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Internal Standards for Absolute Quantification of Large Molecules (Proteins) from Biological Matrices by LC-MS/MS

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Abstract

Internal standardization plays a critical role in the performance of a bioanalytical method. There has been a tremendous increase in the popularity of using liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for quantitative bioanalysis of protein molecules. Protein, being too large to be directly analyzed by LC-MS/MS, is proteolyzed and a characteristic peptide is used as a surrogate analyte for quantification. Internal standardization in small molecules' analysis is straightforward, i.e., either a stable labeled isotope (SIL) form of the analyte or a structural analogue is used. As protein quantification involves protein digestion to yield peptides, there are more options for internal standardization. Currently, internal standard selection is based on the availability of the internal standards and the sample preparation workflow. A SIL-form of the analyte protein is the ideal internal standard. However, its use is limited due to cost and commercial availability. Alternatively, a SIL form the surrogate peptide analyte or a cleavable SIL-peptide can be used as an IS. For preclinical bioanalysis of humanized IgG antibody-based drugs, a universal SIL analogue protein has been effectively used as an internal standard. This chapter focuses on internal standardization for the quantitative analysis of proteins, such as biotherapeutics and biomarkers, using LC-MS/MS.

Keywords: internal standards, protein bioanalysis, LC-MS/MS

1. Introduction

Mass spectrometry, as a quantitative tool, was largely restricted to the evaluation of small molecules until the 1990s. This was due to the lack of good-soft ionization techniques that are required for large molecule quantification. The development of soft ionization techniques such

as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) facilitated the use of mass spectrometry for analysis of peptides and proteins [1, 2]. At the beginning of the twenty-first century, large molecule quantification was restricted to immunoassays. The advancements in mass spectrometric instrumentation and better quantification strategies have resulted in a shift of large molecule analysis from immunoassays to mass spectrometry [1, 2]. In the last decade, several methods have been reported for quantification of protein biomarkers and protein biotherapeutics. This popularity of LC-MS/MS methods can be attributed to its inherent selectivity, high dynamic range, shorter development time, and multiplexing capabilities.

Protein quantification using **targeted** mass spectrometry-based quantification from biological matrices is challenging owing to the high molecular mass and high protein background in biological matrices. Direct LC-MS/MS analysis of an intact protein analyte can be performed on proteins having molecular weights below 10 kDa. Proteins tend to ionize with multiple charges during electrospray ionization resulting in a complex spectrum with many precursor ion peaks corresponding to each charged state. Because of the complex precursor ion spectra, any single chosen precursor ion signal for quantitation will only represent a very small fraction of the total ionized analyte signal. This will result in lower signal response and reduce the sensitivity of the method. The complex ion spectrum also impacts the method specificity due to the presence of many overlapping signals arising from naturally occurring isotopes. In addition, larger protein analytes tend to be undergoing inefficient or non-existent collision-induced dissociation, in turn, impacting targeted mass spectrometric analysis. These challenges are overcome by enzymatic proteolysis of the analyte protein to yield one or more characteristic peptide fragments (i.e., signature peptides), which can be used as surrogate analyte(s) for quantification. In some methods, immunoaffinity enrichment is used for sample clean particularly for methods requiring detection limits below 100 ng/ml. These enzymatic digestions and immunoaffinity isolations are a potential source of variation and need to be controlled.

An internal standard is added to the sample during absolute quantification to compensate for variability encountered in sample processing and instrumental analysis. For mass spectrometric assays, a stable isotope labeled (SIL)-form of the analyte is an ideal internal standard it mimics the analyte throughout the method. However, SIL-proteins are difficult to produce with sufficient purity, and hence substitute internal standards such as SIL-peptides or protein structural analogues are used. In this chapter, we discuss the commonly used strategies for protein quantification using appropriate internal standards.

2. Overview of mass spectrometric-based protein quantification

Before we evaluate the different internal standardization options for protein quantification, it is essential to understand the various sources of variability during protein bioanalysis. Method variability arises during sample preparation or instrumental analysis. Protein bioanalytical quantification can encompass complex sample preparation steps such as protein analyte enrichment,

protein analyte proteolysis, and surrogate peptide analyte enrichment. Instrumental analysis comprises of chromatographic separation and mass spectrometric ionization. This section briefly summarizes various process encountered in protein quantification by LC-MS/MS.

2.1. Signature peptide selection

Selection of a signature peptide is a critical part of method development for protein bioanalysis. Peptides containing amino acid residues with potential post-translational modification (PTM) sites are usually avoided due to a potential change in peptide mass that would affect reproducible quantification. However, if the intended purpose is to quantify a post-translational modification, a tryptic peptide containing the specific PTM is selected [3–5]. Tryptic peptides containing amino acids susceptible to oxidation such as methionine and tryptophan are avoided as chemical modifications of these molecules can result in a change in its mass and thus can affect method reproducibility. Usually, tryptic peptides containing cysteine residues are avoided as they undergo iodoacetamidation. However, methods using signature peptide containing a cysteine residue have been reported after accounting for any mass change occurring prior to mass spectrometric detection [6, 7]. Missed cleavages can result inconsistent production of the signature peptide in turn impacting quantitation. Peptides containing ragged ends or dibasic ends next to each other (such as in Arg-Arg, Lys-Lys, or Arg-Lys) should be avoided as they are known to result in missed cleavages [8–10].

