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Special Topic Commentary

Dilemmas With Absolute Quantification of Pharmacologically Relevant Proteins Using Mass Spectrometry

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ABSTRACT

Determination of abundances of proteins involved in uptake, distribution, metabolism and excretion of xenobiotics is a prerequisite to understand and predict elimination mechanisms in tissue. Mass spectrometry promises simple and accurate measurements of individual proteins in complex mixtures using isotopically labeled peptide standards. However, comparisons of measurements performed in different laboratories have shown considerable discrepancies in the data generated. Even when very similar approaches are compared, the results differ significantly. An alternative method of measuring protein titers is global proteomics. Depending on sample type, this allows quantification of hundreds to thousands of proteins in a single analysis. It enables system-wide insights by providing protein copy numbers and cell sizes. Regardless of differences, the workflows of both the labeled standard-based and the proteomic approach share several steps. Each can be critical. Selection of optimal techniques is the prerequisite for accurate and reproducible protein quantification.

Introduction

In pharmacological sciences, measuring the fate of xenobiotics in a cell is of a pivotal importance for drug development and calculation of medication dosages. Absorption of drugs is facilitated by transporter proteins which are embedded in the plasma membrane. In a cell, drugs are metabolized by enzymes and the metabolites are excreted by another group of the transporters. In many cases, drugs are immediately excreted without any action of enzymes. To follow and understand these processes, quantification of the transporters and enzymes is necessary.

Determination of protein concentration has never been an easy task. This is due to the heterogeneity in composition and structure of proteins. Variability in amino acid composition and their unequal accessibility in a folded or coiled assembly of polypeptide chains, are the essential complications in unequivocal and reproducible measurement of protein abundance. In a similar way this difficulty affects measuring the concentration of a single protein as well a complex mixture of proteins. Quantification of a protein in a tissue sample is even more challenging because of the presence of other cell components. Nucleic acids, carbohydrates and lipids often interfere with applied analytical methods. Additionally, limited solubility of many proteins in aqueous solutions complicates analytical workflows.

Advances in mass spectrometry technologies have enabled quantification of individual proteins in complex mixtures such as cellular lysates. In an apparently uncomplicated way, concentrations of many proteins of interest can be measured in a short time. Nevertheless, this attractive possibility often becomes a tough task. Conversion of tissue into analyte as well as its quantitative mass spectrometry hides a high potential for inaccuracies. As quantitative mass spectrometric analysis of entire proteins is in reality not possible, the main strategies in quantitative proteomics are based on analysis of peptides originating from digested proteins. This method of analysis is termed 'bottom up'. A parallel measurement of spectral intensities of sequence specific peptides and labeled standards allows quantification of proteins. This type of analysis is termed targeted analysis. In this method the number of quantified proteins is limited to availability of standards. An alternative way of protein quantification allows computation of protein concentration without selecting targets prior to analysis. This can be accomplished by either using a spectral signal of identified peptides in relation to a total spectral signal or the signal from unlabeled references. Dependent on sample type, this approach allows quantification of thousands of proteins without defining proteins of interest at beginning of the analysis.

The workflows of the targeted and quantitative global approaches involve several common preparative and analytical steps (Fig. 1) (Table 1). Practically, each of them is prone to errors. Subcellular fractionation procedures, tissue lysis and protein extraction, digestion of protein, isolation of peptides, LC-MS analysis and

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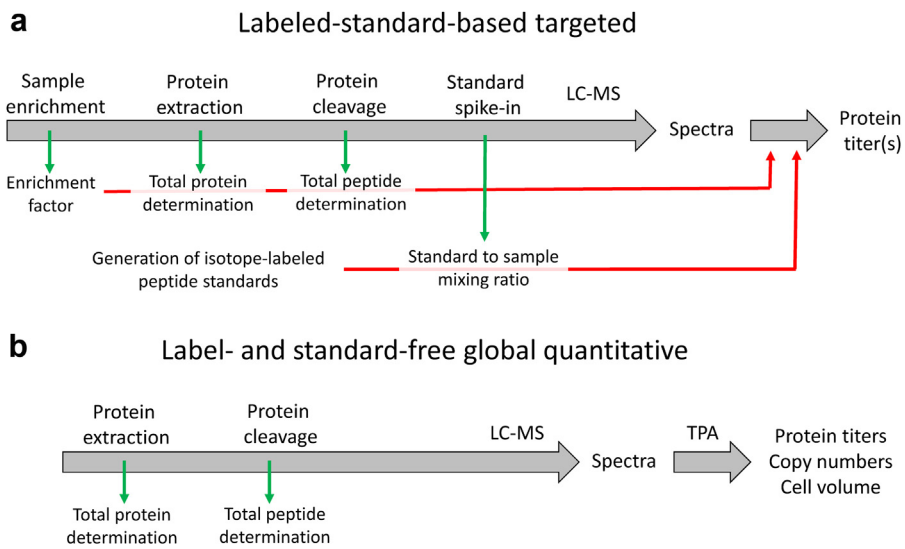

