Isolation and Identification of Process Related Impurities and Degradation Products from Pharmaceutical Drug Candidates, Part I

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The objective of this two-part review article is to provide guidance for isolating and identifying process related impurities and degradation products from pharmaceutical drug candidates. The identification of degradation products can provide an understanding of impurity formation and define degradation mechanisms. If the identification process is performed at an early stage of drug development, there is adequate time for improvements in the drug substance process and drug product formulation to prevent these impurities and degradants long before the filing stage. Impurity and degradant structure elucidation is a collaborative effort involving the analytical chemist, process chemist and/or formulator as well as the degradation, mass spectrometry and NMR experts. The process described in this two-part article uses a designed approach for the impurity and/or degradant identification, which focuses on efficiency so that the success of data collection is maximized. There are a number of activities other than collecting experimental data, even though the experiments are central to the process. Part I of this article describes a process for isolating unknown impurities and degradants, while Part II will illustrate the role of mass spectrometry and NMR in the identification process.

The Process

The process of identification of impurities and/or degradants begins early in drug development. Early brainstorming sessions should involve the analytical chemist, the process chemist, the formulator and the degradation chemist, as well as the mass spectrometry and NMR experts. It is imperative to involve all that are familiar with the project of interest. The group meets to assess the timelines for completion and to gather all pertinent information. This initial planning and discussion effort can save significant time in the experimental stage. A few questions that need to be answered at this early stage are: Is this an impurity or degradant problem? At what level is the impurity/degradant present? Is it a process related impurity (PRI), and if so, at what step of the process is it formed? Is it a degradant, and if so, under what degradation condition is it formed? Are enriched samples with the unknown impurity/degradant available? By gathering all relevant information, the most efficient method of isolation and identification can be selected.

The first step of the process is to determine at what level the unknown is present. According to the ICH Guidelines on Impurities in New Drug Substances1:
The studies conducted to characterize the structure of actual impurities present in the new drug substance at a level greater than 0.1% (depending on the daily dose, calculated using the response factor of the drug substance) should be described. Note that all specified impurities at a level greater than the identification threshold in batches manufactured by the proposed commercial process should be identified. Degradation products observed in stability studies at recommended storage conditions should be similarly identified. When the identification of an impurity is not feasible, a summary of the laboratory studies demonstrating the unsuccessful effort should be included in the application.

According to the ICH Guidelines on Impurities in New Drug Products2:
Degradation products observed in stability studies conducted at recommended storage conditions should be identified when present at a level greater than the identification thresholds (1% for a maximum daily dose of <1 mg to 0.1% for a maximum daily dose of > 2 g). Identification of impurities below the 0.1% level is generally not considered to be necessary unless the potential impurities are expected to be unusually potent or toxic1. Therefore, it is imperative to determine the level of the unknown impurity and/or degradant early in the process. If the unknown is below the 0.1% threshold, then a discussion will need to take place among the project team members in order to determine if isolation and

identification are necessary. However, if the unknown is at or above the 0.1% limit, then effort should be put forth for identification.

Once a decision has been made to identify an unknown, the next logical step is to evaluate all known process related impurities, precursors, intermediates, and degradation products. By observing the relative retention times (HPLC) of all known process related impurities, precursors and intermediates (if available), one can quickly determine whether or not the impurity of interest is truly unknown. If the relative retention time of the unknown impurity matches that of a standard, then the unknown can be identified using HPLC with ultra-violet (UV) photodiode array as well as mass spectrometry (MS) detection. The identity is confirmed by correlating the retention time, UV spectra and mass spectra of the unknown impurity with that of the standard.

Identifying an unknown by using a standard, as described in the above paragraph, is a quick and easy process. However, what happens when the relative retention time of an unknown does not match that of a standard? The next step is to obtain molecular mass and fragmentation data via HPLC-MS. It is essential to determine the molecular mass of the unknown. Not only does the molecular mass help in the identification of the unknown, but it also enables one to track the correct peak by HPLC if isolation becomes necessary. In order to run LC-MS, a mass spectrometry compatible HPLC method must be available. The mobile phase should contain volatile buffers that are HPLC-MS-compatible. (Note: A discussion of mass spectrometry compatible mobile phases will be discussed in part II). If such a method is not available, then one must be developed, which adds time to the identification timeframe.

If the mass spectrometry data evaluation yields sufficient structural information, this eliminates the need to isolate the impurity in question. If standards of the proposed structures are available, they can be correlated with the unknown as previously described. If standards are not available, which is usually the case, the proposed structures can be discussed with the project team. The project team can then decide if the information is suitable for their needs, or if isolation is required.

