TECHNICAL BRIEF

Immunoassays and Mass Spectrometry: Transitioning Technology

This technical brief presents data obtained when measuring eicosanoids by immunoassay and mass spectrometry. It briefly considers the limitations of each technology and describes the potential benefits of combining these two technologies, using antibodies to improve the sensitivity of mass spectrometry-based assays.

Immunoassays and Mass Spectrometry: Transitioning Technology

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BACKGROUND

Immunoassays, such as enzyme-linked immunosorbent assays (ELISAs), rely on the specificity of the antibody-antigen interaction for the detection and quantification of biomolecules of interest. It has recently come into the spotlight that poorly characterized antibodies have given rise to erroneous results in the research community (and in some cases, clinical labs). It is also known that structurally related molecules or interferents can alter binding of the analyte of interest with well characterized antibodies causing misleadingly high or low (incorrect) results. This has created a challenge for researchers quantifying small molecules and biomolecules such as eicosanoids, proteins, and peptides in biological samples.

Researchers and clinicians alike are now turning to mass spectrometry (MS) as an alternative analytical technique because of its potential advantages over ELISA-based methods. MS offers an inherent specificity advantage as well as multiplexing capabilities, albeit with significant start-up costs. The value of rapidly quantifying multiple targets simultaneously with accuracy and certainty makes MS an increasingly popular method of choice.

Regardless of the assay that is chosen, it is also important to understand the limitations of the methods being used within the context of the experiment or study. It is important that the assay is tested and validated in the relevant matrices to avoid irreproducibility and inaccuracy.

ELISA

ELISAs are plate-based immunoassays used to detect and quantify analytes of interest (proteins, peptides, hormones, *etc.*) in biological matrices. This technology combines the specific interaction between an antigen and an immobilized antibody with detection using reporter enzymes to quantify molecules of interest. There are two types of ELISAs, competitive and sandwich.

Competitive ELISAs are most commonly used to measure small molecules (*i.e.*, lipids, hormones, and small peptides). They are based on the competition between the analyte of interest and an enzyme-conjugated version of the same analyte (the tracer) for a limited number of specific antibody binding sites. The amount of tracer that binds to the antibody is inversely proportional to the concentration of the analyte in the sample (Figure 1).

Sandwich ELISAs are suitable for proteins and peptides greater than 20 amino acids in length. This assay format involves two antibodies that "sandwich" the analyte of interest between them. A specific capture antibody coats the assay plate (immobilized), the analyte of interest binds to the capture antibody, and a detection antibody is added to the well. The amount of analyte present is directly proportional to the amount of detection antibody, which is typically quantified by its enzyme conjugate (Figure 1).

