

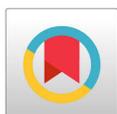
REVIEW

Quantitative Bioanalysis of Intact Large Molecules using Mass Spectrometry

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ABSTRACT

As biologic drugs become an increasing segment in the overall pharmaceutical market, it is important to develop accurate and reliable methods to analyze these drugs in biological matrices. With advancements in technology, biologics' complex molecular structures can now be selectively distinguished and quantified using high-resolution mass spectrometry and deconvolution software. Intact (top-down) mass spectrometric techniques have been established as alternative or complementary bioanalytical techniques for instances when ligand binding assays (LBAs) alone were not well suited, as it can provide additional structural information, in its pharmacologically active form.

KEYWORDS: LC-MS, bioanalysis, intact protein analysis, mass spectrometry, Orbitrap

INTRODUCTION

In the past decade, there has been a shift in the pharmaceutical industry towards the development of biologic based therapeutics such as peptides, fusion proteins/peptides, PEGylated proteins/peptides, monoclonal antibodies, antibody drug conjugates, and many other constructs. As a result, more than half of the top selling drugs in the world are biologics. The rapid growth of biologics has also brought about a significant increase in the molecular complexities. Bioanalytical scientists are challenged to develop accurate and reliable methods to analyze these complex therapeutics in biological matrices (i.e., plasma, urine, tissue, and other body fluids) [1-3], which is critical for assessing drug efficacy and toxicity. This is necessary for appropriate interpretation of drug efficacy and toxicity during pharmacokinetic and immunogenic evaluations during pre-clinical and clinical trials. However, biologics molecular structure complexity can present challenges during bioanalytical method development. Traditionally, ligand binding assays (LBAs) have been employed in the pharmaceutical and clinical industries due to their high sensitivity, ease-of-use, high throughput, low equipment costs, and their general acceptance. However, these techniques rely heavily on expensive customized capture and detection

antibody reagents that typically take several months to generate. In addition, LBAs often result in cross-reactivity and selectivity issues, especially with regards to biologic drugs due to the potential presence of anti-drug antibodies. Lastly, since LBAs often measure the drug at the binding sites, some information, that could change the function of drug, such as glycan attachment, may not be detected by these indirect techniques [4-6].

More recently, mass spectrometric techniques have been established as alternative or complementary bioanalytical techniques for instances when LBAs alone were not well suited. Mass spectrometric methods can provide additional selectivity and reduced interference, generally take less time to develop, and can provide additional information beyond what is possible with LBAs, such as information about isoforms, PTMs, and degradation products. In addition, further data mining can be explored after initial analysis. For smaller proteins and peptides (generally less than 10,000 Da), traditional, 'small molecule' techniques may be employed. However, for larger molecules, additional sample preparation and mass spectrometric analysis steps are required, due to the complexity of the drug structures. These mass spectrometric techniques can further be broken down into several sub-categories: bottom-up, middle-down, and top-down [6-8].

Currently, the most common alternative to LBAs is bottom-up analysis using mass spectrometry. This technique varies, but usually it employs several general steps: non-specific (or specific) immunocapture of the drug, followed by denaturation, reduction, alkylation, and enzymatic digestion of the drug, resulting in a signature (or surrogate) peptide(s) (i.e. unique amino acid sequences, not found in other proteins) that is used as a surrogate for quantitative analysis of the intact drug. The coupling of non-specific or specific immunocapture (clean-up/enrichment) techniques with liquid chromatographic separation and selected reaction monitoring mode (SRM) mass spectrometry (Hybrid LBA-LC-MS/MS), often minimizes interferences, improves selectivity, leading to improved cost / time-efficiencies. In addition, since low resolution instruments can be used for bottom-up analysis (typically triple stage quadrupole mass spectrometers, QqQ), it is possible to produce high throughput methods, without much modification to current lab settings (triple quadrupoles are utilized commonly in both biopharmaceutical and clinical industries). Analyte responses are normalized using internal standards, which are added in the same concentration to each sample. Bottom-up techniques generally use stable isotope labelled peptides as the internal standard (similar to the signature peptide, added at the end of extraction) and proteolytic enzymes, such as trypsin, Glu-C, or Lys-C [7, 9, 10-13]. Trypsin cleaves proteins on the C-terminal side of arginine or lysine (unless proline on the C-terminal side). Lys-C cleaves proteins on the C-terminal side of lysine. Glu-C cleaves proteins on the C-terminal side of glutamic acid or aspartic acid. However, middle-down techniques typically use similar internal standards but digestive enzymes such as IdeS, IdeZ (IgG specific proteases), pepsin, or papain, which partially digest the molecule into large subunits (i.e. Fab and Fc, Fab2, light and heavy chain, etc). IdeS, IdeZ, and pepsin cleave immunoglobulins at the hinge region, producing Fab2 and Fc fragments. Papain cleaves immunoglobulins above the hinge region, producing 2 Fab fragments and a Fc fragment. Depending on the size of the fragment formed, bottom-up or top-down mass spectrometric techniques may be used for analysis. Middle-down techniques can provide additional structural information, not possible using surrogate peptide methods, especially when evaluating multiple fragment types at once [7, 14-16].