2.2. Sample preparation techniques for protein quantification LC-MS/MS

Biological samples, especially plasma and serum, are complex mixtures comprising of proteins, lipids, and salts in addition to the analyte molecule. Biological samples require pre-treatment such as analyte enrichment or proteolysis prior to protein LC-MS/MS analysis. Sample preparation depends on the analyte physiochemical properties as well as the required level of selectivity and sensitivity. For example, proteins having a low molecular weight (<10 kDa), can be analyzed without proteolysis using protein precipitation and/or solid phase extraction. High molecular weight proteins (>10 kDa) require proteolysis to yield peptide fragments compatible for mass spectrometric analysis. These samples may require enrichment before and/or after proteolysis to achieve required detection limits. Routinely used sample preparation techniques are described below.

2.2.1. Non-selective protein enrichment techniques

For proteins smaller than 10 kDa, various non-selective protein enrichment techniques can be employed. Partial protein precipitation, using organic solvents along with surfactants, is used as a simple sample purification technique to deplete the endogenous plasma proteins. Partial protein precipitation eliminates larger proteins but leave smaller ones in solution [11–13]. Protein precipitation could have low recovery due to losses as a result co-precipitation, which would be a drawback. As it is simple but a crude clean-up technique, the resulting extracts are usually complex containing high concentration of salts and lipids. Matrix effects are commonly observed with these extracts, which can be a source of variability.

Solid phase extraction (SPE) is another purification technique that is employed solely or along with other purification techniques for sample clean up wherein the analyte is a smaller protein or peptide [12, 14–17]. Several mixed mode SPE cartridges, combining reversed phase stationary phase along with strong cation exchange or weak anion exchange, are commercially available for peptide analysis. These are usually available in 96-well microelution plate format. Microelution SPE offers many advantages including increased sensitivity due to low elution volumes, analysis of limited volume samples, significantly cleaner samples compared to extracts obtained after protein precipitation and higher reproducibility. The low elution volume avoids the need for sample extract evaporation and reconstitution, which can result in peptide instability.

2.2.2. Abundant protein depletion

Several commercial kits are available which use immunoaffinity depletion to selectively remove serum albumin, immunoglobulins, and other high abundant proteins from biological matrices [18–21]. These kits have shown to reduce protein content by up to 85% [22]. The enrichment technique is best suited for methods that have multiple protein analytes typically seen in biomarker research. The high costs of these kits and recovery issues have been the major drawback of this approach [23, 24]. Abundant protein depletion has been used in several biomarker quantification methods [23, 25, 26]. Liu et al. showed that isopropanol with 1.0% trichloroacetic acid was effective in removing 95% of the total albumin in human plasma samples while retaining 60–100% of the three analyte proteins that were evaluated. The recovery using this approach was found to be better than commercially available albumin depletion kits [27].

2.2.3. Immunoaffinity enrichment

Use of immuno (or affinity) capture for isolation of the analyte protein or its signature peptide is a highly selective enrichment technique. Combining the selectivity of an immunoaffinity capture with the selectivity of a LC-MS/MS system can allow a 1000-fold enrichment in comparison to conventional techniques [24]. Although this technique requires specialized antibodies, it provides sufficient purification to achieve quantification of low abundance proteins from plasma [24, 28–31]. Low recoveries and cross reactivities are some of the issues seen during immunocapture enrichment [32, 33]. Immunoaffinity isolations may be carried out with single or multiple antibodies depending on the availability of analyte-specific antibodies and the desired detection limits. The capture antibodies are immobilized in a 96-well plate or on to the surface of magnetic beads prior to the immunocapture. This immobilization is achieved using biotinylated antibodies and streptavidin-coated plates or magnetic beads. Alternatively, Protein A or G coated supports may be used for immobilizing the antibody using the Fc region of the antibody. The wide variety of immunocapture techniques can roughly be categorized into three categories based on the type of capture reagent.

2.2.3.1. Immunocapture using protein-specific antibodies

The simplest approach is to use monoclonal or polyclonal antibody that is specific to the target analyte for immunocapture enrichment. Most of the protein biomarkers will have commercially

available antibodies while biotherapeutic drug molecules have specific antibodies developed for immuno-purification or screening during early pharmaceutical discovery and development. Due to the high specificity of this enrichment process, only a SIL-protein internal standard can be used as an internal standard. However, in methods using a capture antibody, that has an epitope present on or near the signature peptide region of the analyte protein, an external SIL-peptide can be used as an internal standard [34].

2.2.3.2. Immunocapture using peptide-specific antibodies

Anderson et al. introduced the stable isotope standards with capture by anti-peptide antibodies (SISCAPA) strategy wherein immunocapture enrichment is directed toward a signature peptide after digestion using anti-peptide antibodies [35]. This technique allows high sensitivity and precision. However, these signature peptide-specific antibodies may not be commercially available and require inhouse development increasing method development cost and time. Some methods have employed this type of immunocapture online using specialized columns containing analyte-specific antibodies [29, 36–38]. This workflow is extensively used for multiplexed biomarker assays [24, 39]. Methods using dual enrichment, i.e., analyte protein enrichment and surrogate peptide enrichment post digestion, have also been reported. Multiple enrichment steps require the selection of an appropriate internal standard to compensate for method variability arising within each enrichment step.