An alternative to isolation is small-scale synthesis. If possible structures have been proposed from the mass spectrometry data, one can study the process chemistry and determine at which step of the process the impurity and/or degradant is most likely to be formed. By knowing the process chemistry, the feasibility of the proposed structures can be evaluated. Proposed structures can then be synthesized if a reasonable synthesis is available. It is easier to synthesize and identify the unknown if the chemistry works quickly (i.e. one step/straight-forward chemistry). If small-scale synthesis is chosen, the synthesis must be the most efficient route.

At this stage of the process, it is frequently necessary to isolate and characterize the unknown. One of the most important factors to consider when approaching an isolation experiment is the sample origin. It is vital to determine whether the unknown is an impurity and/or degradant, and to locate a sample that contains an enriched quantity of the unknown. Isolating low level impurities can prove to be very cumbersome and time consuming. Therefore, the ultimate goal is to find a sample that contains an enriched quantity of the unknown. Two great resources of enriched samples are retained mother liquor samples and purposeful degradation/stability samples. If the unknown is a drug substance degradant, then the degradation reaction can be scaled-up to generate a large quantity of the unknown. If it is a drug product degradant, then effort should be put forth to form the degradant in the drug substance so that extraction from the excipients is not required. Whenever enriched samples are not available, the unknown must be isolated from the bulk drug substance or drug product.

A number of methods can be used for isolating impurities and/or degradants. Three of the most utilized techniques are thin-layer chromatography (TLC), flash chromatography (column chromatography), and preparative high performance liquid chromatography (HPLC). The actual technique used depends upon the nature of the impurity and/or degradant, including the amount present in the original material from which it must be isolated. A good starting point is to assess the separation that is currently being used by the analytical chemist. Does the current methodology provide optimum resolution of the impurity/degradant from the main band and other impurities, and if so, is that method by TLC or HPLC? This is a key factor in determining which technique to utilize. Each of the three techniques will be discussed separately.

Isolation Techniques

Thin-Layer Chromatography

Thin-Layer Chromatography (TLC) is a good technique to use when normal phase solvents provide optimum separation. Typical thin-layer separations are performed on glass plates that are coated with a thin layer of stationary phase. The stationary phases used in TLC encompass all modes of chromatography including adsorption, normal and reverse phase, ion exchange, and size-exclusion chromatography. The equipment required is simple and inexpensive. It is an ideal technique for the isolation of compounds because of its simplicity. However, in order for TLC to be successful, the impurity and/or degradant should be at or above the 1% level. Anything below this level is very difficult to isolate on a TLC plate due to higher detection limits.

The steps involved in preparative TLC are: (1) application of the sample onto the plates, (2) development of the plates, (3) detection and location of the compound of interest, and (4) extraction of the compound of interest. Detection is usually by ultraviolet light. When the separated compound of interest is located on the plate, the band is scraped and the impurity is extracted from the stationary phase (i.e. silica gel) with an appropriate solvent. The extracted material is filtered or centrifuged, and the solvent collected is evaporated to yield the isolated material. It is essential to remove silica gel and other interferences that may inhibit the identification of the compound. The isolated material is then submitted for LC-MS analysis.

One of the main disadvantages of using TLC for preparative isolations is that limited amounts of material can be isolated from the plates. Using preparative TLC plates can circumvent this problem. Preparative plates contain thicker films of stationary phase, thus allowing larger amounts of sample to be applied. Even in cases where preparative plates are used, chances are good that not enough material can be isolated to obtain traditional NMR analysis (including 1H and 13C NMR). Therefore, TLC is most useful when an impurity and/or degradant is identifiable by LC-MS. In cases where NMR analysis is essential for identification, flash chromatography and/or preparative HPLC are more suitable techniques.

Flash Chromatography (Column Chromatography)

When an existing normal phase TLC method provides adequate resolution of the impurity/degradant to be isolated, then flash chromatography can be a useful technique. Flash chromatography is a simple absorption chromatography technique for the routine purification of organic compounds. It allows for separations of samples weighing 0.01-10.0 g in 10 to 15 minutes. Flash chromatography is a rapid, inexpensive and easily performed technique with a large sample capacity (approximately 5 times the load of reverse phase packing materials). Normal phase flash chromatography is ideal when the sample is soluble in nonpolar or moderately polar solvents such as hexane, chloroform, and dichloromethane. These volatile solvents allow easier concentration of impurities and degradants. Before choosing flash chromatography as the separation technique, use chemistry knowledge to assess the potential stability of the isolated product prior to isolation in order to determine if special collection conditions are necessary. For example, collect thermally unstable products in chilled flasks.

