HOW TO APPROACH THE IDENTIFICATION OF ANOMALOUS PEAKS
DURING PHARMACEUTICAL SAMPLE ANALYSES

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The sudden appearance of an unknown peak during an HPLC analysis of a pharmaceutical product can be a critical finding causing delays and requiring considerable resource (both time and money) to resolve. The immediate need to investigate an unknown peak is a common problem and is generally accompanied with aggressive timelines and stressful conditions and the issue can be caused by many things which can range from simple sample preparation contamination, all the way to unexpected degradation/stability properties of the product. In any case, it is essential that a rapid and effective investigation take place to quickly determine the identity of the anomalous peak and determine the root cause of its presence in the product.

The source of an unknown peak can be attributed to:

- Laboratory sample contamination
- Instrument related peaks
- Method resolution (e.g. a vendor modification to HPLC column packing altering the column performance revealing a previously undetected entity)
- Raw material impurities or contamination
- Product or aliquot degradation (a material stability issue or reaction with another excipient)
- Leachables/extractables (from a manufacturing step or a container closure system)
- Manufacturing process cross-contamination
- Some other unknown source

Regardless, once an impurity analysis on a drug product reveals that an anomalous peak causing a failure of a specification, it jeopardizes the release of material into production, or even has the potential to initiate a product recall.
As a first step, one needs to assess if the peak is real and not an artifact. Once confirmed, it is critical to determine the identity and the source of the unknown peak in order to ascertain the impact this peak may have on the affected product and ultimately the patient. However, determining the identity of the peak can be challenging because:

1. The level of the peak is usually small (0.05 – 0.2% area percent of the main peak)
2. There is limited knowledge on the source of the peak
3. Without a structure, spectral properties such as relative response factor are unknown
4. The peak may not be well resolved from other peaks in the analysis

While each investigation is situation specific, these are some general guidelines and strategies that can be utilized to achieve success in these endeavours. In a typical study, the strategy for obtaining the necessary data to identify the unknown peak is not "set in stone". While there is certain flexibility to the plan, the basic tools necessary to make a conclusive identification include (but are not limited to) diode array, LC-MS/MS, accurate mass MS, and NMR.

BEFORE ATTEMPTING PEAK IDENTIFICATION

The isolation and identification of an unknown peak is not a trivial exercise and if done incorrectly, can cause more problems. Prior to making the decision to begin the isolation and identification process, some in-lab investigations and standard OOS practices should be performed.

1. Re-perform the sample preparation in scrupulously pre-rinsed glassware to rule out laboratory contamination
2. Re-analyze the sample on a different instrument
3. Analyze the raw materials used in the manufacturing process individually using the drug product impurity methodology in an attempt to identify the source of the peak
4. Review historical data to determine if the unknown entity had been present (maybe at lower levels) in prior batches
5. If cross contamination is suspected, review batch and equipment records to assess potential candidate components

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STAGE 1 – DIODE ARRAY ANALYSIS

Once the decision is made to initiate the unknown peak investigation, the first data that should be generated in an unknown peak investigation is a UV spectrum using a diode array detector. This serves three purposes: 1) the UV spectrum can give some critical structural information about the unknown peak, 2) it may also give an indication if the peak is related to the parent molecule being analyzed and 3) it enables peak tracking capability during situations where method modifications are required.

STAGE 2 - USE OF VOLATILE BUFFERS TO ACHIEVE AN LC-MS COMPATIBLE METHOD

In the event that the method being used utilizes volatile buffers that are compatible for LC-MS analyses, then method development is not required and one can proceed directly to performing mass spectral analyses. However, many methods in the pharmaceutical industry use phosphate buffer systems in HPLC mobile phase. Phosphate buffers are not compatible with LC-MS analyses so the first step in the unknown peak investigation is to substitute a volatile buffer into the mobile phase. For low pH situations, formic acid or ammonium acetate are suggested as mobile phase modifiers. Also, attempt to adjust the pH to match to the original method pH to minimize shifts in retention times. Lastly, avoid using sodium salts, if possible, in either the buffer or pH adjustment process as counter-ion adducts in MS analyses make data interpretations difficult. Once the mobile phase has been adjusted, the analysis needs to be re-run (using diode array detection) to confirm that the chromatographic profile remains relatively unchanged from the original mobile phase composition and the unknown peak remains well resolved from interferences. The diode array data also ensures that the unknown peak continues to be the target of the investigation.

STAGE 3 – LC-MS/MS AND HIGH RESOLUTION MS ANALYSES

Mass spectrometry is a powerful tool during the investigation of unknown peaks but caution must also be employed when interpreting the data generated using this technique. Points to consider when analyzing the MS data include:

1. The unknown peak being analyzed may not be homogenous
2. The response of the peak in the MS detector may not correlate with the UV data
3. In cases where the peak is not homogenous, the MS will afford data consistent with the moiety in the peak that is the most stable ion, and not necessarily the largest component

That being said, MS and MS/MS data combined with structural libraries can be an effective way of accomplishing a structural elucidation of an unknown peak. In addition, the determination of a high resolution MS data may aid in the investigation. The use of a 10 ppm tolerance for suggested molecular formula matches may result in multiple hits. Therefore, information such as the presence of heavy atoms is important to help narrow down the search for the molecular formula of the unknown peak. Generally, at this point, a holistic analysis considering the UV profile, molecular weight of the unknown peak and how that compares to the main active pharmaceutical ingredient or excipients, fragmentation patterns, and any other available information or historical data should be performed.

In some cases, GC-MS may also prove to be a valuable tool but should also be used with caution due to thermal degradation or lack of volatility.