2.2.3.3. Non-antibody capture of antibody-based biotherapeutic drugs

The majority of the biotherapeutic drugs are monoclonal antibodies or antibody-based molecules such as antibody drug conjugates. Antibodies have a constant tail region also known as the fragment crystallizable region (Fc region) and a variable region also referred to as antigen-binding region (Fab) region. The Fab region contains the complementarity determining region. Antibody-based drugs can be isolated using Protein A or G coated supports to bind to the Fc. This technique requires minimum time and resources for development and can achieve high throughput with adequate sensitivity. If additional selectivity is required, anti-Fc antibodies may be used depending on the analyte and the biological matrix. This technique is useful for the quantitation of humanized biotherapeutic drugs in animal models. Quantitation of humanized biotherapeutic drugs in human biological matrices requires the use of anti-idiotypic antibodies as capture agents to achieve significant detection limits. In some methods, the target antigen is used as a binding agent for the selective capture. Dubios et al. described an immunoaffinity coupled LC-MS/MS method wherein the analyte Cetuximab was isolated using its target antigen (soluble epidermal growth factor) as a capture reagent [40].

2.2.4. Enzymatic proteolysis

Most protein quantitative LC-MS/MS methods involve enzymatic digestion of the proteins to yield smaller peptides which can be easily quantified by commercially available quantitative mass spectrometers. A typical protein digestion procedure involves denaturation, reduction, and alkylation followed by proteolysis. Denaturation is carried out to unfold the protein so that it can be easily accessible to the proteolytic enzyme. Urea is the most commonly used for denaturation during protein quantification. Alternatively, denaturation has been achieved using

other chaotropic agents such as guanidine HCl, surfactants such as sodium deoxycholate, organic solvents such as methanol and heat (95°C) [13, 17, 26, 41, 42]. RapiGest SF, an acid-labile surfactant, is a Waters proprietary product that has gained high popularity for protein bioanalysis due to its compatibility with mass spectrometric detectors. This detergent is easily precipitated out by lowering the pH during the termination step of the enzymatic digestions. Reduction of the protein is carried out using dithiothreitol or TCEP (tris(2-carboxyethyl)phosphine) to break the disulfide linkages between cysteine residues. The resulting free thiol groups are then derivatized using an alkylating agent such as iodoacetamide or iodoacetic acid in order to prevent reformation of disulfide linkages.

Trypsin is the most commonly used enzyme for protein digestions primarily as tryptic peptides have a c-terminal basic residue that favors ionization. In addition, average tryptic peptides have lengths suitable for detection on commonly used quantitative mass spectrometers. However, other enzymes such as Lys-C, Arg-C, pepsin, chymotrypsin have been used when a specific cleavage is required [43–46]. To improve digestion efficiency, different approaches have been illustrated including high temperature, microwave-assisted digestion, and use of organic solvents [31, 47].

The “pellet digestion” method is a simplified method in which the proteins are precipitated using an organic solvent like acetonitrile to form a pellet and the supernatant containing interfering molecules such as phospholipids are discarded. This method provides an easy, efficient way of performing a fast clean-up and has resulted in improved digestion efficiency in comparison to direct digestions [48–51].

2.3. Chromatographic separation

Liquid chromatography is used for separation of the protein or peptide mixture prior to mass spectrometric detection. For most peptide and protein analytes, reversed-phase column chemistry, typically C18 columns, allows separation of structurally and chemically similar molecules using mobile phases that are compatible with ESI. Hydrophilic interaction liquid chromatography (HILIC) has also been used to separate hydrophilic peptides. The mechanism of separation for small molecules is based on partitioning between the mobile and stationary phases. However, proteins and large peptides are not able to fully penetrate the pores and instead adsorb to the surface, and are desorbed at a critical concentration of organic solvent. Columns with larger pore stationary phases (~300 Å) allow improved penetration of larger molecules as well as the use of higher flow rates with reduced band broadening, and therefore provide greater selectivity for some peptides and proteins.

Two-dimensional chromatographic separations, such as ion-exchange chromatography (IEC)-RPLC or RPLC-HILIC, have been utilized to fractionate and clean up samples, thus improving sensitivity of detection [16, 24, 46, 52, 53]. Additionally, many reported methods have used column trapping prior to analytical separation. The trap column retains the analytes and removes salts and other highly hydrophilic peptides [25, 28–31, 39, 54]. Some methods have used columns with antibodies for online immunoaffinity LC has been reported to achieve exceptional selectivity with minimal sample clean-up [31, 37, 38].

2.4. Mass spectrometric detection

Triple quadrupole (QQQ) and Quadrupole Ion Trap (QTrap) using the multiple reaction monitoring (MRM) mode are the most widely used mass analyzers for protein quantification. Proteins and peptides under electrospray conditions generally ionize to several charge states. For smaller peptides and proteins, $[M + 2H]^{2+}$ is usually the most abundant species, however, for larger molecules, $[M + 3H]^{3+}$, $[M + 4H]^{4+}$, and so on may also form, distributing the signal over several charge states and reducing the achievable limits of quantification. It is essential that the charge distribution across multiple samples is reproducible or will introduce method variability.

MRM mode allows measurement of multiple transitions and can be used for quantification of multiple analytes. Besides the signature peptide used for quantitative evaluation, additional characteristic peptides maybe monitored as monitoring peptides or qualitative peptides. Based on their location in the protein amino acid sequence, these monitoring peptides can provide valuable insights about the integrity of the analyte protein.

Mass spectrometric detection using electrospray ionization is highly susceptible to matrix effects. Samples with elevated concentrations of phospholipids, such as glycerophosphocholines and lysophosphatidylcholines, exhibit increase in ionization suppression when compared to normal plasma [55]. Lipid-related interference is generally not an issue with immunocapture-based methods, but it can be source of concern with methods that use non-selective isolations such as partial protein precipitation.

