LC Troubleshooting

Stability-Indicating Assays

How do you prove everything is separated?

During the past couple of months, I've had several readers write with questions about stability-indicating assays for pharmaceutical products, so I'd like to focus on that topic in this installment of "LC Troubleshooting." Stability-indicating assays are some of the most difficult types of liquid chromatography (LC) separations to develop, because chromatographers can't be sure how many components are present and need to be separated.

Let's look at what these separations are all about and how to develop high-quality assays.

Stability Indication

If you look at the label on your favorite prescription or over-the-counter pharmaceutical product, you'll see a *use before* date. Before this date, the product should remain fully effective under normal storage conditions. The product's shelf life is determined using standardized storage conditions of controlled temperature and humidity, which can be translated into accepted product lifetimes. To determine this shelf life, you must measure two different aspects of the drug after it has been stressed. First, determine its potency, or the amount of active ingredient. It is easy to understand that a significant loss in the amount of active ingredient would make a drug less effective. This measurement is fairly simple because relatively speaking, a large amount of drug is present. Second, determine the degradants or impurities that appear as a result of aging. This determination is much more difficult, because you must anticipate what degradants might be present, and you must measure all degradants that appear at concentrations of more than 0.1% of the parent drug. This portion of the assay falls in the realm of trace analysis.

Developing a stability-indicating assay requires consideration of three aspects of the method: obtaining a representative sample, choosing the separation technique, and selecting the detector. Let's look at each of these aspects.

The Sample

A good deal of the effort in developing a stability-indicating assay is generating a sample that can be used for method development. You want a sample that is a balance between the worst-case scenario and one that realistically represents a naturally degraded sample. Each research group has a slightly different approach, but analysts generally use a set of samples for method development instead of a single sample. For simplicity, most workers focus on the drug substance — the pure drug compound — instead of the drug product, which is the drug substance plus any excipients used to formulate the drug. This process is justified by the argument that the breakdown products of the drug are more likely to be toxic than the inert ingredients added to facilitate formulation, so the degradants should be the primary focus.

One way to prepare samples is to obtain all the compounds that you might expect to be present in the drug substance before it is formulated. Knowledge of the drug's synthetic pathway will help guide you toward selecting compounds in the synthetic scheme that could be present in trace quantities in the final product. Sometimes you can expect that chemical degradation will occur in the reverse order of synthesis, so one or more of the drug's precursors could be good choices to include in the mixture of test compounds. Other sources of potential breakdown products are failed synthetic batches and the mother liquor from a purification step.

Another way to obtain samples for developing stability-indicating assays is to place the pure drug substance under stress intentionally. This process often is called *forced degradation* or *purposeful degradation*, and every laboratory has its favorite recipes, but the general approach is the same. The drug is subjected to acid, base, heat, light, or oxidation. Usually, the goal is to degrade the parent drug by 10–20% or so. Degradation much greater than 10–20% could result in secondary degradants that will complicate the development process.

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This stage of development can be quite time-consuming. For example, you could start by dissolving a portion of sample in 0.1 N hydrochloric acid for the acid degradation experiment and then collect samples at intervals of 1, 2, 4, 8, and 24 h. The reaction then would be quenched in base, and the resulting samples could be analyzed by a simple method that would measure the loss of parent drug. If the degradation was insufficient or too great, you would need to repeat the experiment with stronger or weaker acid, perhaps for different time intervals. For acid, base, and oxidation solution experiments (for example, with peroxide), you can set up the reaction in an autosampler vial, make injections at regular 1-h intervals, and observe the sample change in time. After a week or so of effort, you'll have an acceptably degraded sample from each of these pathways. Sometimes, you'll need to eliminate one pathway if the drug is recalcitrant to its degradation; for example, light.

The Separation

Reversed-phase LC is the method of choice for stability-indicating assays because the samples are generated in aqueous solutions. The polarity of the degraded samples can vary widely; for example, when a nonpolar drug is degraded into smaller polar components. For this reason, you should choose gradient elution for sample screening. The goal will be to obtain separation conditions that allow determination of as many distinct peaks as possible from the set of test samples.

The most common separation variables include solvent type, mobile-phase pH, column type, and temperature. At the beginning of the method development process, you should make changes in variables to force dramatic changes in the separation. The time for fine-tuning will come later. Acetonitrile and methanol are the best solvents; tetrahydrofuran, although often responsible for large changes in selectivity, is incompatible with the low-wavelength detection required for most pharmaceutical compounds.

Low and intermediate pH generally are obtained by using phosphate buffer in the pH 2.5–6.5 range. If your method will be used with a mass spectrometry (MS) detector at some point, it is important to select buffers that are MS-compatible such as 0.1% trifluoroacetic acid (pH $\approx$ 1.9) and ammonium formate (for applications at higher pH values).

Choose two or three column types, such as C8, embedded polar, and cyano phases. Changing from one chain length to another, such as from C8 to C18, or from one manufacturer's C8 column to another's usually provides insufficient change in selectivity at this stage of development.

The column temperature can be a powerful tool to control selectivity, especially with gradient elution. Temperatures of 35–50 °C are good choices because they can be obtained with most equipment and do not put undue stress on a column. It is easy to see that testing all combinations of the variables mentioned would be very time-consuming. Instead, most workers start with a judicious selection of conditions and use the results of each set of experiments to lead them toward additional experiments.