Fig. 1. Workflows of LC-MS based protein quantification by targeted (a) and global proteomics (b). In the targeted approach calculation of protein concentrations involves experimentally derived enrichment factor, total protein and/or total peptide values and standard to sample ratio (red lines and arrows). For calculation of protein concentrations by global proteomics using the "Total Protein Approach" (TPA) only spectral intensities are required. In both approach the total protein and peptide determinations are important for adjusting of the protein digestion and LC-MS.

methods for protein quantification have to be carefully selected and optimized. This requires expertise in different areas of biochemistry and mass spectrometry and therefore designing of a confidently working proteomic workflow is not easy.

Methods and workflows for protein quantification were recently comprehensively described and discussed in Prasad et al.[1] The aim of this article is not to review the existing proteomic techniques and methods which have been applied to analysis of pharmacologically relevant proteins involved in absorption, distribution, metabolism and excretion (ADME proteins) of xenobiotics. The goal of this work is more to highlight critical steps, to stress advantages of using one technique over another and to provide critical views that can be used for planning and carrying out quantitative proteomic measurements of proteins.

Subcellular Fractionation

As sensitivity and dynamic range of mass spectrometers is limited and often not sufficient to quantify proteins with lower abundances such as the majority of the drug transporters, subcellular fractionation to enrich these proteins is commonly used. Most frequently, membrane fractions are prepared from whole tissues. However even though methods for membrane isolation have been developed and continuously improved for more than a half century, a universal protocol for this does not exist. The fundamental drawback of such methods is their low efficiency. In most cases a significant amount of a protein of interest is not present in the isolated fraction, but remains in the undisintegrated tissue. As a consequence of this, quantitative determination or protein titers in tissue becomes more complex. Recoveries in subcellular fractionation vary between different sample types. Usually isolation of subcellular fractions from cultured cells is more efficient than from tissue. Human biopsies are often difficult to handle.

To manage this, a calculation of 'correction' factors has often been suggested. It assumes that some abundant plasma membrane proteins of a 'housekeeping' function such as (Na^+,K^+)ATP-ase are uniformly distributed in the plasma membrane. By measuring their abundances in the isolated fraction and in the rest of the sample, a ratio is calculated and used for extrapolation of the abundance of proteins of interest. There are surely situations where this strategy can be correct but it can also be wrong when the cell surface of analyzed cells is not homogenous. For example, hepatocytes in tissue have at least two distinct types of plasma membrane, the canalicular and basolateral. Quantitative studies on human liver fractionation showed that transporters of xenobiotics are differentially enriched in the subcellular fractions, and that phase I and II

Table 1
Comparison of the Targeted and Non-Targeted Approaches.

Step	Targeted	Global TPA	Global Top 3
Standardization			
Standard selection	Required	Not required	Not required
Labeled standards	Required	Not required	Not required
Calibration range of measurement	Required	Not required	Required
Sample preparation			
Importance of complete digestion	Yes	No	No
Determination of total peptide affects quantification	Yes	No	Yes
MS analysis			
In-depth proteome analysis	No	Required	Required
Analysis time	Short	Long	Long
Data processing			
Use of archival data (LC-MS analyses) possible	No	Yes	No
Quantification of proteins sharing high homology	Possible	Not always possible	Not always possible
Number of peptides used in quantification of a single protein	1–2 (3)	All identified	3

metabolizing enzymes, the cytochrome P450s and the UDP—glucuronyl transferases, have complex subcellular distributions.[2] These findings unveil a potential bias in quantification of proteins with an aid of subcellular fractionation and correction factors. In addition, when analyses are conducted with human tissue, the use of the housekeeping proteins for data normalization can lead to inaccuracies in quantification, which are related to inter-individual variability.

For this reason, subcellular fractionation should be avoided wherever possible. If the fractionation is an inevitable step, any data extrapolation should be done with the highest care. Microsomal and cytosolic preparations are often 'gold standards' for assaying metabolic activities using commercially available reagents. For this reason, in many cases proteomic analyses have to be conducted with these preparations. Measuring of protein abundances in parallel in subcellular fractions and whole lysates may provide more reliable correction factors.

