trypsin was reconstituted in 50 mM acetic acid and added at a 1:25 trypsin:protein ratio to each sample. Samples were incubated with trypsin for 15 hours at 37°C unless specified otherwise. Trypsin digestions were quenched with 20 μ L ice cold acetonitrile with 8% trifluoroacetic acid, samples were centrifuged at 3,000 g for 25 minutes at 4°C and the supernatants transferred to a 96-well plate for LC-MS/MS analysis. The peptides were quantified using an AB Sciex 5500 qTrap Q-LIT mass spectrometer (AB Sciex, Foster City, CA) equipped with an Agilent 1290 UHPLC (Agilent, Santa Clara, CA), an Aeris Peptide XB-C18 column (50 $\times$ 2.1 mm, 1.7 μ m) and a SecurityGuard Ultra UHPLC C18-peptide cartridge (Phenomenex, Torrance, CA). Gradient elution at 400 μ L/min with H₂O (A) and acetonitrile (B) both with 0.1% formic acid was used at 40°C as follows: 3% B until 3.5 min, increased to 40% B by 12.0 min, then to 95% B by 12.1 min and kept at 95% B until 15.0 min, returning to 3% B at 15.1 min with run time of 18 min. The peak areas were determined using Analyst 1.5.1 (AB Sciex, Foster City, CA).

Evaluation of signature peptides and matrix effects

To assess analytical sensitivity of the candidate peptides, purified RDH11 (200 nM), ALDH1A1 (600 nM), and ALDH1A2 (200 nM) were spiked to buffer and trypsin digested for 15 hrs before LC-MS/MS analysis. The four peptides with the largest peak areas were selected for further analysis. To test for matrix effects, RDH11 (200 nM) was spiked in duplicate into buffer or 2 mg/mL mouse liver or insect cell microsome matrix. ALDH1A1 (600 nM) and ALDH1A2 (200 nM) were spiked in duplicate into buffer or 2 mg/mL mouse liver or insect cell cytosol. Blank matrices were digested together with the spiked samples. The samples were trypsin digested for 0, 0.1, 2, 4, 6, 10, 15, and 24 hours. After the 24 hour trypsin digestion, aliquots of the digest were stored at 4°C, 23°C, and 37°C and analyzed after 12 or 24 hours of storage. Peptides that displayed > 20% change in signal were not considered further.

As RDH11 needs to be incorporated into membrane for appropriate reference, the effect of membrane fractions from different species to the formation and detection of SIL-VVV and SIL-MLS peptides from SIL-RDH11 was determined using mouse and human liver microsomes. SIL-RDH11 (50 nM) was spiked into buffer or human or mouse liver microsomes (2 mg/mL) and incubated at room temperature for 20 minutes to allow incorporation of RDH11 into the membrane. The samples were then digested in quadruplicate for 15 hours and analyzed by LC-MS/MS. The endogenous VVV and MLS peptides were also quantified.

Internal standard selection, identification of sources of variability and method validation

[¹³C₆ ¹⁵N₂]-lysine or [¹³C₆ ¹⁵N₄]-arginine labeled peptides (>95% specified purity) were used to optimize the mass spectrometric parameters for the signature peptides (Table 1). The parameters established with the SIL peptides were applied to the SIL and unlabeled peptides for the protein quantification and method validation experiments. The SIL peptides, a SIL-VVV extended peptide (VVVVTGANTGIGKETA) and SIL-RDH11 were evaluated as

internal standards as shown in Figure 1. The digestion variability (preparation and analysis of replicate samples) and instrumental variability (12 analyses of a sample pooled from replicate digestions) and were evaluated at 50 nM RDH11, 325 nM ALDH1A1 and 40 nM ALDH1A2 in the mouse tissue matrix as depicted in Figure 1. For method validation, internal standards for each protein were added at 50 nM (RDH11), 35 nM (ALDH1A1) and 17.5 nM (ALDH1A2) and replicate samples were analyzed according to the workflow shown in Figure 1. RDH11 calibration curve (7.5-150 nM purified RDH11) and quality control (QC) samples had 2 mg/mL mouse liver microsomes. ALDH1A1 (50-1000 nM) and ALDH1A2 (5-100 nM) calibration curves and QC samples contained 0.2 mg/mL mouse liver cytosol. For validation three to eight replicates/day of QC samples were digested and analyzed on three separate days.