While bottom-up and middle-down mass spectrometry practices provide valuable and reliable information, there has been increasing interest in analyzing fully intact biopharmaceutical drugs using mass spectrometry, designated top-down mass spectrometry. Historically, top-down mass spectrometry methods were thought to only be useful for qualitative characterization or relative quantification in proteomics [17, 18]. While this provides very valuable analytical information about biopharmaceutical drugs, recent technological advances have made it possible to use these techniques for absolute quantitative bioanalysis. In order to evaluate these complex molecules at the intact level, high-resolu-

tion instrumentation, such as Orbitrap (linear-Orbitrap, Q-Orbitrap), Time-of-Flight (QTOF, TOF-TOF), or Fourier Transform ion cyclotron resonance (FTICR) mass spectrometers, are necessary [19, 20]. However, the sensitivity obtained using bottom-up techniques has not yet been replicated for top-down methods. If high sensitivity is not necessary, quantitative analysis of intact biologic drugs (top-down) can provide quantitative information while preserving structural information (such as post-translational modifications and isoforms), allowing better differentiation between closely related molecules. For certain methods, removal of the digestion step (when deglycosylation is not necessary) or the denaturation steps (native conditions), will also allow for easier and shorter sample preparation. However, obtaining appropriate internal standards can present challenges with top-down techniques. Best practices involve fully intact stable isotope labelled (SIL) internal standard biomolecules, but this is often difficult to make, expensive, and time-consuming. Non-specific SIL molecules, such as SILu™Mab for monoclonal IgG antibodies, are available for purchase and can be valuable for normalization in generic methods in animal matrices. Although not as good at tracking compared to SIL IS's, analog internal standards may be used in situations where SIL IS's are unavailable or cost prohibitive. While the data processing for intact analysis is more complicated than SRM, as it involves deconvolution to the zero charge state, most companies manufacturing high-resolution instrumentation have developed commercially available software capable of deconvoluting the very complex, but valuable, data [20, 21]. **Figure 1** shows a schematic overview

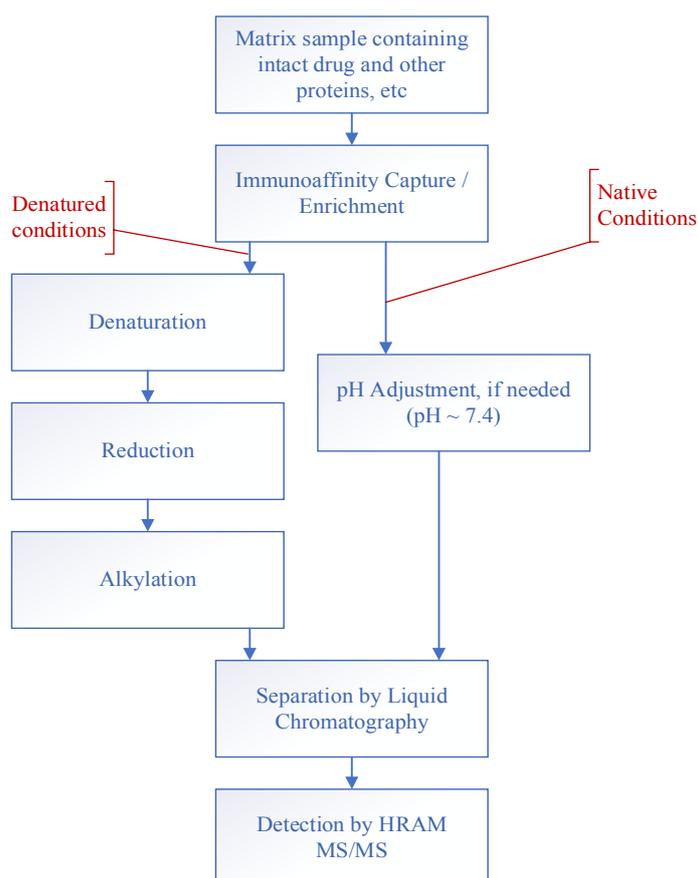


Figure 1. Schematic overview of the sample preparation for top-down intact analysis of biologics under denatured and native conditions.