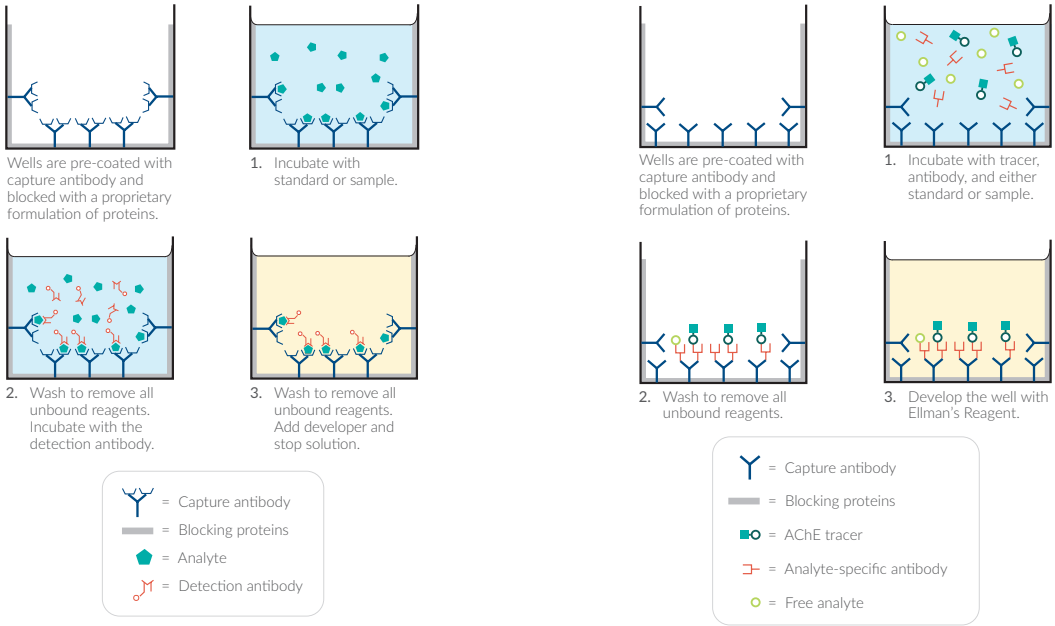

Figure 1 – ELISAs

Quantification in ELISA is obtained by comparing the amount of detection antibody that is bound (measured in optical density) to unknown samples with the amount of detection antibody that is bound to known quantities of the analyte that has been used to prepare a standard curve (Figure 2). This is most often the analyte of interest being detected and measured in the assay.

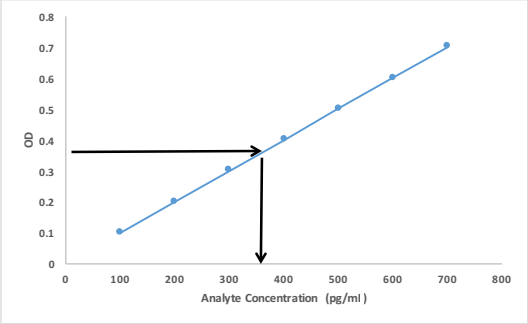

Figure 2 – Example ELISA Standard Curve

MASS SPECTROMETRY

Mass spectrometry is an analytical technique that enables the identification and quantification of known and unknown compounds and biomolecules by revealing their structural and chemical properties. MS measures the mass-to-charge ratio (m/z) and abundance of gas-phase ions. Most MS is now performed with a chromatographic separation of the sample prior to the mass analyzer and utilizes tandem MS (MS/MS). In liquid chromatography-tandem mass spectrometry (LC-MS/MS), precursor ions that are analyzed in the first mass analyzer (MS1) are fragmented by one of a number of methods of dissociation (or fragmentation) in a collision cell. All resultant product ions (or transitions) are then analyzed in a second mass analyzer (MS2) and collected in the detector. LC-MS/MS is most commonly used for identity confirmation and quantification (Figure 3).