The first step in developing a flash chromatographic separation is to determine the optimum solvent composition by analytical TLC. A solvent system is chosen that provides good resolution and moves the desired impurity and/or degradant to Rf = 0.354. TLC can provide a guide to suitable solvent systems, but development work may be needed in order to optimize the separation when it is scaled-up to the larger I.D. columns used in flash chromatography. A suitable column is selected based upon the resolution of the impurity and/or degradant and the amount of sample to be purified. The amount of sample that can be purified on a column is dependent upon the resolution of the impurity and/or degradant, and it is proportional to the cross sectional area. If less resolution is required to separate a key degradant or impurity, sample load can be significantly increased. Typically, each run on a flash column can chromatograph gram quantities of material depending on the column size. The column load is typically much higher than that of reverse phase chromatography.

The columns are generally packed with silica gel. In order for the separation to be successful, the size of the silica gel should be 40-63 mm4. A concentrated solution of the sample is prepared. The sample solution is applied at the top of the column, and the walls of the column are washed with a few milliliters of eluent. Solvent is added to the column, and air pressure is applied at a flow rate of 2 inches/minute to rapidly elute the desired impurity and/or degradant. Separation is based upon the differential interactions between the solute molecules and the adsorbent surface of the silica gel. Fractions are continuously collected and monitored by chromatographic techniques (HPLC with UV detection, GC, or TLC). The fractions containing the compound of interest are combined and evaporated to dryness. The isolated material is cleaned (post-isolation cleanup, such as small scale column or analytical HPLC re-injection, is essential) and submitted for LC-MS and NMR analysis.

Flash chromatography is a fast and inexpensive technique for isolations requiring only moderate resolution4. Typically, compounds having Rf > 0.15 can be cleanly separated using this technique, and separations at Rf @ 0.10 are possible. In cases where high resolution is required, flash chromatography can be used as a preliminary purification step. It can be a good method of concentrating and partially purifying complex mixtures, thus making the final isolation much easier by preparative HPLC. For example, the main band can be isolated from the rest of the mixture; therefore, the impurity mixture needing optimum resolution can be injected with higher load on HPLC. In addition, automated flash chromatographic systems with UV detectors and fraction collectors are available that further simplify the isolation process.

Preparative HPLC

Preparative chromatography is the process of using liquid chromatography to isolate a sufficient amount of material for other experimental or functional purposes. This section describes the use of preparative HPLC in isolating tens of milligrams of pure unknown compound(s) for the purpose of structure elucidation by spectroscopic techniques, which is often referred to as semi-preparative HPLC. This section will focus primarily on preparative HPLC methods with the following parameters:

Column Length: 15-50 cm
Column Width: 10-40 mm
Particle Size: 5-60 mm
Column Load: 10-1000 mg
Flow Rate: 5-100 mL/min

The scale of preparative HPLC is normally larger than that of conventional HPLC. Therefore, a practical starting point is to develop an analytical separation that optimizes the isolation conditions. Optimization of the analytical method implies seeking conditions which combine maximum resolution of the peak of interest and minimum elution time, under the restriction of a limited pressure drop. The optimized conditions determine the column, mobile phase, flow rate and sample loading capacity for the particular column. The conditions may be either normal phase or reverse phase. The mobile phase should be chosen carefully to avoid salt complexation with the compound to be isolated. Volatile acid salts such as trifluoroacetic acid, formic acid and acetic acid are acceptable mobile phase additives, and the ammonium counter-ion is preferred for pH adjustment to any of these acids.

Once the analytical scale method conditions are optimized, the next step is to choose a column and scale-up the analytical HPLC parameters so that preparative chromatography can be performed and the unknown compound(s) isolated for identification by MS and NMR. For ease of transition, a preparative column consisting of the same packing material and particle size should be chosen. The column is the most important component of the process. The column determines the amount of material that can be loaded onto the column for the desired purity and recovery. An important step in the scale-up procedure is determining the maximum load on the analytical column. The maximum analytical load is essential in determining the loading capacity of the preparative column. When an appropriate column is chosen, the analytical isolation can be scaled up using Equation 1:

The scale-up factor is used to predict the loading capacity and flow rate for the preparative column. For example, if a separation optimized on a 4.6 mm x 150 mm column was scaled to a 20 mm x 300 mm column, the scale factor for the sample load would be 38. Thus, the scale-up factor multiplied by the maximum analytical load estimates how much material can be loaded on the preparative column (see Equation 2).
To maintain the same resolution when scaling-up a method, the flow rate also needs to be scaled proportionally. The preparative flow rate can be estimated by using the scale-up factor. For this estimation, the scale-up factor is multiplied by the analytical flow rate to estimate the preparative flow rate (see Equation 3).

YMC Inc. has simplified the process of scaling-up an analytical separation for preparative isolation by developing matched R&D column sets6. Each R&D column set contains an analytical methods development column and a preparative isolation column packed from the same lot of packing material. This provides assurance that any separation developed on the analytical column will scale-up directly on the matched preparative column without further method modification. The use of these column sets eliminates potential selectivity differences caused by different types of silica and different particle and pore size packings by providing matched columns.