of the sample preparation and analytical techniques for top-down intact biopharmaceutical drug quantitation. As shown in **Table 1**, several research groups have developed top-down methods for quantitative bioanalysis of biologics, mostly peptides and proteins, both in pharmacokinetic and biomarker applications. Intact bioanalysis has been evaluated in both animal and human serum / plasma and as well as human tissues. Molecular weights of these compounds range from 4.4 to 150 kDa; with lower limit of quantifications (LLOQ) ranging from 0.001 to 25 µg/mL [4, 7, 11, 19-21, 23, 27, 41, 42, 47].

Sample preparation

Unlike bottom-up or middle-down based techniques, which require digestive enzymes, top-down methods can be performed without the need for digestive enzymes. Based on review of the literature on quantitative intact biopharmaceutical analysis, samples are typically prepared for two main types of analysis: denatured or native conditions. Under denatured conditions, the molecule is unfolded, removing tertiary and secondary bonds, so that its entire primary structure is available for analysis. When the molecule is unfolded, this exposes more sites for protonation, allowing for more, as well as higher charge states, during mass spectrometric analysis. This can be helpful, especially when analysis is done on an instrument which has a specific mass-to-charge ratio (m/z) range. An example of this is hybrid quadrupole-Orbitrap instruments, which are commonly used for intact analysis. However, if higher charge states are not necessary for analysis (within instrument m/z range), native conditions can be useful in providing additional information regarding the pharmacologically active form of the drug. This can allow for both higher confidence with regards to the quantitative technique, since the active form of the drug is being evaluated (direct analysis), as well as provide valuable information about binding interactions and structural confirmations [22].

Under denaturing conditions, samples are prepared by first undergoing an immunocapture step. Capture antibodies (or other capture reagents) are added to aliquots of subjects' samples, in biological matrix, where the analyte of interest (drug or biomarker) will selectively bind to it. In order to separate the capture reagent-analyte complex from the rest of the matrix components, the capture reagent is usually bound either with magnetic beads (such as streptavidin beads bound to biotinylated capture antibody-analyte complex) or to a microwell coated plate (such as a 96-well polypropylene plate incubated overnight with streptavidin to form a streptavidin coated-plate; which can then bind to (and immobilize) biotinylated capture antibody-analyte complex). The bound complex is then washed to remove other matrix components from the sample. Binding of the capture reagent, analyte, and beads or coated-plate can occur simultaneously, or separately in various combinations. The order in which the incubations will occur is dependent on the molecules involved and should be evaluated during method development [23, 24]. Depending on sample matrix complexity, the capture step may not be necessary; however, most biological matrices would require a capture step to clean up the final extract. The capture reagent may specifically bind to the drug (such as an anti-idiotypic antibody), or it may be more universal (non-specific); for example, for monoclonal antibody (mAb) based drugs, Protein A, Protein G, or anti-human Fc (in animal matrices) can be used. After the beads or plate has been appropriately washed, samples are subjected to denaturing conditions [21, 22]. Denaturation can occur in a variety of ways. One technique involves exposing the sample to alkaline or acidic conditions, mild enough to be amenable to mass spectrometric analysis, such as 1% formic acid. Other techniques involve using high temperatures and or surfactants to denature the molecules. Using this technique, reducing and alkylating the molecule after denaturation is required, to ensure it does not refold. Reduction involves addition of a reducing agent, such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP), to break disulfide bonds, and alkylating agents, such as iodoacetamide (IAM) or iodoacetic acid (IAA), are added to prevent disulfide bonds from reforming [9]. Alkylating agents will change the molecular weight of the mol-

Table 1. Summary of Current Literature on Quantitative Intact Large Molecule Bioanalysis by LC-MS/MS.