3. Internal standardization for protein bioanalytical methods

Internal standards need to track the analyte during all stages of sample analysis that includes sample preparation, chromatographic separation, and detection. Immunoaffinity capture, enzymatic proteolysis, and mass spectrometric ionization are the three major steps that are susceptible to variability during protein bioanalysis by LC-MS/MS. The internal standard may be added at different step(s) of the extraction process depending on the availability of the internal standards and the sample extraction workflow. **Figure 1** describes various commonly used workflows for protein quantification and the internal standard (IS) options. Protein internal standards are added prior to analyte protein enrichment. Protein analyte enrichment can be immunoaffinity isolations or a non-selective process such as partial protein precipitation, SPE, and abundant protein removal. Smaller proteins can be directly analyzed after protein analyte enrichment. Larger proteins are proteolyzed to yield signature peptide(s) that can be used as surrogate analytes. Peptide internal standards when added prior to enzymatic proteolysis can only track peptide instability and volume recovery during the proteolytic process as well as mass spectrometric ionization during analysis. Additionally, cleavable internal standard peptides may be able to track the digestion variability when added prior to proteolytic incubations. Methods requiring low detection limits utilize peptide enrichment post enzymatic proteolysis. SIL-peptides are required to track immunoaffinity-based peptide enrichment processes. Currently used internal standards for protein bioanalysis are described below.

Figure 1. Internal standardization strategies for different LC-MS/MS protein quantification workflows.

3.1. Protein internal standards

3.1.1. Stable isotope labeled protein internal standard

A stable isotope labeled (SIL) form of the analyte protein is the most ideal IS for absolute quantification of proteins. As the analyte protein and SIL-protein internal standards have the same physiochemical behavior, this IS will be able to track the analyte protein throughout the entire analytical procedure. SIL-proteins are added at the start of the sample extraction and can account for immunoaffinity isolation(s), enzymatic digestion, pre-analytical treatments as well as the mass spectrometric ionization.

The EMA guidelines recommend the use of a SIL-protein IS whenever possible for LC-MS/MS methods. However, a major restriction in the use of SIL-proteins as internal standards for protein quantification is their commercial unavailability or the high cost of production. The complex structure consisting of specific intramolecular folding of amino acid chains as well as intramolecular di-sulfide linkages and presence of post translational modifications makes it difficult to synthesize these proteins in a reproducible manner. If the analyte protein is small, a SIL form of the protein can be chemically synthesized using solid-phase synthesis [16, 56]. However, for most protein analytes, the production of its isotopic labeled form requires a cellular environment. Two methods are described below for the generation of isotopically labeled proteins: metabolic labeling using whole cells and the cell-free approach using cell lysates.

3.1.1.1. Cell culture production

All cell-based labeling approaches rely on the metabolic conversion of labeled precursors into a protein. The labeled precursors used in cell-based systems may be amino acids or they may be

more fundamental precursors which serve as carbon or nitrogen sources for the synthesis of amino acids prior to their incorporation into protein.

SILAC is a popular technique for production of SIL-proteins by incorporation of SIL-amino acids into the target protein. This technique is popularly known as stable isotope labeling by amino acids in cell culture (SILAC) [57] or stable isotope labeling with amino acids (SILAA) [58]. SILAC is a straightforward procedure in which essential amino acids are left out of culture media and replaced by deuterated, carbon-13, and/or nitrogen-15 labeled variants of these amino acids. After multiple cell duplication cycles, these SIL amino acids get incorporated in the entire proteome. Heavy labeled variants of lysine and arginine that provide ample spacing between isotopic envelopes of light and heavy tryptic peptides (e.g., 10 Da using $^{13}\text{C}_6$ $^{15}\text{N}_4$ -Arg and 8 Da using $^{13}\text{C}_6$ $^{15}\text{N}_2$ -Lys) are the most commonly used amino acids for production of SIL-proteins using SILAC. Arginine and lysine on an average they occur at every tenth position in a protein sequence. Trypsin, the major proteolytic enzyme used in protein quantification assays, cleaves at lysine and arginine ensuring that at least one of the labeled amino is present on each tryptic peptide.

Cell culture production is highly useful for generating SIL-labeled proteins for biotherapeutics. Biotherapeutics are produced by genetically modified cell lines, yeast or bacteria. To obtain a SIL-protein, the cells producing the biotherapeutic protein are grown in a medium containing labeled precursors for the desired protein. The stable isotope labeled amino acids gets incorporated in the proteins, thus resulting in production of a SIL-protein. These SIL-proteins are then purified and can be used as internal standards.

The cell culture method is an easy process of producing labeled proteins, but requires a cell culture equipment and a sterile laboratory. A major disadvantage is that in cell culture other endogenous proteins will be simultaneously produced having the incorporated label, and hence a more elaborate purification is required. Also, incomplete labeling can occur when the pools of labeled amino acids are diluted with amino acids newly synthesized by the cell.

3.1.1.2. Cell-free production

SIL-proteins can also be made by *in vitro* protein synthesis in a cell free system [59]. Cell-free translation systems are largely supernatants obtained by centrifugation of the crude lysate of either *E. coli*, wheat germ, or rabbit reticulocytes at 30,000 g. The cell lysate supernatants, also referred to as S30 fraction, contain the cell's protein synthetic machinery consisting of ribosomes, translation factors, aminoacyl-tRNA synthetases, and tRNAs.

Cell-free systems can be operated in three different modes: batch mode, continuous flow cell-free (CFCF) mode, and CECF mode. In batch mode, the reaction is carried out in a tube to which all components of the reaction are added. It is easy to step-up and can be useful for fast and easy production of small amounts of protein. Its disadvantage is it is a closed system and hence has limited capacity.

