Liquid chromatography-high-resolution mass spectrometry for regulated bioanalysis: bile acid and oligonucleotide quantitation as a reference

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Historically, LC coupled with triple quadrupole (QqQ) MS has been the gold standard for quantitative analysis, while ion trap and Q-TOF MS have been exclusively used for qualitative analysis (e.g., metabolite identification). The main reasons why QqQ-MS has dominated the quantitative analysis field for decades include its great sensitivity, excellent selectivity with SRM mode and wide dynamic linear range. However, it is also known for low resolution (1500 FWHM or 0.7 Da) and limited mass range (up to 2000 m/z , generally). These are the limitations that hinder its application for large molecule quantitation, and is increasingly being challenged by an emerging generation of new high-resolution mass spectrometry (HRMS). In 2011, it was predicted that there will be a paradigm shift from SRM-mode QqQ-MS to HRMS for bioanalysis [1]. Since then, the applications of HRMS can be found frequently in journal articles, conference podium presentations and meeting posters. While we are embracing the new technology for bioanalysis, we have to admit that the knowledge we gained comes along with some level of confusion. These HRMS are better engineered, have smaller footprints and are easy to use for simple experiments, but also require sophisticated and complicated software. For many bioanalytical chemists who are skilled in small-molecule quantitation using QqQ-MS, we do not fully understand

the implications and potential that HRMS brings to regulated bioanalysis. Even the terminology is easily misunderstood. Often, it seems that the terms high-resolution accurate MS (HRAM or HRAMS) or HRMS are used interchangeably [2–4]. There is also a lack of understanding about this technology by external regulatory experts, corporate compliance and scientists, which causes unnecessary anxiety and controversy regarding the regulatory aspects of HRMS. At the end of the day, HRMS is simply another MS detector.

There are many reasons why HRMS is becoming popular for quantitative bioanalysis. Newer technologies with state-of-the-art engineering, made the traditionally expensive and large footprint of Q-TOF or Orbitrap MS smaller, more affordable and user friendly. The faster scan speeds are now comparable to QqQ-MS, making it feasible for high-throughput bioanalysis [5]. The continuously evolving technology is echoing market demands. The ultimate drive for the growing need for HRMS is the complicity of new drug candidates, and the availability of additional analyte information retrospectively (i.e., data mining) using full-scan mode to collect any ions in the designed mass range. Two decades ago, the majority of the drugs in development were small synthetic chemicals. Currently, the shift for new drugs includes biologics, monoclonal antibodies (mAbs),

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antibody–drug conjugate (ADC) peptides and oligonucleotide (ON) therapeutics. These molecules often possess high MW (>10,000 Da) and, are by design, meant to mimic endogenous compounds in order to reduce toxicity and increase bioavailability. The quantitation of these large molecules is challenging using low-resolution QqQ-MS because of endogenous interference and complex isotope contributions. From the early 2010s, scientists started to use HRMS for quantitative purposes [7]. Initially, this started from within discovery and DMPK groups and was based on a strategy of ‘kill two birds with one stone’. In 2013, the perspective of using HRMS for regulated bioanalysis started to draw attention. At the American Society for Mass Spectrometry 2013 annual meeting, a dedicated session for this topic was held [6]. This was followed by an increasing application of HRMS for the quantitation of peptide, PEGylated protein, mAbs, biomarkers and so on [8–10].

Our laboratory specializes in regulated bioanalysis. The very first successful case study from our laboratory using HRMS was to resolve a poor sensitivity issue for a bile acid and its metabolites biomarker assay that resulted from the lack of fragmentation using QqQ-MS [11]. Not only were we able to achieve better S/N and lower LLOQ using HRMS, it was also noticeable that the overall accuracy on HRMS is much better than QqQ-MS. For a panel assay of up to 16 bile acids, there was seldom a run failure and the CV was typically single digit. We can speculate that HRMS is a good choice for steroid-based compounds or any compound lacking fragmentation. We also learnt that full-scan mode is not only easier to operate but provides better accuracy than target single ion monitoring (T-SIM) mode, in particularly for low end of calibrators and QCs. For example, for both T-SIM and full-scan modes using same average gain control (AGC, $1e6$) and maximum injection time (IT, 200 ms), the scan numbers for taurodeoxycholic acid (tDCA) at LLOQ of 1.00 ng/ml is 30 for full scan mode versus 5 for T-SIM mode and the latter significantly impaired precise measurement [12].