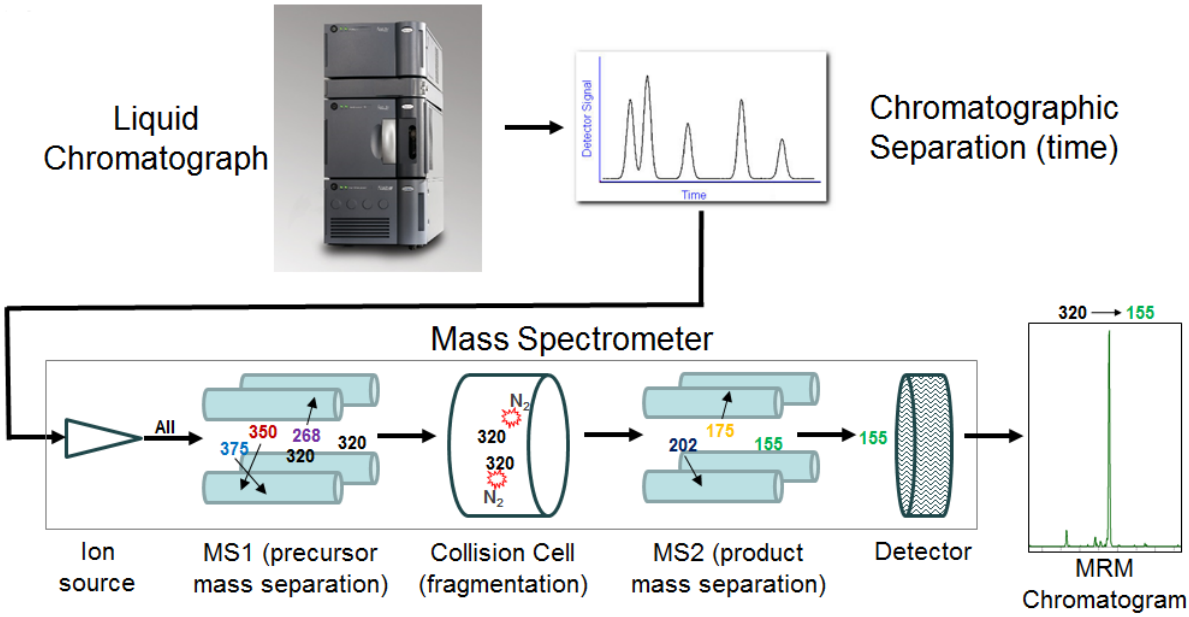

Figure 3 – LC-MS/MS Schematic

Targeted MS/MS, also known as selected reaction monitoring (SRM), is the mode of MS that is most equivalent to ELISA in that it is used for the detection and quantification of known analytes in biological samples. SRM is a targeted assay with high sensitivity and absolute specificity that allows one to detect and quantify analytes (small molecules, lipids, proteins, peptides, and other biomolecules) of interest. When this assay is multiplexed (used for multiple analytes in a single analysis), it is known as multiple reaction monitoring (MRM).

MRM assays have high multiplexing capability. For example, an MRM assay can be set to monitor as many as 50 to 100 (or more with sophisticated methods) analytes in a single assay. These assays can provide relative or absolute measurement of the selected analytes. Relative measurements are accomplished by comparing the same precursor and transitions between samples. Absolute measurements can be obtained with external calibrators and internal standards (ISs). An external calibration curve of known concentrations is generated for each analyte in buffer or relevant matrix matching the samples in the particular study. Stable isotopically-labeled IS are added directly to the sample in known quantities. The labeled (synthetic) and unlabeled (native) compounds have the same physicochemical properties but are isotopically distinct, differing by a known mass, and therefore can be easily distinguished in the mass spectrometer. IS calibration is typically required for accurate quantitation in MS due to processing variability between samples and matrix derived interferences that affect ionization. The IS elutes at the same retention time as the endogenous form of the analyte and its ionization in matrix is identical. The resulting ratio of analyte to IS in each sample allows for accurate quantification.

EICOSANOID MEASUREMENTS BY ELISA AND LC-MS/MS

To compare the capabilities of ELISA and LC-MS/MS, measurements of seven primary eicosanoids in relevant biological matrices were performed. Prostaglandin E_2 (PGE_2), leukotriene C_4 (LTC_4), 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), thromboxane B_2 (TXB_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), leukotriene B_4 (LTB_4), and prostaglandin D_2 (PGD_2) were measured in conditioned media, mouse liver, and lung employing ELISA and LC-MS/MS methods.

METHODS

Conditioned media samples were prepared according to Hong, J. *et al.* Carcinogenesis **25(9)**:1671-1679. Briefly, RAW 264.7 (mouse leukemic monocyte macrophage cell line) cells were plated at a concentration of 1.6×10^6 cells/well on 6-well plates in DMEM containing 10% FBS plus penicillin and streptomycin. After 24 hours, the growth medium was replaced with serum-free DMEM. Cells were incubated overnight and the media was replaced with fresh serum-free DMEM containing 2 µg/ml lipopolysaccharide (LPS) or vehicle. The conditioned media was collected after an 18 hour incubation and centrifuged at 10,000 x g for 10 minutes. The media was transferred to a clean tube, snap frozen, and stored at -80°C until used.

Tissue homogenates were prepared as follows: 100 mg mouse lung or liver was homogenized in a Precellys® Evolution tissue homogenizer in 1 ml 0.1 M potassium phosphate, 10 mM EDTA, to give 10 µM indomethacin, pH 7.0. Each tissue homogenate was added to four volumes of ice-cold acetone and kept at -20°C for at least 20 minutes. The samples were centrifuged to pellet the debris [and proteins]. The clarified supernatants were transferred to clean tubes. Approximately 20% of each sample was reserved for LC-MS/MS analysis and the remainder was dried under a stream of nitrogen for use in the ELISAs.