Quantification of hepatic RDH11 and ALDH1A1

RDH11 and ALDH1A1 were quantified in four human livers with two quantification methods using the peptides listed in Table 1 as quantification peptides. For RDH11 human liver microsome samples (40 µg, 2 mg/mL) were used for quantification. For ALDH1A1 human liver cytosol (4 µg, 0.2 mg/mL) was used. The first method used recombinant purified RDH11 and ALDH1A1 to generate a six-point calibration curve in mouse liver microsomes (2 mg/L) or mouse liver cytosol (0.2 mg/L) as described for method validation. SIL-peptides corresponding to the VVV (50 nM) or ANN-1A1 peptide (35 nM) were added in the quench as internal standards. The unlabeled/SIL-peptide peak area ratio (VVV/SIL-VVV for RDH11 and ANN-1A1/SIL-ANN-1A1 for ALDH1A1) measured from each sample was used with the calibration curve to determine the protein concentration in the samples.

The second method was based on the AQUA method where the SIL-peptide within the sample serves as the calibration standard and no standard curve is prepared.³² For the AQUA method, the concentrations of RDH11 and ALDH1A1 were calculated by multiplying the unlabeled/SIL-peptide peak area ratio in each sample by the known concentration of the SIL-peptide (50 nM for SIL-VVV and 35 nM for SIL-ANN-1A1) in the sample.

RESULTS AND DISCUSSION

In silico signature peptide identification and assessment of LC-MS/MS sensitivity

To identify signature peptide candidates, each target protein was digested in silico with Skyline. As a result, 13 peptides were identified as potential signature peptides for RDH11, 21 for ALDH1A1 and 20 for ALDH1A2 (Supplemental Figures 1-3). Two of the identified peptides for ALDH1A1 and four of the ones for ALDH1A2 contained a proline residue on the carboxyl side of the trypsin cleavage site and were discarded as potential signature peptides. In addition, one of the peptides from RDH11 and four of the peptides from ALDH1A1 were conserved between mouse and human (Supplemental Figures 1 and 2), and were not considered further to allow use of mouse tissue as a blank matrix for method validation. Two peptides for RDH11, three peptides for ALDH1A1, and five peptides for ALDH1A2 were present in multiple proteins in human proteome (Supplemental Figures 1-3) and were excluded to ensure selectivity. After excluding also reported natural variants from

signature peptides, nine peptide candidates for RDH11, eleven for ALDH1A1, and eight for ALDH1A2 were identified in the in silico screen (Supplemental Tables 1-3).

Prediction of relative LC-MS/MS ion abundances of peptides in silico is challenging and not entirely reliable, and hence the detection sensitivity of the peptides was monitored after trypsin digestion of protein standards (Figure 2). The relative magnitude of LC-MS/MS response varied up to 200-fold between detected peptides demonstrating the importance of assessing the analytical performance of peptides experimentally. The four peptides with the largest peak areas for each protein were chosen for further evaluation. After trypsin digestion of the purified proteins 78%, 83% and 100% of the candidate signature peptides identified in silico for RDH11, ALDH1A1, and ALDH1A2, respectively, were detected (Supplemental Tables 1- 3). The peptide coverage of the ALDH1A enzymes was greater than that observed for membrane bound RDH11. RDH11 may aggregate more readily than ALDH1As decreasing peptide coverage as formation of protein aggregates has been shown to negatively affect the coverage of trypsin digestion.³³ Membrane-associated regions in RDH11 may also impede digestion coverage even after protein denaturation. Lack of coverage may also be due to poor sensitivity or instability of the formed peptides as three of the four peptides that were not detected contained methionine, tryptophan, or histidine residues that are prone to oxidation.

Trypsin digestion, peptide stability and matrix effects

The digestion kinetics and stability of the generated peptides varied considerably between peptides even when these proteins were digested in buffer. For some peptides peak detection was observed already after a 2-hour digestion and for some (YIL) not even after 24-hour digestion (Figure 3). This is not surprising as trypsin has been shown to preferentially cleave proteins at certain cleavage sites³⁴ and peptide specific digestion time courses have been shown before.^{8,35} Yet, a need for a long trypsin digestion (>15 hours) was considered a liability as it will increase the probability of non-specific protein cleavage³⁶ and peptide modifications.^{37,38} Therefore, the YIL peptide was excluded as a signature peptide. A decreasing signal with increasing digestion time was observed for one of the four candidate peptides for both RDH11 (LAN) and ALDH1A2 (LAF) (Figure 3), excluding these peptides as signature peptides. While the instability of the LAF peptide could be predicted from its tryptophan residue, LAN peptide did not contain any predictive markers of instability. The decreasing signal is likely due to nonspecific cleavage by trypsin as most of these peptides were stable at 4°C, 23°C and 37°C for 12 and 24 hours (Supplemental Figure 4). Only the EMG-1A2 peptide showed signal deterioration following 24 hour storage at 4°C suggesting the 25% decrease in signal for this peptide after 24 hour trypsin digestion compared to 6 hours may be due to lability of its methionine residue rather than nonspecific digestion. This instability led to excluding this peptide from further consideration.