The CFCF mode is an open system which requires the continuous supply of fresh substrates and removal of by-products by a continuous flow of a feeding solution into a reaction chamber. The CFCF mode was first developed by Spirin and co-workers [60]. The total volume of the

reaction is maintained constant by having the volume flowing out of the chamber equal to the volume flowing in. This system can be used for continuous production of labeled proteins.

The CECF mode is an open system with two chambers separated by a semi-permeable membrane. The first chamber is the feeding chamber and contains the substrates and the energy system. The second chamber is the reaction chamber and contains the enzymes and DNA. The substrates permeate through the semipermeable membrane and are converted to proteins in the reaction chamber. This can be easily set-up using a simple dialysis bag as the reaction chamber. The bag can be immersed in a feeding solution inside of a tube larger than the dialysis bag creating a simple two-chamber device.

3.1.2. Derivatized protein internal standard

Derivatization allows one to easily generate a protein internal standard that is physiochemically like the analyte protein. Winther et al. reported an LC-MS/MS method for quantification of progastrin-releasing peptide (ProGRP), a small cell lung cancer biomarker, in human serum using an acetylated form of the protein as an internal standard [30]. The IS was made in-house by specific acetylation of the lysine side chains in ProGRP (31–98) by using N-hydroxysuccinimide-based ester acetic acid N-hydroxysuccinimide (AA-NHS) as the acetylating reactant. The acetylated ProGRP (31–98) signature peptide NLLGLIEAK gets converted to NLLGLIEAKacENR, which was used as a peptide internal standard. The extraction procedure involved protein precipitation with acetonitrile followed by pellet digestion with trypsin prior to analysis.

This acetylated ProGRP internal standard mimicked the analyte ProGRP through extraction steps including tryptic digestion and hence compensated for any variations during extraction. However, acetylation of the IS-peptide, results in chromatographic differences between the signature peptide and IS-peptide. Differences in retention time can result differences in ionization due to co-eluting matrix interferences. The derivatization also caused ionization differences in the precursor ion charge states. NLLGLIEAK had +2 as the most dominant charge state while NLLGLIEAKacENR had +3 as the most dominant charge state of the precursor ion. The addition of IS to the samples improved the coefficient for both the linear and the polynomial calibration curve and the intra- and inter-day accuracy. However, the high intra-day precision values (%CV of 12–25.2%) displayed some unaccounted variability in turn questioning the performance of the internal standard in this method. An important consideration while using derivatized internal standards is to ensure that impurities of underivatized analyte protein or derivatizing reagent are not present in the purified internal standard.

3.1.3. Universal stable isotope label protein internal standard for quantification of antibody-based biotherapeutics in non-human matrix

Drug development involves bioanalytical testing in non-human species. During bioanalysis of humanized IgG antibody based biotherapeutics in animals, selected peptides from the constant region (Fc) of the antibody can be used as signature peptides. These peptides will be present in the humanized immunoglobulin (IgG)-based biotherapeutic drugs, but will not be present in antibodies found in the animal biological fluids. In methods using signature peptides obtained from the constant region, a stable labeled analogue monoclonal antibody (mAb)

can be used as an internal standard. SILuTMMab internal standard is a commercially available stable labeled IgG1 monoclonal antibody and has been metabolically labeled with ¹³C₆ ¹⁵N₄-Arginine and ¹³C₆ ¹⁵N₂-Lysine was expressed in CHO cells. SILuTMMab is used as a universal internal standard in quantitation of humanized biotherapeutics in non-human biological matrices. **Table 1** lists out the various characteristic SIL-peptides that can be generated after proteolysis of SILuTMMab with trypsin.

The universal applicability of SILuTMMab is exemplified in an immunoaffinity coupled LC-MS/MS method reported by Kaur et al. [61]. A generic method was developed for mAb-1 and its universal applicability was demonstrated with the additional six mAbs. SILuTMMab was used as the common internal standard in all seven methods. The extraction involved immunoaffinity enrichment followed by tryptic digestion. For three mAbs, the immunoaffinity capture was carried out using anti-human Fc antibody attached to magnetic beads. This immunoaffinity capture allows selective binding to a humanized mAb in nonclinical matrices. Alternatively, for remaining four mAbs, the affinity enrichment was performed using a less selective reagent, Protein A/G. After immunocapture, the analyte and IS bound to the magnetic beads were denatured, reduced, and alkylated. The isolated analyte mAb and IS were digested with trypsin prior to LC-MS/MS analysis. The method for mAb-1 in cynomolgus serum was found to have a linear response over the nominal concentration range of 0.100 to 25.0 µg/ml with high precision (%CV < 3%) and good accuracy (%DFN ± 9%). The high precision and accuracy of the method validates the effectiveness of the internal standard to compensate for any variability during extraction and analysis. The method performance was evaluated with additional six mAb as well as in rat and mouse sera. All the assays showed good precision (%CV < 20%) and accuracy (%DFN ± 20%).

The generic method, using a universal internal standard that tracks the analyte throughout extraction and instrumental analysis, circumvents the method development challenges for biotherapeutic mAbs. This internal standard use is restricted to non-human biological matrices. In human matrices, the signature peptide will lose its selectivity due to the presence of high concentrations of endogenous mAbs.