STAGE 4 – PEAK ISOLATION AND NMR ANALYSIS

In the event that MS data are inconclusive, further investigation will be required to identify the unknown peak. At this point, it is suggested that NMR be employed to determine the structure

but this exercise is not trivial. A substantial peak collection effort is required to collect enough of the unknown peak to perform an effective NMR analysis. On a case by case basis, various peak fraction collection strategies can be used to obtain enough sample for NMR. Unknown peaks (~0.1%) that are well resolved from interferences and have maximized the on-column load of the material may still require anywhere from 20-40 injections/collections to obtain the necessary material for 1D and 2D NMR experimentation. However, in an ideal situation, it is beneficial to collect enough material so that a weight can be measured. Once weighed, the extinction coefficient of the unknown peak at the detection wavelength can be determined which enables the appropriate quantitation of the unknown peak in the HPLC impurity analysis.

Upon collection, the mobile phase can be removed using nitrogen flow. The use of heat is not recommended as the chemical stability of the unknown peak is not established. Once dry, the sample can be submitted for NMR analyses. Consultation with an NMR scientist will establish the appropriate NMR strategy to pursue. The utilization of 1D and 2D techniques combined with spectral prediction software should afford the structure of the unknown peak.

DISCUSSION

The point to remember is that each situation is unique and will require an individualized case-by-case approach to identifying the unknown peak. However, the general strategy to identifying an unknown peak is summarized in the flowchart (figure 1).

It is also very advantageous to collect as much available information before initiating the investigation. Information such as structure of the parent molecule, excipient formulation, known degradation pathways of the parent, and historical data (i.e. forced degradation study data) can all aid in the strategy development. Also, it may be beneficial to assess any subtle changes that may have occurred including:

- Changes in raw material suppliers
- Changes in manufacturing location or process
- Any deviations or issues during the manufacturing process
- Changes in the container closure system or supplier

Lastly, the discovery of an unknown peak usually causes a great deal of stress. The resolution of the unknown peak issue will be time critical and there will be pressure to complete the investigation in an expedited manner. It is important to understand that this type of an investigation takes time and there are many technical hurdles that may arise during the course of the work. A reasonable time estimate for the full study is anywhere from a few days to ~4 weeks.

However, performing a due diligence of all the available information prior to starting the work and following the strategy outlined in Figure 1, helps to ensure a successful outcome and efficient utilization of time and resources. Once the identification of the peak is obtained and

the real amount present is known, a plan forward can be developed to resolve the issues to: accept or reject the batch, amend the specification to control the impurity level, and potentially release the product to market.

CONCLUSION

The identification of an unknown peak is a valuable capability to have in ones troubleshooting arsenal. It requires planning as well as the assimilation of many pieces of data. The combination of DAD, low and high resolution MS, MS/MS, and NMR data will contribute to the successful structural elucidation. Each situation will offer unique challenges and considerations, so the overall strategy can not be a "one size fits all" approach. Different investigational tools may be needed and alternate strategies may be mapped out, but in general, using this guidance as a basis for initiating the anomalous peak investigation will afford the framework needed for expeditious success.

FIGURE 1: **FLOW CHART FOR DETERMINING THE STRATEGY OF UNKNOWN PEAK INVESTIGATIONS**
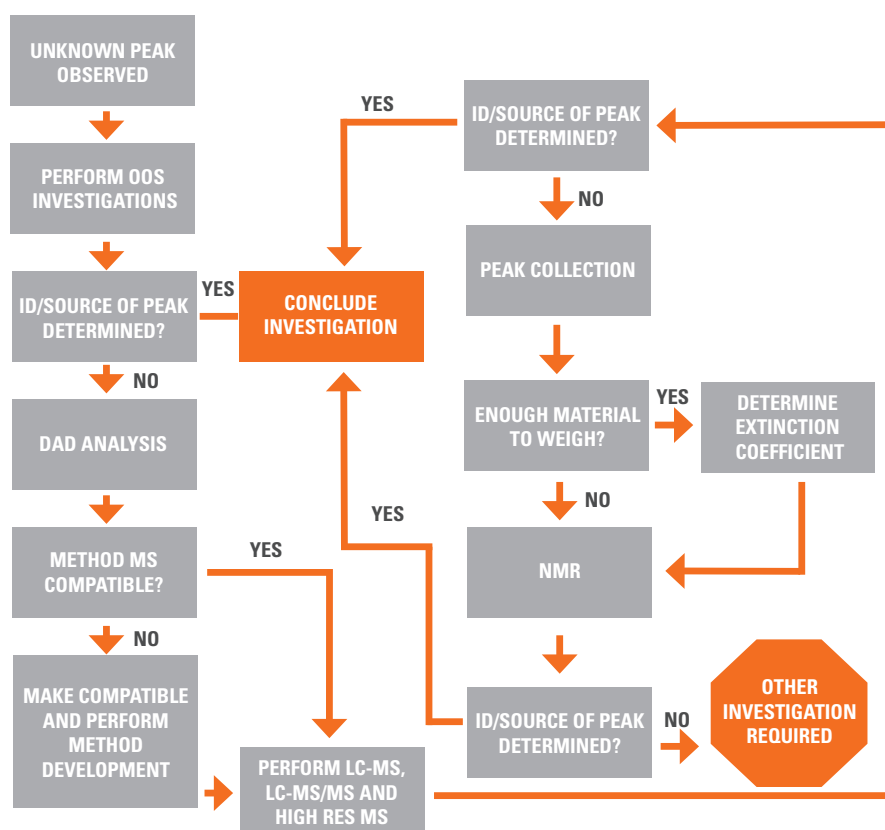
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