Matrix effects on signal intensity and stability were observed for several peptides generated from RDH11 and ALDH1A spiked to mouse and insect cell fractions (Figure 3). The signal for the VVV and LAN (RDH11), ANN-1A1, IFV and YIL (ALDH1A1), and LAF (ALDH1A2) peptides was considerably reduced when the proteins were digested in insect cell microsomes and cytosol in comparison to buffer (Figure 3). For IFV (ALDH1A1) and

MLS (RDH11) peptides the signal was also reduced in mouse tissue matrix when compared to buffer, while mouse liver microsomes or cytosol had no consistent effect on the signal intensity for the other peptides analyzed. The signal decrease in insect cell fractions appeared to be due to various reasons including rapid degradation of the peptides during the digestion (VVV, LAN, ANN and LAF) and consistent suppression of digestion. The effect of insect cell matrix on peptide stability is of major concern, as many proteins are expressed in baculovirus infected insect cells,³⁹ and insect cell preparations containing an overexpressed protein of interest are often used as quantification standards for LC-MS/MS.^{40,41} The data shown here suggests that insect cell fractions containing the overexpressed protein of interest are not an ideal reference for method development. The fact that the signal intensity varies with time in peptide and matrix specific manner can likely explain the common observation that two signature peptides used with a method relying on peptide standards result in discrepant quantification. This data also shows that it is important to establish digestion time courses for any given protein of interest in the matrix of interest rather than in buffers.

As RDH11 is a membrane protein it is possible that matrix effects on RDH11 are more pronounced due to differences in incorporation of the spiked protein to membranes. Indeed, the signal intensity for the SIL-MLS peptide was significantly lower when SIL-RDH11 was digested in mouse or human liver microsomes than in buffer while the SIL-VVV peptide was unaffected by matrix (Figure 4). There were no observable differences in the detection of the labeled peptides between mouse and human liver matrix, demonstrating that mouse liver could be used as a matrix for method validation. It is unlikely that the decrease in MLS peptide detection is due to incorporation of the spiked protein to the membrane as the ratio of the signal intensity for VVV and MLS peptides was not different for the endogenous RDH11 present in human liver and SIL-RDH spiked into human liver microsomes (Figure 4). The SIL-EIQ peptide from 50 nM RDH11 or the EIQ peptide from endogenous RDH11 was not detected in the buffer, mouse or human liver microsomes, demonstrating inadequate sensitivity of this peptide. Together this data show that matrix effects on digestion and signal intensity are peptide specific and evaluation of the peptide detection from digested protein using matrix is critical for accurate quantification.

Sources of variability and method validation

Based on the assessment of peptide sensitivity, protein digestion and matrix effects, the VVV, ANN-1A1, and ILE peptides were chosen as the quantification peptides for RDH11, ALDH1A1 and ALDH1A2, respectively. The MLS, VAF and EEI peptides were included as secondary verification peptides for RDH11, ALDH1A1 and ALDH1A2, respectively. The sources of variability and overall method performance were evaluated using the primary quantification peptides and the workflow shown in Figure 1. Previously, LC-MS/MS analysis has been reported to introduce up to 13% variability in peptide quantification between samples (instrumental variance), and 17% variability over two weeks of continued analysis (instrumental instability).²⁴ In this study, the instrumental variability, sample preparation variability and the overall variability were all below 10% as measured based on the detection of the quantification peptides from digested reference proteins (Figure 5). However, for the SIL-VVV extended peptide sample preparation variability was much

higher resulting in 14% variability in the analysis of the quantification peptide from the extended peptide. This highlights the challenges in the use of extended peptides as calibrators and internal standards. When SIL-peptide internal standards were added to the samples, the variability in the measurement of the quantification peptide increased (Figure 5). However, when instrumental instability and signal deterioration was introduced by repeat injections of single sample (Supplemental Figure 5), the inclusion of a SIL peptide as an internal standard rectified the observed signal deterioration allowing for consistent quantification.