Universal SIL-peptide	Antibody isotype	Peptide location
DTLMISR*	IgG1, IgG2, IgG3, IgG4	Heavy chain
FNWYVDGVEVHNAK*	IgG1	Heavy chain
VVSVLTVLHQDWLNGK	IgG1, IgG3, IgG4	Heavy chain
NQVSLTCLVK*	IgG1, IgG2, IgG3, IgG4	Heavy chain
GFYPSDIAVEWESNGQPENNYK*	IgG1, IgG4	Heavy chain
AGVETTTPSK*	IgG1, IgG2, IgG3, IgG4	Light chain (Lamda)
YAASSYLSLTPEQWK*	IgG1, IgG2, IgG3, IgG4	Light chain (Lamda)

*Stable isotope labeled amino acid (¹³C₆ ¹⁵N₄-Arginine or ¹³C₆ ¹⁵N₂-Lysine).

Table 1. Tryptic SIL-peptides of SILuTMMab.

3.1.4. *Analogue proteins as internal standards*

A structural protein analogue can also be used as an internal standard. This internal standard is the least favorable as it may not truly mimic the protein analyte during proteolysis or the signature peptide during mass spectrometric ionization. The advantage of using an analogue protein is ease of availability and low cost. As it accounts for volume losses, it can be an appropriate IS for methods that involve simple sample preparation such as partial protein precipitation. An unlabeled analogue protein is chosen based on the similarities in physiochemical properties, such as size, hydrophobicity, and isoelectric point, with the analyte protein and its surrogate peptides obtained after proteolysis. Some methods have been reported wherein the chosen internal standard was able to track the analyte efficiently throughout the method.

Halquist et al. reported an LC-MS/MS method for quantification of Alefacept, a therapeutic protein for treatment of psoriasis, in human plasma using horse heart myoglobin a protein analogue internal standard [11]. The method used partial protein precipitation to selectively precipitate background proteins while retaining the analyte protein and the internal standard protein in solution. The isolated proteins were proteolyzed using trypsin. A 20-h-incubation was chosen for proteolysis to ensure least digestion variability for both proteins. The signature peptides from analyte and IS were then separated using reversed phase chromatography and detected using tandem mass spectrometry with electrospray ionization. The chromatographic gradient conditions were adjusted to ensure the surrogate analyte peptide and the IS-peptide had the same retention time. This enabled the IS-peptide to compensate for any matrix effects during ionization.

3.2. Peptide internal standards

3.2.1. *Stable isotope labeled peptide internal standard*

When a SIL-protein is unavailable for use as an internal standard, a stable isotope labeled form of the signature peptide can be used. A SIL form of the signature peptide (SIL-peptide) is the most commonly used internal standard during protein quantification. SIL-IS peptides are variants of the signature peptides having one or more stable isotope labeled amino acids. Stable isotope labeled amino acids are obtained by substitution of certain atoms (N,C,H) with their heavy variants. The most frequently used stable isotopes are ^{13}C (carbon-13), ^{15}N (nitrogen-15), and ^2H (deuterium). A SIL-peptide can be created by using solid-phase peptide synthesis [56, 62]. Due to the widespread use of SIL-peptides for protein bioanalysis, several laboratories provide commercial services for production of customized SIL-peptides at reasonable prices.

SIL-peptides are physiochemically identical to the signature peptide but can be easily distinguished on a mass spectrometer due to the mass shift from the heavier isotopes. A minimum mass difference of at least 6 Da between SIL-peptides and the signature peptide is recommended. This will ensure an adequate resolution between the mass of SIL-peptide and signature peptide even for peptides having a dominant charge state of +2. SIL peptides can effectively compensate for extraction recovery, peptide instability and LC-MS/MS variability. However, unlike a SIL-protein it does not track immunoaffinity and proteolytic digestion

steps. The major advantage of using a SIL-peptide is that these can be synthesized at relatively low cost.

A SIL-peptide internal standard can be added before and after proteolysis. However, if the signature peptide is suspected to undergo degradation, it is recommended to add the SIL-peptide prior to digestion. Also, if a recombinant form of the protein is not available, protein concentrations are calculated stoichiometrically, solely based on the known molar concentration of the SIL-peptide used. In such instances, the internal standard is added post digestion to obtain reproducible peptide quantification [24, 63].

3.2.2. Extended stable isotope labeled peptide internal standard

Variation in digestion efficiency can be accounted for with the use of an extended SIL-peptide which has cleavable groups flanking either side of a SIL-peptide [29, 64, 65]. Generally, the cleavable groups consist of three to six amino acids residues from the original protein sequence at both the N- and C- terminus [29, 65, 66]. The addition of an extended SIL-peptide IS prior to digestion provides a more cost-effective alternative to compensate for variability in digestion efficiency, peptide stability, volume recovery, and mass spectrometric ionization.

Barnindge et al. were first to report the comparison between SIL-peptide/non-cleavable peptide (NCP) and a dual cleavable peptide (DCP) or “extended SIL-peptide” as internal standards to track protein digestions [64]. They synthesized two peptides each containing a signature peptide sequence from amino acids 318 to 323 in human serum albumin (HSA). The non-cleavable peptide (NCP) was labeled with a stable isotope labeled alanine residue, i.e., NYA*EAK, whereas the other peptide had two tryptic cleavage sites and two stable isotope labeled alanine residues, i.e., DVAK-NYA*EA*K-DVFLG. Different concentrations of HSA were digested along with equimolar concentrations of NCP and DCP. Prior to digestion the samples were reduced for 30 min at 30°C using 10 mM dithiothreitol (DTT) followed by alkylation using a concentration of 30 mM iodoacetamide (IAA) with the reaction going to completion in the dark at room temperature for 30 min. Each sample was then digested with trypsin for 12 h at 30°C in a shaking water bath using an enzyme-to-substrate ratio of 1:10. The reaction is terminated after 12 h with trifluoroacetic acid. The samples were analyzed using LC-MS/MS. The results showed that a cleavable internal standard peptide could give similar results to a non-cleavable internal standard peptide. Timed digest experiments showed that the digestion rates for dual cleavable peptide and analyte protein, i.e., HSA were different with the DCP proteolysis coming to completion faster (approximately 1 min) than HSA (approximately 20 min). From these results, they concluded that although an internal standard with a cleavage site provides understanding of the digestion process, a SIL-peptide truly cannot replicate the proteolysis conditions experienced by the analyte protein.