The ELISA kits used in this study were from Cayman Chemical: Leukotriene C_4 ELISA Kit (Item No. 501070), a Leukotriene B_4 ELISA Kit, Prostaglandin D_2 ELISA Kit (Item No. 512031), Prostaglandin E_2 ELISA Kit – Monoclonal (Item No. 514010), Prostaglandin $F_{2\alpha}$ ELISA Kit (Item No. 516011), 6-keto Prostaglandin $F_{1\alpha}$ ELISA Kit (Item No. 515211), and Thromboxane B_2 ELISA Kit (Item No. 501020).

The conditioned media samples were thawed on ice. Each sample was assayed in triplicate wells at three dilutions (diluted in DMEM). The standards were prepared with DMEM and run to generate standard curves according to each specific ELISA kit instructions for use (IFU).

The dried clarified tissue supernatants were resuspended in 0.8 ml ELISA Buffer (supplied with the kits). Each sample was assayed in triplicate wells at three dilutions (diluted in ELISA Buffer). The standards were prepared with ELISA buffer and run to generate standard curves according to each specific ELISA kit IFU.

Each ELISA assay was run according to its IFU and followed the general protocol shown in Figure 4 and plate layout shown in Figure 5.

Step	Reagent	Blank	Total Activity	Non-Specific Binding	B_0	Standard/Sample
Add Reagents	ELISA Buffer	--	--	100 µl	50 µl	--
	Standard/Sample	--	--	--	--	50 µl
	Tracer	--	--	50 µl	50 µl	50 µl
	Antibody	--	--	--	50 µl	50 µl
Incubate	Cover plate and incubate overnight or two hours – assay specific					
Wash	Rinse all wells 5 times with Wash Buffer					
Add Reagents	Tracer	--	5 µl	--	--	--
	Ellman's Reagent	200 µl	200 µl	200 µl	200 µl	200 µl
Incubate	Cover plate and incubate 60-90 minutes at room temperature with gentle shaking					
Read	Wavelength between 405-420 nm					

Figure 4 – General ELISA Protocol

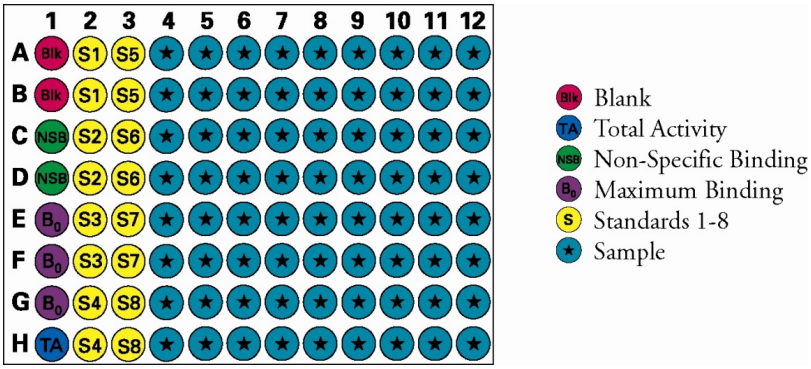

Figure 5 – Plate Map for ELISAs

The LC-MS/MS methods employed in this study were developed and optimized at Cayman Chemical and analyzed on a Waters ACQUITY UPLC® and Waters Xevo TQD triple quadrupole mass spectrometer. The conditioned media samples were thawed on ice. The vehicle treated samples were prepared by mixing 100 µl of each sample, 200 µl H_2O, and 100 µl blank methanol. The LPS-treated sample had to be diluted 100-fold (10 µl LPS-treated sample, 990 µl methanol) in order to bring the levels of PGD_2 into the working range of the assay (empirically determined). The LPS-treated samples were prepared by mixing 100 µl methanolic diluted LPS-treated sample, 200 µl H_2O, and100 µl DMEM. Calibrators were prepared by mixing 100 µl methanolic calibrators (8 calibrators spanning 0.1-50 ng/ml), 200 µl H_2O, and 100 µl DMEM. 25 µl of IS solution (25 ng/ml) was added to each sample, calibrator, and blank and mixed well.