Although definite validation criteria have been established for small molecule, ELISA and RIA assays,^{42,43} validation guidance and method acceptance criteria for LC-MS/MS based protein quantification is still emerging.⁴⁴ The validation performance of the different internal standard options available was determined (Table 2). For all three proteins adding SIL-peptide internal standard prior to digestion resulted in >15% variability or error in quantification and hence was considered unacceptable (Table 2). In contrast, adding the SIL-peptide after sample preparation during the quench of the trypsin digestion provided sufficient accuracy and precision for all three proteins at all concentrations analyzed. For RDH11, using SILRDH11 as the internal standard resulted in greater than 15% inter-day variability and 19% error in accuracy at the high QC concentration (Table 2). This higher variability when compared to SIL-peptide as internal standard may be due to variability in the membrane incorporation and extraction of the SIL-RDH11 and aggregation of the purified protein. The SIL-VVV extended peptide as an internal standard had the lowest accuracy, a finding in good agreement with previous studies using extended peptides as internal standards.^{20,45} The intraday variability and accuracy of RDH11 detection is shown in Supplemental Figure 6. Based on these results, SIL peptides were chosen as the optimal internal standard for each enzyme. Collectively the validation results show that a peptide quantification method can feasibly meet published⁴⁴ standard validation criteria.

Quantification of hepatic RDH11 and ALDH1A

RDH11 and ALDH1A expression was quantified in four human livers using the validated method (Figure 6). The average concentration of RDH11 measured in the four livers was 56 ± 12 pmol/mg microsomal protein and the average ALDH1A1 expression was 1180 ± 300 pmol/mg cytosolic protein. Neither of the ALDH1A2 signature peptides was detected in human liver cytosol. Based on the lowest point in the calibration curve for ALDH1A2 (5 nM) and the amount of cytosolic protein digested (4 μ g), if ALDH1A2 is expressed in the liver, its concentration is less than 25 pmol/mg cytosolic protein.

The expression of RDH11 and ALDH1A1 was also quantified using the AQUA method (endogenous/SIL-peptide ratio). The ALDH1A1 concentration measured by the AQUA method was 50% lower (538 pmol/mg cytosolic protein) than that measured with protein standard. Yet, the concentration of RDH11 was similar in 3 of the 4 livers using the AQUA method and purified protein standard. ALDH1A1 concentrations in the standard curve were also underestimated by 50% when calculated by the AQUA method. In contrast, for RDH11 all measurements in the standard curve were within 25% of the true concentration. The lower quantification of ALDH1A1 expression by AQUA method is likely due to incomplete

peptide recovery or digestion as previously suggested.^{46,47} Alternatively, accuracy of the labeling and quantification of labeled peptide could contribute to the error in ALDH1A1 quantification. Importantly, the accuracy of the AQUA method was peptide and protein dependent even with the well-validated peptides suggesting that the AQUA method cannot be used for absolute quantification of protein expression. Taken together this data suggests that recombinant protein standards should be used as calibrators to ensure accurate quantification.

CONCLUSIONS

This study is the first to unequivocally show that different sample matrices have peptide, time and matrix specific effects on protein digestion and absolute protein quantification. The results show that purified protein standards are necessary for absolute protein quantification from biological matrices, as only purified protein standards allow for thorough evaluation of matrix specific effects on digestion of the protein and subsequently quantification of the protein of interest. However, the data shows that labeled proteins do not necessarily capture these matrix effects fully or may be subject to large variability in analysis. Importantly, for endogenous protein quantification, the data presented confirms that corresponding matrix from another species, such as mouse liver microsomes as a surrogate for human liver microsomes, can be used to address matrix effects and optimize peptide selection and sample preparation protocols. The results also suggest that digestion time courses should be tested in sample matrix and peptides showing variability with time should be excluded as matrix effects on digestion are likely to also vary between samples from different individuals. Surprisingly, when the well-characterized peptides were used for method validation the quantification was sufficiently accurate and reproducible to meet validation criteria without any internal standard, suggesting that adding internal standards may increase variability and decrease method performance. Overall, the workflow employed here with identifying the most sensitive peptides, establishing the digestion stability and time course in matrix, and validating the method with appropriate internal standard is applicable for development of absolute peptide quantification methods for any endogenous protein and is likely to identify any potential sources of error and excessive variability in the quantification method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental workflows for determining the precision and accuracy of RDH11 and ALDH1A quantification with different internal standard methods.

Figure 2. Detection sensitivity of the signature peptide candidates generated from recombinant RDH11 (A), ALDH1A1 (B), and ALDH1A2 (C). The peptides are labeled according to supplemental Tables 1-3 and Supplemental Figures 1-3.

