LC TROUBLESHOOTING

It's Gonna Break

Every method will fail sooner or later. Are you prepared?

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From the days of my involvement in the Boy Scouts, both as a youth and a leader, I have no trouble remembering the motto: "Be Prepared." This also is a good motto for those of us involved in using liquid chromatography (LC) methods to get our daily work accomplished. Just because LC is, by some accounts, the most widely used analytical technique in the world, does not mean that it is the most reliable technique. Every LC method will fail, sooner or later. One of the signs of a competent chromatographer is the ability to get that failed method back up and running quickly. Although wisdom and troubleshooting strategies are gained with years of experience, there are some fairly simple practices that can trump years of experience. This is the subject of this month's "LC Troubleshooting" installment. These strategies are all about being prepared — what you can do in advance that will shorten the down time when a method does fail.

It's All About Robustness

The International Conference on Harmonization (ICH) provides a series of documents that provide guidance for workers in the pharmaceutical industry when developing, validating, and using LC methods for the analysis of pharmaceutical compounds and their impurities. Two key documents for the current discussion are "Q2A Text on Validation of Analytical Procedures" (1) and "Q2B Validation of Analytical Procedures: Methodology" (2). Both of these documents, as well as many other regulatory documents, can be found at the Food and Drug Administration's (FDA) website listed in the references. Although these documents are prepared specifically with agency-regulated pharmaceutical methods in mind, the information they contain applies equally well to most LC methods for routine analysis.

The word that describes the task at hand is "robustness." Q2A defines robustness as follows (1):

"The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage."

This is complemented by a statement about robustness testing from Q2B (2):

"The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters."

Both quotations refer to "deliberate variations in method parameters." This implies that some formal testing should be done. We need to deliberately test what will happen when the LC method conditions are changed in a way that might happen by accident or other normal variation in the method. Let's consider which parameters might be good to test.

The Key Variables

Most methods will exhibit more sensitivity to changes in some experimental parameters as opposed to others. What should be tested? Q2B lists mobile phase pH, mobile phase composition, different columns, temperature, and flow rate. These all are parameters that are tested easily by making deliberate changes in the method. We will look at each of these parameters in a moment. Depending upon the method, one or more of these might not apply — and additional parameters can be important.

The important practice here is that testing should occur before the method is put into routine use. This would be

before validation, if you formally validate the method. Testing helps you identify the soft spots in the method, select the best conditions for operation, and develop troubleshooting strategies to correct problems when they do occur. If you know, for example, that the method is very sensitive to small changes in mobile phase pH, you will take extra care when adjusting the mobile phase pH, so as to avoid problems. If you know that the method will perform adequately at any temperature between 30 °C and 40 °C, you can choose to operate at 35 °C so that some tolerance to error is built into the method. If you know that the resolution for a critical peak pair gets worse with lower percentages of acetonitrile in the mobile phase and better when more acetonitrile is used, you will know how to adjust the mobile phase when you see the resolution decrease. All of this knowledge will improve the quality of the method, as well as your skills in running it. Finally, when testing the effect on the method of changing a variable, do not look just at retention time — the resolution of peaks of interest generally is more important than retention.

Mobile Phase pH

If your sample contains ionizable compounds, the mobile phase pH will be a variable that will have a profound effect on retention times. For reversed-phase methods, it generally is advantageous to work at a mobile phase pH that converts the analyte(s) to the un-ionized form. Under these conditions, the analyte will be neutral and, thus, less polar, so it will be more strongly retained. Ionic compounds often are retained poorly under reversed-phase conditions. For an acid, more than 99% of the compound will be un-ionized at 2 pH units below the pK_a. The same holds for bases at 2 pH units above the pK_a. Often the situation is complicated by the presence of acids and bases in the same sample and by multiple pK_as for various analytes, so that a single mobile phase pH will not convert all compounds to the un-ionized form. In addition, the column places some practical restrictions on pH selection. For typical silica-based reversed-phase columns, hydrolysis of the bonded phase takes place at pH < 2, and the silica dissolves at pH > 8. There are specialty columns that tolerate conditions outside the $2 <$ pH < 8 limits, especially on the high end.