Another new frontier for HRMS application is the quantitation for therapeutic ONs. ONs are short, single- or double-stranded DNA or RNA molecules consisting of strands of 10–50 nucleotides. By targeted modulation of gene expression, ONs can target diseases at their molecular level. Although there are only three ONs approved by the US FDA, many more ONs have been or are currently being developed to treat a wide range of diseases. Historically, we have used QqQ-MS for ON quantitation in order to compete with the traditional bioanalytical technologies such as hybridization ELISA, real-time-qPCR or

HPLC-UV/fluorescence methods. While LC-MS/MS is inevitably more specific and selective as compared with the above technologies, we also recognized its limitation. Not necessarily the MS technology itself, but more likely related to the inherent chemical structures of ONs. Using SRM mode, the precursor ions of ONs are typically multiply charged and vary depending on the length of strand and modification of the ONs. However, the product ions are universally phosphate ion, m/z 95, under negative ion mode. This SRM transition is not ideal. The interference from endogenous ONs has been observed quite often. Additionally, the SRM transition is too exclusive. The information on $n-1$ or $n-2$ metabolites can be completely lost. In real-life situations, some companies may choose ELISA or LC–UV over LC–MS/MS even when presented with compelling LC–MS/MS data. Currently, we heavily use HRMS for quantitative bioanalysis in our laboratory. We found that an increasing number of companies prefer HRMS over QqQ-MS. This is not necessarily for more sensitivity or selectivity, but more for the potential capability of metabolite information post data collection. We have performed many side-by-side comparison evaluations using QqQ-MS and HRMS and found that the sensitivity on both platforms is comparable with the exception of the bile acid assay. However, the full-scan mode for HRMS opens another window of opportunity, which is impossible for QqQ-MS. It possesses the advantage of the selectivity and specificity of QqQ-MS, but preserves the information for metabolite data mining. It was demonstrated by a successful example using HRMS for ONs quantitation and metabolite data mining presented in this year’s ASMS annual meeting [13]. RG-125 (AZD4076) is a GalNAc-conjugated ON targeting microRNA-103/107 for the treatment of Type II diabetes/prediabetes. Five major metabolites with modified conjugate portion or truncated ON lengths were identified *in vivo* at the early discovery stage. The GLP validation and sample analysis was conducted to quantify the active drug and two major metabolites. Two additional metabolites were not included in the formally validated methods. The concentrations of the two metabolites were obtained purely via computation and data mining post the GLP data analysis and after the GLP data were locked.

During our data mining exercise, we also recognized that there is a limitation for data mining. Clearly, there is a difference between metabolite profiling from the metabolite ID point of view and data mining from a purely bioanalytical purpose. For metabolite profiling, the objective is to identify every potential metabolite. Thus, a simple sample preparation such as protein

precipitation extraction is utilized in order to preserve every metabolite in the extract. It typically requires a long cycle time with shallow gradient programs to ensure adequate resolution. For data mining, the extraction and the LC conditions are optimized for just the intended analytes. Some metabolites may be excluded unintentionally during the extraction. They are either eluted in the void volume or remain on the LC column beyond the analytical run time.

Future perspective

After nearly 6 years of implementation and advocating by individuals from both pharma and CRO laboratories, and many excellent research data and publications, we should put any debate regarding the suitability of HRMS for regulated bioanalysis behind us. It is precise, reproducible and can reduce the operational cost if we use the massive amounts of full scan data appropriately. In terms of future perspective, I predict that there will be increasingly more regulated bioanalysis performed using HRMS. There should be no

limitation for the types of molecules, but presumably more benefit for large molecules such as PEGylated protein, mAbs, ONs and biomarkers. I also see the need for data mining using full-scan mode. Of course, from a regulatory point of review, the intent of the data mining needs to be described in protocol *a priori*. For clinical trials, a consent form should be signed from patients and volunteers. Finally, the detection limit for HRMS needs to be improved from an engineering point-of-view. Otherwise, it can never completely replace QqQ-MS.

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