Figure 3.

Matrix effects on RDH11, ALDH1A1 and ALDH1A2 digestion, peptide stability and detection. The time course of VVV (A), LAN (B), MLS (C) and EIQ (D) peptide formation from RDH11 was observed in homogenization buffer or 2 mg/mL mouse liver or insect cell microsomes. The time course of VAF (E) ANN (F), IFV (G), and YIL (H) peptide formation from ALDH1A1 and EMG (I), EEI (J), LAF (K), and ILE (L) peptides from ALDH1A2 was observed in homogenization buffer or 2 mg/mL mouse liver or insect cell cytosol.

Figure 4.

Comparison of mouse and human liver matrix for RDH11 quantification. The detection of SIL-VVV (A) and SIL-MLS (B) peptide in buffer, mouse (MLM) and human (HLM) liver microsomes is shown. The ratio between VVV/MLS peptide signal from SIL-RDH11 and endogenous RDH11 is shown in (C). ANOVA analysis with student's t-test as a post hoc test was used to test for differences in SIL-VVV and SIL-MLS peptide formation from SIL-RDH11 in different matrices, *: $p < 0.05$.

Figure 5.

Variability introduced to peptide detection at various stages of analysis as measured following digestion of reference proteins (shown on x-axis). The samples had 50 nM RDH11, 325 nM ALDH1A1, 40 nM ALDH1A2 and 50 nM SIL-RDH11 and SIL-VVV extended peptide.

Figure 6.

Quantification of RDH11 and ALDH1A1 in human liver tissue fractions. Chromatograms of the peptides with two m/z transitions detected from recombinant RDH11 (A) and ALDH1A1 (B) spiked into mouse liver microsomes or cytosol, respectively, and representative chromatograms of detection of endogenous RDH11 (C) and ALDH1A1 (D) in human liver fractions. The quantified expression levels of RDH11 (E) and ALDH1A1 (F) in four human livers are shown using either recombinant protein standard (Black) or the AQUA method (Red) as calibrators. Each point depicts a replicate measurement of the sample. The measured concentration of ALDH1A1 was significantly ($p < 0.05$, Student's t -test) lower using AQUA method than protein standard.

Table 1

Optimized mass spectrometric parameters for the quantification peptides and their SIL-peptide internal standards. The mass spectrometric parameters were optimized using the SIL peptides. Each SIL peptide contains a [$^{13}\text{C}_6^{15}\text{N}_2$]-lysine

Protein	Peptide Sequence	Precursor Ion (m/z) [SIL-Precursor]	Fragments (m/z) [SIL-Fragments (m/z)]	Declustering Potential	Collision Energy
RDH11	VVVVTGANTGIGK	607.9 [611.9]	818.4, 717.4 [826.5, 725.4]	75	30
ALDH1A1	ANNTFYGLSAGVFTK	795.4 [799.4]	1042.6, 879.5 [1050.6, 887.5]	120	35
ALDH1A2	ILELIQSGVAEGAK	714.4 [718.4]	846.4, 959.5 [854.4, 967.4]	120	30

Table 2

Method validation data using different internal standard options. Accuracy is expressed as the percent measurement in comparison to true concentration. Precision is shown as the percent CV for replicate analyses over three days of measurement. The SIL-VVV extended refers to the extended peptide, digest refers to adding the internal standard prior to digestion and quench to adding the internal standard after trypsin digestion. For RDH11, the mid-QC was prepared as a pooled sample of human liver microsomes from four donors.

Internal Standard	QC (low)		QC (mid)		QC (high)		r ² for calibration curve
	Accuracy (%)	CV%	Accuracy (%)	CV%	Accuracy (%)	CV%	
RDH11	22.5 nM		2 mg/mL pooled HLM (n=4)		55 nM		
SIL-RDH	89	17		16	81	15	0.97± 0.01
SIL-VVV extended	153	16		12	122	11	0.91± 0.10
SIL-VVV in digestion	120	10		13	113	15	0.96± 0.01
SIL-VVV quench	98	8		8	94	8	0.98± 0.01
ALDH1A1	150 nM		325 nM		600 nM		
SIL-ANN-1A1 in digestion	74	33	93	23	127	20	0.96± 0.02
SIL-ANN-1A1 quench	110	10	111	14	111	11	0.99± 0.01
ALDH1A2	10 nM		32.5 nM		80 nM		
ILE in digestion	109	26	120	4	116	16	0.96± 0.02
ILE quench	105	14	92	11	89	15	0.98± 0.01