The net effect of all of these variables is that the mobile phase pH often is closer than 2 pH units to the pK_a of at least some compounds in the sample. This means that the method will be susceptible to changes in pH. Once you have determined the best pH for the method, check the effects of a change in pH on the separation. I recommend checking at least ± 0.5 pH units from the desired pH. When you find the pH range that is tolerated, generally it is best to run the method in the middle of the range so that some small variation in pH can be tolerated.

A couple of other pH-related comments should not need to be made, but it is surprising how often they are ignored. First, use a buffer that is effective at the pH you have chosen. As a rule, a buffer is effective ± 1.5 pH units from the pKa of the buffer. For LC methods using UV detection, most com-

monly phosphate is used for $2 < pH < 3.5$ and $6 < pH < 8$, whereas acetate is chosen for $3.5 < pH < 6$. Also, remember to adjust the pH of the buffer before adding organic to the mobile phase — pH meters do not give the same readings when organic solvents are present as they do in water. Finally, the mobile phase pH and the pK_a of sample compounds will change when the temperature is changed, so be sure to thermostat the column.

Mobile Phase Composition

During method development, usually you will identify the organic solvent that gives the best separation by testing acetonitrile, methanol, and sometimes tetrahydrofuran. Then you will adjust the mobile phase organic concentration to get the best isocratic separation or the starting and ending concentration plus the slope for the best gradient separation. Data you gather during this development process can be used to help you understand the sensitivity of the method to a change in the organic composition of the mobile phase. I often refer to "The Rule of Three," which states that a 10% change in organic solvent will result in approximately a three-fold change in retention factor k, or for well-retained compounds, retention time. This converts to a 10–20% change in retention for a 2% change in organic. For your method, you should quantify this approximate rule, so you will know the impact of a small error in mobile phase preparation. I recommend checking ±2–5% organic for isocratic separations. For gradients, check −2–5% initial organic with +2–5% final organic, and vice versa. As with the other parameters, it generally is best to center your method conditions in the middle of a region where a small increase or decrease the in the parameter setting can be tolerated.

Different Columns

Whereas the mobile phase composition can be varied in a continuous manner and is under the operator's control, column changes are discrete, and under the control of the column manufacturer. Eventually, the LC column will no longer provide a satisfactory separation and must be replaced. You would like to be sure that the next column you buy will give an equivalent separation — thus, the "use column X, or equivalent" statement in many methods. Just what is equivalent? First, you should check to make sure the column you have chosen will really do the job. A check of three columns is a good place to start. This should include two columns from the same batch and one from another batch. By batch, I mean the silica particles to which the bonded phase is attached. This test will give you an idea of the column-to-column variability you will encounter under normal conditions. Column reproducibility is much better with today's high-purity, type B silica columns than the type A columns that were more common 15 years ago. Hopefully, your understanding of the effect of the other variables (pH, percent organic, temperature, and so forth) will help you decide how to adjust the conditions to achieve a successful separation when minor changes in the column are encountered.

Depending upon the method and your company policies, you might need to have a second source of columns, in case

the primary supplier can no longer provide them. If you have been in the LC business for very long, you know that all C18 columns are not created equal. A separation on one manufacturer's column might look substantially different on one from another manufacturer. Recent publications on the comparison of column selectivity (for example, references 3 and 4) and column vendor's comparison guides can be used to help guide you in selection of a truly equivalent column as a backup for your primary column.