Protein Extraction

For quantification, proteins have to be extracted from tissue. As a large portion of proteins are tightly bound to cellular membranes, harsh reagents are required for this. To this group of proteins belong transporters and many enzymes involved in metabolism of xenobiotics. For this purpose, chaotropes, such as urea or guanidine hydrochloride are often used. The advantage of these reagents is that they do not stick to the proteins and can be easily removed at any further steps. The shortcoming of using these reagents is their limited strength, allowing only partial extraction of proteins embedded in the membranes. In contrast to urea or guanidine hydrochloride, detergents facilitate disintegration of membranes. This enables quantitative extraction of proteins. However, in contrast to urea and guanidine hydrochloride, detergent depletion can be tedious. Since removing of these substances is essential for a protein digestion and the following identification of the peptides by mass spectrometry, protein extraction conditions and sample treatment methods have to be carefully chosen. Moreover, it has to be considered in advance how the amount of extracted protein will be determined and processed for mass spectrometric analysis.

Total Protein

Determination of total protein content is the prerequisite for measuring of a specific protein concentration. This task can be conducted in many ways but can lead to different values. Except for quantitative amino acid analysis - which for most laboratories - is difficult to manage as a routine method, there is no other assay providing unbiased results. UV-absorption and colorimetric assays allow only relative determination of total protein. In addition, other critical factors such as sample dilution and presence of substances used for protein extraction may severely affect the measurements.

Presence of tryptophan and tyrosine allows detection of proteins using absorption of long-wavelength UV light. However, in contrast to other commonly quantified nucleic acids, there is no single formula for all proteins that allows determinations based on UV absorption. In addition, UV absorption allows quantification of total protein only in relatively concentrated solutions (above 0.1%), this is due to a contribution of light scattering from the solvent to the spectrum. Presence of detergents and nucleic acids may also complicate the direct measurements in the UV light. For this reason, colorimetric assays are the preferred methods, where the bicinchoninic acid assay (BCA) and Bradford are the most widely used. Each of the methods has advantages and weaknesses.

BCA is only a little sensitive to presence of detergents but should not be applied to samples containing reducing agents, such as dithiothreitol, 2-mercaptoethanol, or glucose. In addition, urea and guanidine hydrochloride at high concentrations should also be avoided. In contrast, the Bradford assay becomes unreliable for samples containing higher amounts of detergents, but is almost unaffected by reducing agents.

Generation of standard curves is the key step in the colorimetric assaying of proteins. Since the properties of the standard protein are only seldom shared by other proteins, the assays are biased. Dependent on selection of a standard protein, the results obtained by BCA and Bradford can vary several times.[3] The bovine serum albumin (BSA) that is commonly used as a calibration as a standard, is known to give misleading results in the assays.

In conclusion, commonly used total protein determination must be considered as a serious reason behind inaccurate protein quantification. The results can deviate due to many factors. These are mainly the presence of substances other than protein, the type of the assay and protein standard used.

Sample Preparation for LC-MS

Depletion of non-proteinaceous substances and conversion of proteins into peptides are the central tasks in sample preparation in the bottom-up proteomics. Although enzymatic digestion appears as a simple preparative step, efficient protein cleavage and generation of peptides is difficult to achieve. There several reasons for this. Activity of enzymes used for cleavage of proteins can be affected by presence of denaturants, detergents and other substances used during protein extraction. Dilution of the extracts is commonly used as a remedy allowing retaining of a portion of the enzyme activity. However, dilution can impair protein cleavage. When concentration of the substrate, which is protein is falling, the rate of polypeptide bond digestion can decrease. In the classical proteomic method proteins are separated, cleaned from a detergent and digested in a polyacrylamide gel. A drawback of this method often is a limited sample recovery. Many alternative methods have been proposed, where separation of substances interfering with digestion is carried out by precipitation,[4] adsorption-binding,[5–8] ultrafiltration[9] or combination of these. In quantification of transporter proteins, a filter aided sample preparation (FASP) appears to be a good choice.[10] However, decent results can be obtained using a gel assisted sample preparation method (GASP)[11] as well.[12]