In my laboratory, my colleagues and I take advantage of automation and separation optimization software to reduce the amount of chemist time required for the screening process. I'd choose a 150 mm $\times$ 4.6 mm, 5-μm d_p column because it generates a sufficient number of theoretical plates to separate complex mixtures but can be operated at 2 mL/min for short run times. The core set of experiments should be four runs for each sample. These runs comprise a short and a long gradient at low and high temperatures. Typically, 10- and 20-min gradients and temperatures of 35 °C and 50 °C are used because they provide good chromatography and reasonable run times. The results from these runs can be fed into DryLab separation optimization software (LC Resources Inc., Walnut Creek, California), and they enable the optimization of gradient or isocratic runs throughout the tested temperature range. As many as six columns can be mounted in a column oven for automatic selection of both the column and temperature by the system controller. A program set with one solvent, two or three columns, two temperatures, and three to five samples plus blanks can require 12–24 h to perform but requires no operator intervention.

The hard work starts after the screening runs are completed. Now, you must match the peaks between runs so that each compound can be tracked as the conditions change. Although each sample might contain only four to six significant degradants, different degradation conditions can produce some of the same compounds in addition to unique degradants. For force-degraded samples, peaks with less than 1–2% of the parent drug's area should be ignored to simplify the tracking process. A skilled chemist might need several hours to sort out these peaks; a beginner might require several days.

The Detector

The mass spectrometer is becoming the detector of choice for many LC methods, particularly for determining drugs in biological samples. The sensitivity and specificity of these assays justifies the added price and complexity of MS detectors. However, the UV detector remains the detector of choice for stability-indicating assays. By definition, stability-indicating assays must be able to determine sample components within at least a 1000-fold concentration range from 100% to 0.1–0.05% of the parent drug. This range is well within the reliable performance of UV detectors, whereas it could push the linear range of a mass spectrometer. An MS detector, however, can be very useful in identifying unknown peaks in the final method. For this reason, many workers try to make their stability-indicating assays MS-compatible so that an LC system can be moved to a mass spectrometer and mass spectral information can be obtained for each peak in the run without additional method modification.

Diode-array UV detectors often are used during the development of a stability-indicating assay. A variable-wavelength detector could be used for routine applications, but the diode-array detector is an advantage during development. Stability-indicating assays typically use the parent compound's wavelength of maximum absorbance as the detection wavelength. (With a diode-array detector, each compound could be detected at its absorbance maximum by using individual maxima for routine detection, which raises a whole set of other issues beyond the scope of my current discussion.) By collecting a UV spectrum for each peak in the chromatogram, peak tracking can be simplified. Some manufacturers claim that peak matching in these cases is trivial, and peak-purity parameters can determine if one peak has another hiding under it. In my experience, when the size of the peak approaches the background noise intensity, the automatic peak matching routines are of limited use. Spectral information, however, can be very helpful when manually matching peaks between runs.

The Final Method

After completing the screening runs, you should have a good idea of how many dis-

tinct compounds are present in the sample set. This estimate should allow you to reduce the number of samples from the full set to a subset that contains all the components. The earlier you can reduce the number of samples, the fewer runs will be required in the rest of the development process. The next step is fine-tuning the separation. With the help of optimization software, experience, and luck, you should be able to find conditions that separate all the compounds of interest.

At this point, you can adjust the pH a bit, try another manufacturer's column, or try a solvent blend to pull a couple of recalcitrant peaks apart. Sometimes you cannot separate all the peaks, so choose conditions that separate all the minor peaks from the parent. If one of the overlapped peaks appears in a real sample, you probably can find conditions that will separate those two peaks and ignore the overlap of other compounds. With luck, the drug will be stable, and you never will observe any degradants in real samples, so you won't have to deal with an overlap of minor peaks. When you are happy with the separation, you should rerun all the original degraded samples to be sure you still can separate every peak. You also could try another column, preferably from a second manufacturing batch. Because the stability-indicating assays are designed for complex samples, column-to-column differences can be critical. It is better to know before the method is validated, rather than after it is put in routine operation.

Identifying each peak in a chromatogram might not be necessary, depending upon the size of a peak. Using known compounds from the synthetic pathway, LC–MS, and other techniques can help identify unknown peaks.

The validation stage follows. Validating stability-indicating assays is more vigorous than validating some other assay types because of the complexity of the separation. Validation usually calls for testing the influence of each separation variable to determine the limits within which the separation must be performed.

After the method has been validated, you can use it to support studies of sample stability under specific temperature and humidity conditions and establish shelf life and other product characteristics. Often, you can use a stability-indicating assay with few or no changes as a method to determine impurities during manufacturing or to determine the potency of a drug substance or formulated product.

Conclusions

There's nothing magic about stability-indicating assays. They're just LC methods for analyzing trace components. Many of the same method development techniques would be used to develop methods for determining trace environmental compounds or minor components in an industrial manufacturing process. Many combinations of test variables are possible, so it is important to use a systematic approach for developing a complex separation. First look at the variables that are both easy to change and likely to yield the most dramatic changes in selectivity. Early elimination of variables with little effect upon the separation will greatly reduce the work involved in method development. For example, early selection of mobile-phase pH or column type could cut the number of experiments by one-half or more.

Addendum

Following a recent discussion in "LC Troubleshooting" about ion suppression with LC–MS methods (1), a reader pointed out that I should have referenced the laboratory that is credited with pioneering the infusion experiment technique for ion suppression (2). Reference 3 is another good source of information about ion suppression. As always, your feedback helps improve the quality of the information shared in this column.

References

(1) M.D. Nelson and J.W. Dolan, *LCGC* **20,** 24–32 (2002).
(2) R. Bonfiglio, R. King, T.V. Olah, and K. Merkle, *Rapid Commun. Mass Spectrom.* **13,** 1175–1185 (1999).
(3) R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, and T. Olah, *J. Am. Soc. Mass Spectrom.* **11,** 942–950 (2000).

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