In another study, Faria et al. compared the performance of a SIL-peptide and extended SIL-peptide as internal standards for quantification of human osteopontin [67]. Digestion studies showed that the signature peptide production had a biphasic pattern. This pattern was attributed to the degradation of the signature peptide during digestion with trypsin due to suspected chymotrypsin-like activity. The digestion profile of the protein analyte had three phases, i.e., the “formation phase,” the “transition phase,” and the “degradation phase.” The

formation phase was between 0 and 5 h which was dominant in the formation of signature peptide. The transition phase was between 5 and 10 h where signature peptide formation and degradation processes occur at similar rates. The degradation phase was beyond 10 h in which the degradation of the signature peptide was the most dominant phenomenon. In order to track the analyte protein digestion profile, a SIL-peptide IS and extended SIL-peptide IS were added. From **Figure 2**, we can see that the recombinant protein and the extended SIL-peptide internal standard had similar digestion profiles as they both undergo formation and degradation. SIL-peptide only undergoes degradation and hence only mimics the analyte protein digestion profile during the degradation phase. Validation studies showed that under controlled conditions and long digestion time there was no significant difference in precision when either of the internal standards was used for quantification. However, when trypsin activity was forcibly varied, the extended SIL peptide had higher precision. This difference was more pronounced when digestion was carried out at shorter time intervals.

3.3. Comparison of protein SIL-IS versus peptides IS

Li et al. evaluated the use of SIL-protein, SIL-peptide, and extended SIL-peptide as internal standard for quantification of monoclonal antibodies in preclinical biological matrix by LC-MS/MS [68]. The evaluation was carried out with four mAbs of the same IgG2 isotype as the SIL-IS: α DA-G2, (KLH)-120.6-G2 (α K-G2), 827-435-G2 (827-G2), and anti-DNP-3B1-G2 (α DB-G2). In addition, the test was extended to four more mAbs of a different isotype IgG1: anti-DNP-3A4-F-G1 (α DAG1), anti-KLH-120.6-G1 (α K-G1), 655-341-G1 (655-G1), and anti-DNP-3B1-G1 (α DB-G1). Stable isotope labeled human antidinitrophenol (DNP) IgG2 mAb was used as the IS as it is unlikely to be present endogenously in preclinical species. The whole molecule SIL-IS of clone anti-DNP-3A4-F-G2 (α DA-G2) was produced in cell culture, purified, and characterized

Figure 2. Comparison of digestion profiles of recombinant OPN along with internal standards, i.e., SIL-peptide and extended SIL-peptide. Reprinted from [67]. Copyright (2015) with permission from Elsevier.

prior to use. Synthetic IS peptides with stable isotopic labeled antikeyhole limpet hemocyanin leucine (L*), NQVSL*TCL*VK and REEMTKNQVSL*TCL*VKGFYPSD (six flanking amino acids), were commercially obtained and used as SIL-IS peptide and extended SIL-IS peptide, respectively. The evaluation was carried out in rat plasma and cynomolgus monkey serum.

SIL-protein IS was added to each sample prior to extraction. The analyte mAb and SIL-protein mAb were immunocaptured using an antihuman crystallizable fragment (anti-Fc) that recognizes human mAb biotherapeutics but not the endogenous immunoglobulins in the preclinical sample. After immunocapture, the analytes were eluted with 200 μ L of 50% MeOH and 3% formic acid in water. Samples with IS-peptides were spiked either with SIL-peptide or extended SIL-peptide. The eluate was evaporated to dryness. SIL-peptide and extended SIL-peptide were added to samples with IS-peptides. The samples were reconstituted for reduction and alkylation. The samples were then digested with trypsin. After termination of the proteolytic reaction, the extracts were analyzed using LC-MS/MS.

For the comparison experiment, three sets of QCs each from α DA-G2, α DA-G1, or α K-G2 were analyzed in three replicates along with the three internal standards. The results can be seen in **Figure 3**. Overall, all three mAb were quantified accurately (Bias within $\pm 20\%$) and precisely (%CV within 20%) using the SIL-protein IS. For the extended peptide SIL-IS, the α DA-G2 QCs were also well within $\pm 20\%$. The mid and high QC values of the α DA-G1 were near the $\pm 20\%$ threshold but not the LQC or the QCs of the α K-G2. For the peptide SIL-IS, the QC values of α DA-G2 were marginally acceptable, with higher variability and imprecision for α DA-G1 and the worst results for α K-G2. In addition, the whole molecule IS peak response within the runs was more precise (15.5% CV) than those of the extended SIL-peptide IS (28.1% CV) or the peptide IS (27.7% CV), N = 32. From this data, we can be seen that the whole Ab IS can effectively compensate for any variability during extraction and LC-MS/MS analysis. If the immunocapture and digestion steps were optimized to have high reproducibility, then the synthetic peptide ISs may be adequate for quantification.

