The use of hybrid LC-MS towards quantitation of soluble protein biomarkers

Quantitation of soluble biomarkers provides information on therapeutic efficacy for modalities targeting gene expression. The ability to distinguish small changes to confirm therapeutic efficacy is critical to supporting the need for a new therapy within the market. Routinely, protein biomarkers are quantitated using ligand binding assays due to detection limits that can be achieved with these assays, but is this always the best approach analytically? Researchers should always evaluate what the need is for their assay on a case-by-case basis. What is the limit of quantitation needed to achieve therapeutic relevance? What reagents would be required to achieve those limits of quantitation? Is the detector selective enough to distinguish the changes that are needed to show therapeutic efficacy?

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Analytically, the ability to distinguish small changes in a quantifiable manner is a matter of appropriate sensitivity and selectivity. Fundamentally, sensitivity is defined as the response of your analyte as a function of concentration while selectivity is the ability of your detector to distinguish between a molecule of interest and an interferent.

Historically, liquid chromatography coupled with mass spectrometry (LC-MS) had been an approach not widely utilised for protein quantitation due to structural interactions of proteins or protein complexes, and the need to use harsh organic solvents for ionisation generally resulted in poor sensitivity[1]. Therefore, quantitation of protein biomarkers have long been achieved using ELISA coupled with various spectrophotometric based platforms for detection.

Fluorescence detection is routinely used for ELISA applications in which fluorescent probes are conjugated to molecules of interest directly followed by the addition of a substrate which produces a measurable signal. There are more formats that can be adapted based on experimental design, but to the point, fluorescence offers a sensitive and, in most cases, selective means to detect biological molecules of interest with lower limits of quantitation (sub-ng/mL to pg/mL). Added gains with fluorescence detection have been made with improvements in spectroscopy techniques[2,3]. Specifically, single molecule counting provides sub-pg/mL to fg/mL detection limits by coupling bead-based capture with fluorescently labeled capture antibodies for detection[4]. Perhaps, from an analytical perspective, the largest gain in response is from the use of a confocal laser used on certain single molecule platforms. The narrow beam provides high intensity or a focused beam of photons on a single fluorophore at a time compared to more conventional plate readers. Although advantageous, the selectivity of the response is dependent on the specificity of the critical reagents for the assay and can make changes in protein concentrations at the lower limits of quantitation difficult to distinguish from noise.

Selectivity with respect to fluorometric based applications or spectrophotometric detection with application towards quantitation of biomarkers is directly related to the quality of critical reagents for the assay and variety of commercially available detection probes. Mass spectrometry, specifically high resolution mass spectrometry (HRMS), can distinguish down to 0.001 amu difference between molecules, depending on system specifications, making it highly selective and it's application is not limited to critical reagents available, but instead the mechanism by which ions are separated within the flight path.

Even with the added selectivity of mass spectrometry and unit resolution that can be obtained using HRMS, detection limits are still poor compared to single molecule counting. Recently, Krainer et al. coupled microfluidic separations with fluorescence probes for direct digital sensing[5]. Integration of microfluidic allows for separation of unconjugated or conjugated to probes in solution without the need to bind to a plate. The authors do not elaborate on the ability to multiplex, but conceivably based on chip design multiplexing could be feasible. Perhaps more importantly and as Krainer et al point out is the orthogonal approach to couple a sensitive and selective mode of detection with addition of separation of molecules based on size prior to detection.

Orthogonal means of separation are routinely used in small molecule analysis to reduce or eliminate interferences from matrices. Microfluidic devices can utilise electrophoresis or use a combination of voltage to gate molecules and then maintain separation of molecules using hydrodynamic pressure. Microfluidic devices are however difficult to reproducibly manufacture for environments that require high throughput. But the principles that drive electrophoretic separations are important in the context of how variability in quantitation of protein biomarkers might be improved.

Efficiency or the number of theoretical plates is a theoretical relationship used to describe the mobility of a molecule between the stationary phase and mobile phase. The van Deemter equation describes how efficiency is impacted by Eddy-diffusion (A), longitudinal diffusion (B) and resistance to mass transfer (C), as shown below:

$$H = A + \frac{B}{u} + (Cs + Cm)\, x\, u$$

By reducing the particle size the terms associated with mass transfer and diffusion are decreased, decreasing plate height. This has long been understood as a benefit in capillary electrophoresis where fused silica capillaries are used to separate molecules based on charge to size; in the most simplistic separations silica is protonated or deprotonated based on the charge of the molecule and molecules are then moved through the capillary based on their electrophoretic mobility. In this instance there is no partitioning between the molecule and the stationary phase, limiting diffusion thereby decreasing plate height and decreasing peak width. The use of capillary columns in liquid chromatography (nano liquid chromatography) enable smaller particle sizes to be used with increased column lengths, providing similar theoretical plates as capillary electrophoresis, but offering a more simplistic separation for molecules that are not charged. In addition, the limited solvent volumes required for these separations enable production of smaller droplets. With the reduced droplet size less energy is needed for desolvation making the transition to gas phase easier and thereby improving sensitivity as well as reducing interference from solvents[5]. Additionally, by utilizing nano liquid chromatography samples can be diluted significantly post extraction and response from the protein can be maintained due to high efficiency of the separation.

With the above knowledge the question is, does a hybrid approach need to be used to quantitate protein biomarkers? If using nano-LC-MS/MS or nano-LC with HRMS a hybrid approach might not be necessary. The quality of critical reagents available for the assay may limit the selectivity and in turn the sensitivity of the assay, the same argument could be made for spectrophotometric applications. Protein quantitation using mass spectrometry has the advantage of the added resolution capable of distinguishing between molecules that differ by only a single amino acid. The protein sequence from the biomarker of interest is screened against the human genome to identify peptide sequences that are unique only to the protein of interest. Then, utilising enzymatic digest coupled with nano-LC-MS/MS likely could be capable of achieving sub-picomolar detection limits.

Although critical reagents are necessary for ligand binding assays with mass spectrometry they are only necessary if there is a need to elucidate mechanistic information (e.g. quantitation of protein conjugated to a pay load or to determine the extent of mechanistic action of a therapeutic modality). Beyond elucidation of mechanistic information critical reagents are used more to clean up the sample for improve detection limits.

It is advisable that LC-MS/MS be used as an initial tool for quantitation of protein biomarkers. Significant time in the development of critical reagents can be avoided and exploratory work can be performed to better identify targets for therapeutic efficacy. In addition, the need for decreased detection limits can be assessed as well at minimal cost. The desire and the need for detection limit should be viewed objectively when picking a selecting a platform along with the need for more complex sample processing.

References

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