Prior to injection, the samples were subjected to solid-phase extraction (SPE) on Waters MAX µElution plates. The plates were first conditioned with 100% ACN followed by 25% ACN in H_2O. The samples were loaded onto plates, and the plates were washed with 25% ACN in H_2O and washed again with 100% ACN. Trapping solution (proprietary formulation) was added to the collection plate, and the samples were eluted with 25 µl of 50:50 ACN:IPA and 5% formic acid onto the collection plate. The plate layout is shown in Figure 6.

Figure 6 – Plate Map for LC-MS/MS Assays

RESULTS

The results for the conditioned media are shown in Table 1. As expected, LPS treatment of the RAW 264.7 cells resulted in PG production and release into the media as observed by both assay methods. LTB_4 was not detected in any of the samples by either method and TXB_2 was only detected in the LPS-treated samples.

ELISAs

Sample	LTC_4 (pg/ml)	LTB_4 (pg/ml)	PGD_2 (pg/ml)	PGE_2 (pg/ml)	$PGF_{2\alpha}$ (pg/ml)	6-keto $PGF_{1\alpha}$ (pg/ml)	TXB_2 (pg/ml)
Vehicle 1	24.4	Below LOQ	288.2	65.9	81.5	45.8	Below LOQ
Vehicle 2	52.0	Below LOQ	170.2	44.5	43.2	Below LLOQ	Below LOQ
Vehicle 3	Below LOQ	Below LOQ	353.6	83.2	85.1	40.4	Below LOQ
Average	**38.2**	**Below LOQ**	**270.7**	**64.5**	**70.0**	**43.1**	**Below LOQ**
LPS 1	25.9	Below LOQ	122,109.5	9,029.0	10,686.2	Non-linear	Below LOQ
LPS 2	60.7	Below LOQ	117,127.4	11,052.9	9,116.7	Non-linear	21.0
LPS 3	94.2	Below LOQ	113,539.6	8,926.7	9,610.1	Non-linear	220.3
Average	**60.3**	**Below LOQ**	**117,592.2**	**9,669.5**	**9,804.3**	**>1,000**	**120.6**

LC-MS/MS Assay

Sample	LTC_4 (pg/ml)	LTB_4 (pg/ml)	PGD_2 (pg/ml)	PGE_2 (pg/ml)	$PGF_{2\alpha}$ (pg/ml)	6-keto $PGF_{1\alpha}$ (pg/ml)	TXB_2 (pg/ml)
Vehicle 1	*12	Below LOD	179.9	*42.3	*57.5	*6.2	Below LOD
Vehicle 2	*14	Below LOD	246.4	*37.0	*50.2	*10.5	Below LOD
Vehicle 3	*14.4	Below LOD	378.9	*65.4	*54.8	*4.0	Below LOD
Average	**13.5**	**Below LOD**	**268.4**	**48.2**	**54.2**	**6.9**	**Below LOD**
LPS 1	64.5	Below LOD	122,052.5	9,671.9	8,105.2	349.9	*248.6
LPS 2	59.7	Below LOD	93,520.5	9,453.3	8,125.8	951.9	*218.8
LPS 3	57.5	Below LOD	105,544.3	8,168.9	7,955.7	1,101.9	288.4
Average	**60.6**	**Below LOD**	**107,039.1**	**9,098.0**	**8,062.2**	**801.2**	**251.9**

*Values were below LOQ but above LOD and are extrapolated values.

Table 1 – Summary for Measurements of Seven Primary Eicosanoids in Conditioned Media by ELISA and LC-MS/MS

The data obtained from both methods for LTC_4, PGD_2, PGE_2, and $PGF_{2\alpha}$ matched quite well. We were unable to obtain data with confidence by ELISA for 6-keto $PGF_{1\alpha}$ in the LPS-treated sample so the reported number is estimated. TXB_2 was not detected by either method in the control samples and non-linear in the ELISA. As noted in the table above, many of the values obtained with LC-MS/MS were below the LOQ (but above the LOD) of the assay. In some cases, adjusting the standard curve would allow accurate quantification of these analytes. In other cases, LC-MS/MS alone may not reach the desired sensitivity levels. It took approximately 1-2 hours to run each ELISA kit (7-14 hours total), and LC-MS/MS took approximately 2-3 hours total to complete.