In another study, Bronsema et al. evaluated different internal standardization strategies for quantification of a small protein, salmon calcitonin, which could be analyzed both with and without digestion [69]. Salmon calcitonin comprises of 32 amino acid and has a molecular weight of 3431.9 Da. Eight internal standardization approaches were compared with respect to accuracy and precision in work flows with and without digestion. Both analogue IS standard proteins (eel and human calcitonin), SIL-salmon calcitonin, SIL-salmon calcitonin signature peptide [1–11], and the cleavable SIL-salmon calcitonin peptide [1–11] were commercially obtained. ^{18}O -labeled form of the signature peptide was synthesized in-house by isotope exchange with ^{18}O -labeled water.

The samples were extracted using three different workflows. In work flow A, the samples were extracted using only SPE prior to LC-MS/MS analysis. In workflow B, the samples were either extracted using SPE and the extracts were digested with trypsin prior to analysis. In workflow C, the samples were enriched using SPE, digested with trypsin and then derivatized prior to analysis. Derivatization was performed with a solution containing 10% of deuterated or unlabeled formaldehyde and 10% pyridine-borane complex in methanol. Waters Oasis MCX SPE cartridges were used post derivatization the clean-up prior to LC-MS/MS analysis in work

Figure 3. Accuracy and precision of QCs from 3 mAbs obtained with three different SIL-IS's. (a)–(c): Accuracy of whole SIL-IS, flanking SIL-IS, and peptide SIL-IS, respectively. (d)–(f): Precision of whole SIL-IS, flanking SIL-IS, and peptide SIL-IS, respectively. The dashed lines of 20% are the thresholds of acceptance commonly used by LBAs. α DA-G2, α K-G2, and α DA-G1 QCs are represented by blue, red, and green color bars, respectively. Reprinted with permission from [68]. Copyright (2013) American Chemical Society.

flow C. Internal standards were added at different stages depending on the workflow and internal standard characteristics. The results of precision and accuracy studies using a series of internal standardization routes as per workflows A through C, compared to the same workflows without internal standard, are illustrated in **Figure 4**.

Using workflow A (quantification of the intact analyte) without an internal standard had too much variability at 100 pg/mL, resulting in a bias outside the acceptance criterion of $\pm 15\%$. When SIL-salmon calcitonin was used as internal standard, accuracy, and precision improved significantly at both high and low concentrations. This finding was consistent with the expected performance of SIL-protein IS. The first analogue protein IS, i.e., eel calcitonin (90% sequence homology) introduced high variability at the lower level. When human calcitonin (50% sequence homology) was used as an internal standard, the method performance was severely hampered. Both precision and accuracy were inferior to the results obtained without any internal standard. This illustrates that this internal standard did not correct for variability but rather introduced it into the assay.

In workflow B (quantification of the digested analyte), two internal standardization processes were used, i.e., (1) B-before referring to internal standards that are added prior to digestion

Figure 4. Precision (expressed as error bars) and accuracy for 10 different internal standardization approaches according to workflows A–C as compared to the same workflow without internal standard. The results were obtained by 6-fold analysis of the 100 and 1000 pg/mL samples. The dotted lines indicate the acceptable 15% accuracy limits as set by the FDA-guidance. In workflow B, internal standards were either added before or after the digestion step. Reprinted with permission from [69]. Copyright (2013) American Chemical Society.

and are expected to cover the digestion step, and (2) B-after referring to internal standards that are SIL or structural analogue forms of the signature peptide and will only cover the post-digestion part of the analysis (B-after). Eel calcitonin was not used in this workflow as an internal standard as it yields a signature peptide same as salmon calcitonin. Besides human calcitonin (added before and after digestion), all other approaches tested in workflow B, including the omission of an internal standard, generated acceptable results, which shows that in this workflow all steps were well under control. Again, the best results in terms of precision and accuracy were obtained when SIL-salmon calcitonin [1–32], cleavable SIL peptide internal standard and SIL peptide [1–11] were used as internal standards. Since there was no difference between the performance of a SIL-calcitonin, cleavable SIL-peptide and that of a SIL-peptide for workflow B, it was concluded that the digestion step did not negatively impact method performance. This observation possibly can be attributed to the small size of the analyte protein and absence of any tertiary or quaternary structure. The commercially obtained SIL peptide internal standard and the in-house prepared ¹⁸O-labeled form performed comparably in workflow, thus indicating that the ¹⁸O-labeled peptide can be used as an economical alternative to a chemically synthesized SIL peptide.

In workflow C (quantification of digested and derivatized analyte), it was seen that using a differentially labeled internal standard slightly improved assay performance compared to the results without internal standard. Precision and accuracy of this internal standardization

approach were comparable to the SIL peptide and ^{18}O -labeled peptide approaches for workflow B, which do not include a derivatization and second SPE step. Workflow C is more laborious and has multiple steps without any internal standards, and therefore will have a higher risk of experimental variability.

4. Conclusions

Precision and accuracy of bioanalysis is ultimately improved through internal standardization. The selection of an internal standard is often dictated based upon availability, time, and cost. While SIL-proteins are considered the ideal internal standard, their availability often limits their use. SIL-peptides and extended SIL-peptides are readily available, and serve as good alternatives to SIL-proteins. When using SIL-peptide, it is essential that sample preparation steps, i.e., enrichment and enzymatic digestions that are not tracked by the peptide internal standards are optimized to limit their variability. In the absence of SIL-IS standard, an analogue protein or peptide may be used as an internal standard. The use of stable isotope labeled analogue monoclonal antibody as a universal internal standard has enabled rapid development of accurate and precise methods for quantitative bioanalysis of biotherapeutics in non-human species.

Conflict of interest

The views and opinions in this chapter represent those of the authors only.

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