When the preparative method has been optimized, injections are made and the compound of interest is typically collected using a fraction collector. The fractions are pooled together in a collection vessel. The stability of the isolated product should be assessed prior to isolation in order to determine if special collection conditions are required. In addition, be sure to use clean glassware since contamination can occur if the glassware has not been thoroughly cleaned. Also, carefully select the tubing used for collection of the unknown. For example, avoid using Tygon tubing because it contains phthalates, which can contaminate the isolated compound. The isolated product is concentrated using conventional sample concentration techniques (i.e. distillation at normal or reduced pressure, precipitation, freeze-drying, solvent extraction, and membrane filtration). Rotary evaporation and flash distillation are the two most commonly used techniques to recover isolated products from the mobile phase. After the product has been recovered, it should be dried under high vacuum to remove all solvents. An analytical clean up of the isolated sample is critical prior to NMR analysis. A clean sample improves the purity and quality of NMR data. As was mentioned earlier, volatile acid salts such as trifluoroacetic acid, formic acid and acetic acid are often used as mobile phase additives, which may cause salt formation if the pH of the mobile phase is adjusted (i.e. using ammonium hydroxide). In addition, mobile phase solvents may also contain low level impurities that become enriched during the concentration process. It is essential to remove any salts and/or impurities from the isolated product. A simple purification method for the isolated product is to re-inject it onto the preparative column using a mobile phase without any additives or pH adjustments. By utilizing gradient elution, salts can be removed by incorporating an aqueous rinse at the beginning of the run, and then the organic solvent can be ramped to elute the desired product. Thus, the isolated peak is purified. Solid-phase extraction also offers great potential in purifying the isolated product because of its universality7. Additionally, washing the isolated sample with deuterated solvent several times also helps to prepare the sample for NMR experimentation. Once the sample has been purified, it is submitted for LC-MS and NMR analysis.

As was mentioned earlier, isolation of low-level impurities and/or degradants can be quite labor intensive. Consider a 0.1% level impurity present in a drug substance bulk lot. Based on traditional NMR experiments, 5 mg of the impurity would be needed to obtain structural confirmation. To isolate 5 mg of the impurity from the bulk, a minimum of 5 g of bulk drug substance would be needed, assuming 100% recovery. Because actual recoveries are generally closer to 50% for low level (0.1% range) isolations, 10 g of bulk drug substance would generally be requested.

In addition to requiring significant bulk material, the timeframe to complete the isolation is considerable. If the maximum analytical load for a 4.6 mm x 150 mm column has been determined to be 5 mg of the parent drug substance, assuming the isolation will be performed using semi-preparative chromatography (20 mm x 300 mm column), approximately 190 mg of sample can be loaded onto the preparative column. For a 0.1% level unknown, this translates to 190 mg of unknown injected onto the preparative column. Therefore, a total of 27 injections are required. If the assay time were estimated to be one hour, it would take at least 27 hours to perform the injections needed to obtain 5 mg (once again assuming 100 % recovery). This timeframe does not include the time needed for method scale-up development, concentration and solubility experiments, as well as mass spectrometry and NMR experimentation.

On the other hand, if a sample was available that contained 10% of the unknown, only 1 gram of bulk would be needed and the estimated timeframe of the isolation would be drastically reduced. In this example above, 19 mg of unknown can be injected onto the preparative column (assuming the maximum analytical load does not change and resolution is retained with the higher level impurity). Therefore, only one injection would be needed to obtain the amount necessary for NMR analysis, reducing the time to 1 hour.

Most of the applications discussed in this section deal with semi-preparative HPLC columns using steel as well as radial compression columns. Radial compression uses radial pressure applied to a flexible-wall column to lessen wall effects. Mobile phase has a tendency to flow slightly faster near the wall of the column because of decreased permeability. The solute molecules that happen to be near the wall are carried along faster than the average of the solute band, and consequently, band spreading results. Preparative scale radial compression chromatography columns efficiencies close to those of analytical-scale columns when an adequate radial compression level is used8. Radial compression technology also helps lower the cost by substituting reusable column holders in place of expensive steel columns.

Conclusion

The process described in this article uses a designed approach for the isolation of unknown impurities and degradants in pharmaceutical drug substances. This approach focuses on efficiency so that the success of data collection is maximized. The isolation of pure material is crucial when trying to identify the structure of an unknown impurity/degradant. Once the unknown has been isolated, it can be submitted for structure elucidation using mass spectrometry and NMR. Part II of this article will describe the role of mass spectrometry and NMR in the identification process.

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