Analyte	MW (kDa)	LLOQ ($\mu\text{g/mL}$)	Matrix	Sample Preparation	LC	MS Instrument	Data Analysis	Ref.
SigmaMab (PK)	146	0.2	Mouse Plasma / Serum	IA; formic acid	2D: SEC (PolyHydroxyEthyl A) and RP (PLRP-S)	ESI/QTOF	Deconvolution	[25]
SigmaMab (PK)	146	5	Mouse Plasma / Serum	Overnight IA; pH adjustment using NH_4OH	2D: SEC (PolyHydroxyEthyl A) and IEC (PolyLC)	ESI/QTOF	XIC	[25]
Unspecified mAb subunits (PK)	10-25	0.01, 0.1, or 25	Human and Rat Plasma	IA, DTI, IdeS digestion	RP (C4)	ESI/QTOF	Deconvolution and XIC	[7, 19]
SigmaMab (PK)	146	0.05	Cynomolgus Monkey Plasma	IA	RP (C4)	ESI/TOF	Deconvolution and XIC	[21]
Human Lysozyme (BM)	15	0.5	Monkey Plasma	SPE	RP (C4)	ESI/LTQ-Orbitrap	XIC	[27]
hIgG1A (PK)	149	0.1	Rat Serum	IA, PNGase digestion	RP (monolithic ProSwift RP-4H)	ESI/Q-Orbitrap	Deconvolution and XIC	[4]
Apolipoprotein A1 (BM)	28.3	4 pmol/ μL	Rabbit Plasma	IA	RP (monolithic ProSwift RP)	ESI/QTOF	XIC	[13]
Oxyntomodulin (PK)	4.4	0.0009375 or 0.001875	Rat or Human Plasma	IA	RP (C18)	ESI/Q-Orbitrap	XIC	[42]
SigmaMab (PK)	146	1	Mouse Plasma	IA	RP (C4)	ESI/QTOF	XIC and deconvolution	[11]

Table 1. Cont'd

Analyte	MW (kDa)	LLOQ ($\mu\text{g/mL}$)	Matrix	Sample Preparation	LC	MS Instrument	Data Analysis	Ref.
Alpha-Synuclein (BM)	7.3-14.5	0.0015	Human Tissues	IA x2 (per lysate)	RP (C4)	ESI / LTQ-Orbitrap	XIC	[41]
Trastuzumab emtansine (PK)	150	0.5	Rat Plasma	IA; pH adjustment using NH_4HCO_3	RP (BEH C4)	ESI / QTOF	XIC and deconvolution	[23]
Chicken Lysozyme (PK)	14	0.1	Human Plasma	PPT	RP (BEH C18)	ESI / QTOF	XIC	[20]
Insulin (BM)	5.8	0.01	Rat Plasma	PPT	RP (PLRP-S)	ESI / Q-Orbitrap	XIC	[47]

PK = pharmacokinetic, BM = biomarker, SPE = solid phase extraction, IA = immunoaffinity capture, PPT = protein precipitation, DTT = dithiothreitol, SEC = size exclusion chromatography, IEC = ion exchange chromatography, RP = reverse phase chromatography, ESI = electrospray ionization, QTOF = hybrid quadrupole – time-of-flight mass analyzer, LTQ-Orbitrap = hybrid linear trap quadrupole – Orbitrap mass analyzer, Q-Orbitrap = hybrid quadrupole – Orbitrap mass analyzer, XIC = extracted ion chromatogram

ecule, which must be taken into account. The final extract should be of similar composition to the starting mobile phase conditions [7, 25]. Additional clean up, such as solid phase extraction or molecular weight cut-off filtration, may be included in the procedure, if necessary. Solid phase extraction can most commonly be used in sample clean-up of smaller proteins (10-15 kDa), as it will not only separate compounds based on surface chemistry, but also by size exclusion. In particularly dirty samples, such as blood or plasma/serum, depletion of highly abundant proteins can be employed to improve selectivity [8, 21, 26, 27].

Under native conditions, samples are subjected to similar capture step described in the denatured conditions section above. After that, additional clean up steps, such as solid phase extraction, molecular weight cut-off filtration, dry down/reconstitution, or dilution, may be performed as needed. For native intact analysis, final extracts should be in near neutral solutions with similar pH to physiological conditions (pH 7.4). This can be obtained by reconstitution or dilutions with various buffers, such as ammonium acetate, ammonium bicarbonate, or triethylammonium bicarbonate [28, 29]. A buffer exchange step will likely be necessary in order to remove salts, which are not compatible with mass spectrometric analysis [21, 25]. Water has also been found to appropriately work as a diluent for the final extract for certain intact analysis [30].