The results for the mouse liver and lung samples are shown in Figure 7. The LC-MS/MS assay showed high levels of PGD_2 in mouse liver while PGD_2 was not detected as measured by ELISA[1]. PGE_2 was also significantly lower (about 2-fold) as measured by ELISA as compared to LC-MS/MS. In mouse lung, the data was comparable by both methods except for 6-keto $PGF_{1\alpha}$. The ELISA gave about 2-fold higher amounts of 6-keto $PGF_{1\alpha}$ than LC-MS/MS.

Overall, the data are comparable between ELISA and LC-MS/MS. Further investigations are in progress to understand the observed differences between methods for the tissue samples, but it is evident that complex matrices can introduce significant variability between ELISA and LC-MS/MS.

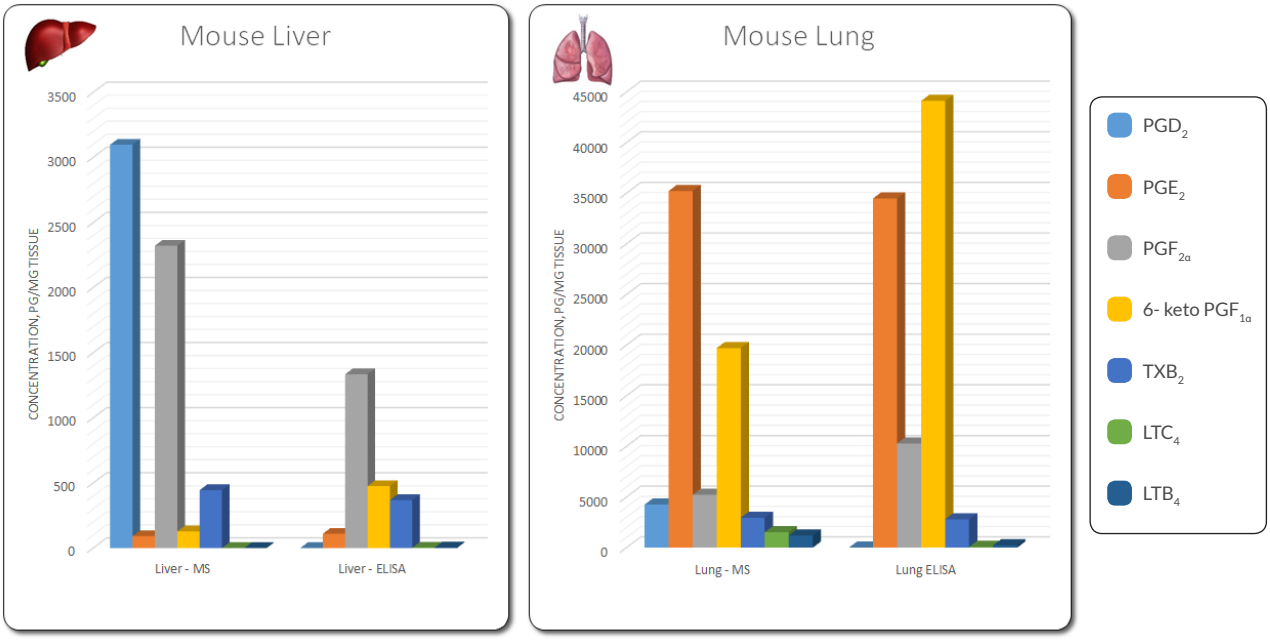

Figure 7 – Summary for Measurements of Seven Primary Eicosanoids in Mouse Liver and Mouse Lung by ELISA and LC-MS/MS

ANTIBODIES, IMMUNOASSAYS, AND MASS SPECTROMETRY

Antibodies are getting a lot of bad press lately, mainly because researchers are finding irreproducible results when working with them (as an example, see Baker, M. Reproducibility crisis: Blame it on the antibodies. *Nature* **521(7552)**, 274-276). Antibodies can be useful tools for researchers, but they are not magical. The most common misconception about antibodies is that they have absolute specificity in any and all biological matrices and experimental conditions. Antibodies often cannot absolutely distinguish structurally related molecules. In addition, different batches of antibodies can have varying performance characteristics. The lack of proper assessment of the specificity and cross-reactivity for the application may be a primary source of variability.