Temperature

I continue to be amazed at how many people operate their LC systems under "ambient" conditions — in many laboratories this can vary by 5 °C or more over the course of a year, and sometimes over a day's time! A good guideline is that retention for reversed-phase separations changes by approximately 2% for each 1 °C change in temperature. It is easy to see that in many laboratories, retention time changes of 30 s or more can occur during a day's run. This could cause a peak to drift out of a data system peak window or to be confused with another closely eluted compound. Furthermore, changes in selectivity as a result of temperature changes can compromise the resolution of a method, especially for ionic compounds. You should include temperature control as a standard part of every LC method. I recommend starting with a temperature slightly above room temperature, so that it is easy to control, such as 35 °C. When you have selected the final temperature, check the impact of the temperature on the separation by checking the method at ±5 °C from the normal setting. Select the final conditions in a region where a few degrees fluctuation will not cause the method to fail.

Flow Rate

For most isocratic applications, mobile phase flow rate merely affects retention, so little impact on resolution will be seen. In gradient elution, on the other hand, flow rate can result in changes in selectivity. However, in either case, the change in flow rate will be much less than 10%, except in cases of severe leaks, check valve failures, or improper instrument settings. For modern 3–5 µm particle columns, these small changes in flow rate will not have any noticeable effect on peak width. Thus, normally encountered flow rate changes are unlikely to have any negative impact on the separation, but it is easy to check to determine the effect of a ±10% change in flow rate.

Validation

Depending on company policy, government regulations, and personal preference, you might or might not have to include robustness testing as part of the validation. If you have shown in your method development process that changes in one or more variables do not negatively impact the method, that is probably ample evidence to obviate testing of the variable during validation. Some workers like to check each variable independently, whereas others use a multiparameter experimental design approach that will minimize the number of experiments required to evaluate the effect of the various parameters. In any event, it is a good idea to include some robustness testing in the method validation process. By showing the effect of changes in the various parameters, and including these data in your method document, you can make life easier on the end user of the method. For example, peaks A and B might move together as the column ages, but you have shown that A and B can be pulled apart by an increase in temperature, as little as 3 °C. Now, when you see the problem, you know how to adjust the method to keep it operating properly — and still be within the allowable method conditions.

Conclusions

If you do your method development with a goal of gaining a better understanding of how peaks move relative to each other as conditions are changed, you will find that troubleshooting will be much easier. You will know what small change in one or more parameters will be required to bring the method back into specifications. A good system suitability test will allow you to evaluate the method performance before you run valuable samples. If you include allowable adjustments as part of the method document, you can make such changes when system suitability fails, and be back up and running quickly. If you do not build flexibility into the method, much more work will be required to test and document even small changes in the method.

Some people look at the ICH recommendations for robustness testing as placing extra requirements on us that inhibit us from doing the important part of our work. A better perspective is that they encourage us to produce a better product — our method. If we do "good science," we should not have to worry so much about regulatory restrictions.

References

(1) Guideline for Industry. Q2A Text on Validation of Analytical Procedures. http://www.fda.gov/cder/guidance/index.htm (March 1995).

(2) Guidance for Industry. Q2B Validation of Analytical Procedures: Methodology. http://www.fda.gov/cder/guidance/index.htm. (November 1996).

(3) J.W. Dolan, A. Maule, L. Wrisley, C.C. Chan, M. Angod, C. Lunte, R. Krisko, J. Winston, B. Homeier, D.V. McCalley, and L.R. Snyder, *J. Chromatogr., A* **1057**, 59 (2004).

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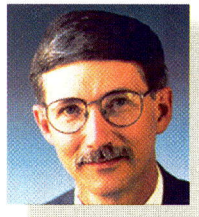

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Erratum

Figure 1 on page 32 of the January 2006 installment of LC Troubleshooting (J.W. Dolan, *LCGC* 24[1], 32 [2006]) was incorrect. The gradient overlays in the two chromatograms were reversed: Figure 1a is for a system with 3.5 mL dwell volume and should have the overlay that is shown in Figure 1b, and vice versa. A discussion of this will be given in the May 2006 LC Troubleshooting column.