In addition, for either native or denatured condition, if glycan analysis is not part of the evaluation, a deglycosylation step, using enzymatic digestion (i.e. PNGase F (glycopeptidase) or EndoS), should be considered. PNGase F and EndoS cleave N-linked oligosaccharides from proteins [10]. This will clean up the final mass spectra and make interpretation of data much simpler [22].

Instrumentation

Once samples have been extracted, analysis is conducted using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Traditional instrumentation used for quantitative bioanalysis in industry, such as reverse phase liquid chromatography and SRM (QqQ) mass spectrometry, while considered highly selective, and therefore well suited for bottom-up techniques, is not generally effective with regards to intact quantification. Different instrumental conditions are required depending on the type of evaluation, native or denatured. In addition, if a deglycosylation step is not performed, shorter hydrocarbon chain reverse phase liquid chromatography columns, such as a C4 column, are typically used, since there is typically an increased hydrophobicity of proteins [8, 14]. Intact (top-down) analysis often requires high-resolution mass spectrometry instrumentation, using full-scan HRMS quantitated using extracted ion chromatograms (XIC), summed XICs, or deconvolution HRMS. Further discussion on instrumentation needed for top-down quantitative analysis will be addressed in the chromatography, ionization techniques, and mass analyzers subsections below.

Chromatography

Traditionally, ion exchange chromatography (IEC) was employed for intact quantitative analysis under native conditions. This was done utilizing a pH gradient (as opposed to changes in salt concentration), to be more compatible with the mass spectrometer. Mobile phases, such as ammonium acetate at different molarities, are run through a weak cation exchange column (such as PolyCAT A, used more for proteins) or mobile phases, such as diethanolamine buffer, run through a weak anion exchange column (such as a monolithic IEC, more often used for oligonucleotides or characterization of post-translational modifications (PTMs) on proteins) [14, 31]. More recently, due to the complexity of large molecule chromatograms, and in order to better separate the different charge states, isotopes, and PTMs, 2D-chromatography has increased in popularity. Most commonly, size exclusion chromatography (SEC) has been coupled with ion exchange chromatography, prior to detection using high-resolution MS or MS/MS. SEC will first sepa-

rate the molecule based on size, using columns such as PolyHydroxyEthyl A or XBridge Protein BEH SEC, followed by IEC separation based on pH [13, 22, 32]. Some research groups have also found success in coupling size exclusion chromatography with reverse phase chromatography, using more traditional mobile phases, such as 0.1% formic acid in water and 0.1% formic acid in acetonitrile, as well as traditional stationary phases, such as C18 columns [33]. For denatured conditions, groups have found some success with 1D reverse phase chromatography using traditional mobile and stationary phases [34, 35], sometimes requiring higher column temperatures [36, 37]. However, this is dependent on the complexity of the molecule, and the selectivity needed for the method being developed. If high selectivity is necessary, 2D chromatography has been shown to provide improved separation. For denatured samples, size exclusion chromatography is often coupled with reverse phase chromatography, in a similar manner to native conditions described above. Some research groups have also found success coupling reverse phase chromatography with HILIC (two-dimensional), to improve overall sensitivity [8]. For both native and denatured conditions, flow rates are typically in the micro or nano range (~5-500 $\mu\text{L}/\text{min}$). Traditional column particle sizes and dimensions can be obtained for this analysis, however, increased pore sizes (200-1000 \AA) are commonly necessary to attain appropriate separation [22]. Pore sizes as high as 4000 \AA may be necessary for large highly complex molecules, such as antibody-drug conjugates [8]. Increased column temperatures (50°C or higher) showed improved separation for large molecule bioanalysis [38]. Peak shape may be improved by specific mobile phase additives, such as trifluoroacetic acid (TFA). While TFA has been shown to cause ion suppression in electrospray ionization based techniques, reduced suppression has been seen in situations where acetic acid (0.5%), formic acid (0.2-0.3%), or propionic acid (1%) were added along with TFA [8].