[1]This may be explained by the fact that the PGD_2 ELISA used was not tested prior to this study in tissue homogenates. Because PGD_2 is rapidly metabolized *in vivo* and forms adducts with proteins, this assay was developed to use with less complex samples such as *in vitro* PGD synthase reactions and conditioned media. We chose the PGD_2 ELISA to assay all samples because it did not seem appropriate to use one assay type for the culture supernatants and a different assay for the tissue samples since the alternative assay would have necessitated processing the samples significantly differently than we did for the LC-MS/MS assay.

Biological samples, such as plasma, urine, and tissue homogenates, are quite complex and contain molecules that can mask the detection of the target molecules by interfering with the antibody-antigen interaction. Frequently, complex sample preparation or methods of separation are employed to partition the target analyte(s) and remove interferents in a sample, which can lead to inaccurate measurements. In immunoassay-based analytical techniques, such as ELISA, the readout is the sum of all the molecules that cross-react with the antibody being used and includes any interfering molecular effects, potentially leading to a falsely reported elevated or reduced analyte concentration.

Plate-based immunoassays can be challenging to multiplex due to the inability to distinguish the various fluorophores or enzyme reporters specific to each antibody in the well. Thus, individual kits may be required for each analyte of interest, increasing the time and cost for measurement of a panel of targets (as shown above in this brief). Bead-based immunoassays have a limited capacity to multiplex, but LC-MS/MS multiplexing capability is only limited by the ability to separate analytes by LC.

Nevertheless, antibodies remain great research tools when we understand their performance characteristics. They can be used as simple partitioning tools, as is the case with plasma, where immunoaffinity is often employed to remove the top 10-20 abundant proteins in a single step in order to allow detection and measurement of the lower abundant proteins since many biomarkers of clinical importance reside in pg/ml to low ng/ml range in plasma. One can also take advantage of antibody specificity, or lack thereof, as antibody promiscuity allows one to isolate and enrich for structurally related molecules and their variants, such as post-translationally modified and unmodified proteins.

The extensive complexity of human biological samples requires analytical methods that can accurately analyze the array of biomolecules, such as lipids, peptides, proteins, and all of their variants present in these samples. The advancement of highly sensitive LC-MS/MS technology is allowing the detection of these entities with higher accuracy, precision, and sensitivity than other methodologies.

However, accessing low level biomolecules in highly complex samples remains a challenge. LC-MS/MS is well-suited for the analysis of complex sample types. In targeted MRM assays, one can detect and quantify analytes present in plasma at pg/ml levels, sometimes with no plasma processing. Separation, isolation, or enrichment strategies often times allow access to low level biomolecules and yields more accurate quantification of these analytes. For example, increased performance characteristics can be achieved with analyte immunoaffinity enrichment prior to MRM-MS.

Antibodies are now being repurposed as analyte enrichment tools prior to LC-MS/MS, otherwise known as Immunoaffinity Mass Spectrometry (IA-MS), giving access to low level analytes in highly complex samples. The antibody specificity issue goes away as LC-MS/MS will confirm identity and quantitate the analyte(s) of interest simultaneously. The promiscuity of the antibody can be taken advantage of as it can potentially enrich multiple structurally related molecules that can be measured in a single assay.

The accuracy and breadth of analytical detail offered by LC-MS/MS is perhaps the most distinguishing advantage of LC-MS/MS over immunoassay-based technology. LC-MS/MS provides a researcher the opportunity to evaluate potentially hundreds of molecular species in a single run. Using an untargeted method of analysis, relative concentrations can be obtained when implementing appropriate internal standards. When using a targeted approach, dozens of molecules can be accurately quantified simultaneously. It is now possible to multiplex in a way that allows researchers to monitor entire cellular pathways.

SUMMARY

ELISA and MS are both successfully being employed in biomarker quantification. Each method has advantages and disadvantages. Immunoassay technology, such as ELISA, for quantification of biomolecules remains a viable tool but often suffers from lack of specificity and accuracy in more complex samples. The development of high sensitivity, accurate LC-MS/MS instrumentation and methodologies has set a new standard for biomarker discovery and monitoring. IA-MS enhances this technology and provides the means for researchers to accurately quantify clinically important biomolecules in a variety of sample types in a time- and cost-effective manner.