Protein digestion is always incomplete. Protein cleavage by an enzyme is just a peptide bond hydrolysis facilitated by a catalyst and the chemical reaction is driven from one to another equilibrium. A good example is trypsin, the favorite enzyme used in proteomics, which in the presence on an excess of a cleavage product turns from the hydrolysis to a condensation reaction leading to joining of peptides together.[13] Peptide bonds are heterogenous. They are formed between various residues with distinct properties. They are located in different environments, which can be polar or hydrophobic. In some cases, their cleavage can be sterically hindered. These properties of the substrate results in distinct velocities of cleavage, where some can be very slow. When using trypsin, the fastest cleaved bonds are between carboxyl site of arginyl and lysyl and small, nonpolar residues, such as seryl, threonyl and methionyl.[14] Efficiency of cleavage is lower when charged residues are at the C-termini of these residues. Peptide bonds involving prolyl residues in this position only occasionally become cleaved.

For protein proteomic digestion, trypsin and endoproteinase LysC are the most commonly used enzymes. Endoproteinases Arg-C and Glu-C, or chymotrypsin are seldom used. Often a combination of the enzymes is used. Frequently a sequential digestion with LysC and trypsin is used. This approach decreases the amount of partially cleaved peptides.[15] A consecutive digestion with these proteinases in a FASP-format (multienzyme digestion: MED FASP)[14] allows creation

of two or three peptide fractions from each sample; these can be analyzed separately. Increased peptide yields and low content of peptides with missed cleavages, are clear advantages of this procedure. Additionally, digestion in the consecutive way improves accuracy of the label-free global protein quantification (see below).[10]

Thiols of cysteinyl residues are the most reactive moieties in proteins. Thiols are routinely alkylated as their tendency to oxidize can complicate MS-analysis. The most commonly used alkylation reagent is iodoacetamide, which is chemically stable and reacts quickly with thiols. However, when the alkylation process is carried out in complex mixtures of tissue lysates, a portion of thiols remains in the reduced form.[16] The incompleteness of the alkylation is accompanied by side reactions with several other moieties, mainly the primary amines. As a consequence, a portion of peptides will escape from routine MS detection and thus can affect quantification. A use of alternative alkylation reagents with narrower specificity, such as chloroacetamide has been suggested as a remedying substitute. However, this reagent increases oxidation of methionine and tryptophan.[17] Recent studies have demonstrated that the alkylation can be removed from sample treatment protocols when complete procedures are carried out under reducing conditions.[16] This approach simplifies and accelerates sample preparation.

Selection of the sample preparation method can be essential for analysis and can be dependent on a type of sample and a downstream analysis.

Total Peptide, Sample Recovery

While total protein determination takes place after tissue extraction and before sample processing procedure, the total peptide has to be measured after enzymatic cleavage of proteins. Quantification of total peptide and comparison to total protein provides information on sample recovery and is a measure of digestion extent. In addition, it allows defining of the digest aliquot for LC-MS analysis and spiking of it with standard at desired ratio. This quantification of total peptides is not always a part of proteomic workflows for two reasons: Firstly, when peptides are a part of the whole digest, it is not possible to discriminate between well cleaved peptides and the remaining undigested sample. Secondly, the total protein assays such as BCA and Bradford are insensitive to small peptides.[3] If quantification of peptides using these methods is implemented in the analysis, it may bias the final results. It is a paradox that the better the protein cleavage is, the lower the amount of product will be observed.

If the peptides are separated from the digestion mixture, by ultrafiltration or another physical method, their quantification can be performed directly using UV-absorption or fluorescence measurements.[18] Notably, following this type of quantification, in the next step the peptide mixture can used for LC-MS analysis. This is not possible when assays based on amine or other residue chemical derivatization or dye-binding are used.

Targeted Proteomics

Targeted proteomics is considered the most accurate method for determination of protein in complex mixtures. However, a number of studies have demonstrated that this is not always true.[19,20] In addition, there is no consensus for conducting protein determination by this means.[21]

Targeted analyses rely on comparison of intensities of ions generated from peptides originating from the protein in the studied sample and standards. The latter are labeled with stable isotopes such as C13 or N15 to make their distinction in the spectra possible. Peptides that can be used for this purpose have to obey several criteria of their composition and size. Sequences containing frequently posttranslationally modified residues such as serine, threonine and tyrosine should be avoided. Target peptides also should not contain residues susceptible to oxidation (cysteine, methionine, and tryptophan) and deamidation (asparagine and glutamine). In addition, the size of the peptide should be between 7 and about 25 residues. Highly hydrophobic peptides, such as those originating from sequences embedded in membranes are not practical because of their limited solubility in water containing solvents. Additionally, a selected peptide should be easily cleavable, out of the protein and well detectable by LC-MS analysis. Considering all these limitations, it is sometimes difficult to find appropriate peptides. In particular, for proteins, which are small or largely embedded in biological membranes. Compromising any of the criteria can severely affect quantification efforts.