Ionization techniques

As described in the introduction, some research groups are attempting to quantify large molecules intact using low resolution instruments, such as triple quadrupole mass spectrometers. However, it is difficult to obtain accurate results without a high-resolution mass spectrometer. This is because of the complexity of the spectra for large molecules, due to the high number of charge states, each of which contains various isotopic peaks. In addition, for proteins, molecular heterogeneity and different PTMs will affect the spectra and create multiple peaks for the same molecule. Due to the advancement in technology, these factors can be selectively distinguished using a high-resolution mass analyzer. In order to quantify large molecules, their m/z must fall within the instrument's m/z range. This means that higher precursor ion charge states (between approximately +5 and +73, with highest charge states being more common with denatured conditions) are necessary in order to fall within most instrument's m/z range (< 6000 m/z) [39, 40]. This requires an ionization source that can create many charges on one molecule (ion). In most cases, an electrospray ionization source (ESI) is required for quantitative work. For proteins, this will typically be done in positive ESI mode [22, 41, 42]. Occasionally, matrix assisted laser desorption ionization (MALDI) has been discussed as an ionization source option for intact quantitative analysis. And although MALDI can reach high m/z ranges, quantitation using this technique is not general practical; and therefore, this ionization source has rarely been applied in actual method development.

Data processing

While SRM (QqQ) is highly selective due to multiple levels of distinguishing the analyte, and therefore is most commonly used for quantitative analysis in industry, it has not been as successful for the analysis of intact large proteins or other large molecule analysis. This may be due to both the charge state distribution and the difficulty of efficiently fragmenting large molecules (ions) in the collision induced dissociation quadrupole (CID).

Often times for large molecules, low collision energy will not result in effective fragmentation, while high collision energy will result in extensive fragmentation, both of which negatively impact sensitivity. An alternative option that can be used on triple quadrupole instruments, is to use pseudo-SRM mode, in which the precursor and product ion are the same. This is more useful in large molecule quantitative bioanalysis, if CID is problematic. However, this technique is not as selective as true SRM mode, and therefore will often not produce reliable results, especially if the method requires a low LLOQ, or if selectivity is an issue, which is common in complex matrices [39, 43].

For these reasons, many researchers have moved away from traditional, lower-resolution instrumentation, in favor of high-resolution instrumentation, such as Q-orbitraps and QTOFs, as discussed previously. When employing high-resolution instrumentation, there are several main modes of analysis currently, full-scan HRMS using extracted ion chromatograms (XIC), summed XICs, or deconvolution HRMS. In full-scan HRMS, the entire mass (m/z) range is scanned in both mass analyzers (quadrupole and orbitrap or TOF), or only the precursor ion is used for analysis. The power of the technique is derived from the selectivity gains obtained from the high-resolution capabilities of the instruments (high resolving power and accurate mass). Since this type of instrumentation can distinguish between very similar molecules (ions), closely related peaks can typically be resolved based on m/z , and the analyte peak can be quantified using area under the curve from the extracted ion chromatogram (XIC). In some instances, it has been shown that summing multiple charge states may be necessary to obtain the sensitivity required for a particular assay [11, 20-22, 39, 43, 48]. In some instances, this has been shown to have no effect. Optimization is required in order to determine the best mode of analysis for each method. Another technique for analysis is called deconvolution. In this mode, software can be used to deconvolute the entire mass spectrum (all charge states and isotopes). The results of this deconvolution are a new spectrum that compares intensity to mass (not m/z), which is obtained from all the charge states available in the original spectra. This can then be evaluated in a similar manner to the full-scan XIC mode. While some studies have shown this mode of analysis is effective, others question whether it can be used to confidently quantify biopharmaceutical products in biological fluids, or if it should be used only for the qualitative characterization of these molecules [20-22, 39, 43, 49, 50].

CONCLUSIONS AND FUTURE PERSPECTIVES

Although traditionally, intact large molecule analysis using mass spectrometry was limited to qualitative characterization (often only in solution, and not biological matrices), in the past decade, advancements in technology have allowed for accurate quantitative bioanalysis of large molecules, at moderate concentrations. Using high-resolution mass spectrometry, structural information can be maintained, while selectively quantifying drug levels in complex biological matrices. As technology continues to advance, intact large molecule quantitative bioanalysis will become a more practical application, which could lend itself to high throughput analysis, in industry. This will not be possible until advancements allow for lower limits of quantification, and selectivity improvements. Advancements in both hardware (such as improved collision cells and wider m/z ranges) and software (such as more advanced deconvolution software) may lead to the improvements needed for practical intact quantitative analysis. Analytical challenges related to biologics, such as instability due to oxidation or protease activity, adsorption, and non-specific binding, will also need to be addressed (case by case). Until then, intact bioanalysis of biopharmaceutical drugs can be employed as a complementary technique to traditional methods, as it is still able to provide additional structural information at moderate concentrations, not obtainable any other way.

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