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Method Development on LC-MS/MS: Challenges and ways to overcome it

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Over the last decade, LC-MS/MS has become one of the most effective and preferred tool for the analysis of chemical compounds. Be it a generic molecule or a New Chemical Entity (NCE): the high sensitivity and ruggedness offered by this technique, combined with the ability to analyze samples in a short time has magnified its popularity as a qualitative and quantitative analytical tool. Applications of LC-MS/MS are myriad, ranging from a simple quantitative or qualitative sample analysis to metabolite identification. Therefore, it becomes imperative that an analyst develops a rugged and robust method for the analysis of the compounds.

Most often the question an analyst asks oneself, "What should be the first basic approach to Method Development (MD)?" One can always search the internet for literature on the same. This effort holds true for a generic molecules. There are a few down sides to it though, which shall be discussed later. Let's appreciate; if the compound is a NCE, published literature could be non-existent. An analyst would then have to rely on his knowledge and skill to decipher the right approach to method development (MD).

The first step to it is gathering as much knowledge about the compound as one could. Molecular weight, solubility, polarity of the compound etc are the basic information one should look into before starting the development. Each of these criteria plays a significant role in planning and developing a robust and rugged method. For instance, let's say a compound has a solubility of less than 10 mg/L in Methanol (MeOH) and less than 1 g/L in Acetonitrile (ACN), it is always preferable to prepare a solution in the latter for tuning, the first step of the MD.

Secondly, while developing a method in LC-MS/MS, tuning of the compound in consideration is perhaps the most important step. Tuning, if termed colloquially, is acclimatization of the compound with the instrument. Ensuring a maximum response of the fragment ions and optimizing the various source and compound parameters goes a long way in ensuring that the method developed is robust and rugged. Ionization of the compound can always be enhanced by either adding a small volume of formic acid or ammonium hydroxide solution to the tuning solution, again, depending on the physico-chemical property of the compound. Often, if the structure of the compound is available, it could be helpful in predicting the fragmentation pattern of the compound. Many times, this would help the analyst in selecting the accurate daughter ions.

After the proper tuning of the compound, the next challenge that an analyst has to face is the development of chromatography. A proper synchronization of a good chromatography coupled with optimum mass parameters is always helpful in the long run. There is no thumb rule approach to developing chromatography. One can always go ahead with the infamous "hit and trial" method, wherein a random selection of mobile phase and columns are used to select a best out of many. However, this process is cumbersome and time-consuming. Instead, a thorough knowledge about the physico-chemical properties of the compound helps in selecting the right column or mobile phase combination for better retention / resolution of the compound. Not a long time ago, 99% of the compounds used to be analyzed using C-18 columns. As technology evolved, chromatography methods also came out with varieties. Presently, an array of different types of columns, ranging from C-4, C-8, Phenyl, Cyano, fluoro-phenyl, and HILIC etc is at the disposal of the analyst. For instance a polar compound is better resolved when a Cyano or Phenyl column is used as compared to a non-polar compound. Slightly polar compound can be effectively separated with columns with lower carbon loading. To be continued in Page 2....

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Particle size, length and internal diameter of the column are some of the other factors that play an important role in Method Development. LC-MS/MS analysis often involves shorter run times and hence approach should always be directed towards using shorter columns with smaller internal diameter. With the advent of Ultra Fast Liquid Chromatography (UFLC), columns with sub-micron particle size are useful for enhanced resolution of the analyte.

As for mobile phases, the strategy "keeping it simple" works out best. Wherever possible, buffers such as Ammonium acetate, Ammonium formate or Ammonium bicarbonate should be avoided in the aqueous portion. However, this is not a thumb rule. Some compounds have been shown to give better results with 2 or 5 millimolar buffers. Similarly, if one uses a buffer, it should be ensured that high concentrations (more than 10 mM) are avoided. Buffers, at high concentrations, generally tend to reduce analyte response over time. This is mostly attributable to the salt deposits over the curtain plate of the mass spectrometer. Thus, most of the time, a combination of Acetonitrile and 0.1% formic acid in water may tend to give better results compared to a combination of Acetonitrile and 10 mM Ammonium formate. The chemistry of the compound must be kept in mind while altering the composition of the organic and aqueous part of the mobile phase.

This is a general overview of method development for a NCE. When the compound in question is a generic molecule, the first step any analyst takes is to look for related literature on the internet. Here is where the paradox lies. The downside of it is that various papers may be available for a specific compound stating various methods and all are validated showing good results. So the dilemma is, "which is the best method out of all?" Let's say for example, a compound 'X' is a generic molecule, and you want to develop a method for the same. You then find 5 different references, each with a different mobile phase and column condition and every one of them is a validated method. Here is where the concept of *Quality by Design (QbD)* comes into play.

QbD is a recent practice, which is being adapted by scientists all over the world to ensure that the method developed is the most robust and effective for a given analyte. It involves the survey of all the literatures available for the compound that needs to be analyzed and trying to replicate the same by taking a few selective trials. So for instance, if you have 5 different mobile phases of different pH for compound 'X', you will then choose three median pH range and perform the trial runs. In other words, if pH range in the literatures varies from 5 through 9, you will select 5, 7 and 9 and perform trials with it. Similar is the case for columns. If suppose the majority of literature suggests the use of a C-18 column, you should choose the same or similar make of column for your trials. If a variety of columns are mentioned, you must select three most comparable ones. This way one can reduce the time and effort spent in developing a new method from the scratch and can, more often, generate better results.

Thus, to summarize, one can always develop a rugged and robust method by simple understanding of the chemical property of the compound in question and a bit of research into similar work performed by other analysts. As previously mentioned, there is no right or wrong approach, nor is there a rule of thumb to develop a method. As long as one develops an efficient method, resolving all the analytes with well-separated retention time, with least interferences with the solvent/matrix, we have achieved the goal!