Once well behaving standards are found, this analytical technique offers fast measuring of abundances of proteins in short time. Routine analyses can be performed in a high throughput mode. To increase the number of quantified proteins in single samples, analyses can be carried out using a set of labeled standard peptides or cleavable oligopeptide constructs with concatenated tryptic peptides.[22] The latter approach was applied to study ADME proteins as well.[23]

A powerful alternative targeted approach to the subcellular fractionation is the enrichment of targets after sample processing. In this method targeted peptides are isolated using group-specific antibodies.[24,25] Interestingly, a comparison of various proteomic quantification methods showed that the best correlation between the immuno-enrichment method and global proteomic approach.[19]

Global Quantitative

Initially global quantitative proteomic approaches were developed with adhering to the rules of analytical chemistry where standards and calibration curves are essential. Best known is the 'top-three' approach[26] where only 3 peptide ions with the highest spectral intensities are used for quantification. In this way ADME relevant proteins were quantified in human liver microsomes[27] and the blood-brain barrier.[28]

An alternative global quantitative approach is standard-free. It assumes that a summed and normalized MS-signal from all peptides belonging to a single protein is proportional to its concentration in a mixture. This method is named Total Protein Approach (TPA).[29] The prerequisite for its application is an extensive proteomic analysis. Sometimes this is considered as a drawback of the method, because it more time consuming and requires additional instrumentation. On the other hand, however, in-depth analyses allow protein quantification using numerous peptides. The complete digestion and undisturbed quantification of a particular peptide, which is the case in targeted approaches, is less critical. In contrast to targeted analyses, various enzymes can be used for parallel, sequential,[15] consecutive,[14] or in a combination of these protein cleavages.[30]

A recent study has demonstrated that multiple digestion strategy allows accurate measurements of proteins involved in transport and metabolism of xenobiotics.[10] A practically complete set of these proteins was quantified in single analyses of liver biopsies. In parallel, titers of more than 2000 other proteins including 243 additional ADME proteins were measured, which offers further insights for specific interest, such as housekeeping metabolism or transcriptional activity.

A limited specificity for isoforms has been often considered a disadvantage of using the label free-approach. However, in many cases the number of observed isoform specific peptides is high enough to discriminate between those. Alternatively, on the basis of isoform-specific peptides a partition coefficient can be derived,

which allows calculation of accurate concentrations of isoforms.[31] The TPA approach also can be applied to various datasets, also those which were not intended to be used for molar protein quantification. For example, Vildhede et al. used MS^2 and MS^3 outputs of a TMT-multiplexed analysis for transporter quantification.[32] Whereas targeted analyses require development of the assay, global proteomics using the TPA method is immediately applicable to any large proteomic dataset, new or archival.

The TPA approach offers not only calculation of protein concentrations. A sum of abundances of histones enables computation of the total protein content pes single cell and protein copy numbers.[33] This "bonus" of the method is unique. It can be used to compare populations of cells, but also can be helpful for monitoring of completeness of protein extraction from cells of known sizes.

High sensitivity is often stressed as an important factor of targeted analysis. However, this is only true when enrichments of a sample fraction precede analysis. In reality for, non-enriched samples the limit of detection is around 0.1 pmol/mg total protein.[34] Similar values were reported for quantitative global proteomic using the TPA[10] or 'top-three' method.[27]

Conclusions

There are two ways to measure protein abundance with the aid of mass spectrometry: the targeted and the global. The targeted, which is armed with standards and calibration curves, reflects a classical workflow of analytical chemistry. It focuses on "target" molecules and does not consider changes in the biological system. Measurements with the proteomic approach encompass proteomes, where proteins of interest such as the ADME proteins, are only a small part of it.

In the global approach measurements of total protein and total peptide are only important for adjusting the amount of protein for the cleavage and controlling the amount of digest for LC-MS analysis. These values do not contribute towards the calculation of concentrations of individual proteins (Fig. 1). This is an important advantage of the proteomic approach, because, as pointed out above, both measurements are prone to inaccuracies.

It is not possible to recommend the one or the other approach for measuring the titer of pharmacologically relevant proteins. Each can provide valuable information on protein titers. The prerequisite for both is understanding and the correct application of the biochemical methods used. However, the targeted approach is technically more challenging.

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