L C T R O U B L E S H O O T I N G

Peak Problems

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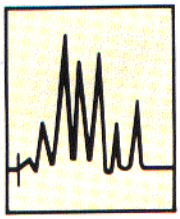

This month's installment of "LC Troubleshooting" addresses two readers' problems with peaks in their chromatograms. The first reader expresses concern about the sudden appearance of a negative peak after months of satisfactory use of a method. The second reader attributes a loss of resolution to a change in the column characteristics. Closer examination, however, indicates several other potential sources of the problem.

NEGATIVE PEAKS

Q: I am analyzing a pharmaceutical preparation by reversed-phase LC with an acetonitrile–water mobile phase buffered to pH 3.5 with a phosphate buffer. The method had been working for months without problems, but one day I noticed an extra peak in the chromatogram. This peak is always negative and has the same retention time from run to run. I have tried changing the water and the acetonitrile, but the peak always remains, even when I inject a reagent blank to which no sample has been added. This is an isocratic assay, using an autosampler, a manually mixed mobile phase, and a UV detector set at 220 nm. What could be causing the problem, and how do I go about isolating its source?

JWD: Negative peaks generally have one of two causes. First, injecting a compound that has a lower absorbance than the mobile phase can give a normal peak, but one that goes below the baseline instead of above it. This phenomenon is intentionally used in indirect photometry, in which a UV-absorbing mobile phase is used to allow the detection of non–UV-absorbing sample components. The second cause is a bit more complicated. If the mobile phase contains a UV-absorbing component or contaminant, the baseline will be elevated. Injecting pure solvent (without the UV absorber) creates a "hole" in the mobile phase (that is, a region with less UV absorbance), which will pass through the column at the same rate as a sample of the UV-absorber. When this region passes through the detector, a negative peak is observed. The stepwise isolation of either of these problems is similar. For a detailed discussion of negative peaks, see reference 1.

The fact that the problem appeared suddenly suggests that something changed in the overall method, so first you should examine your assay records to see if you can correlate your observations with any changes in the method. Look for changes in the mobile phase (a new lot of solvent or a different supplier), changes in the sample, changes in the system (maintenance or breakdown), a different column, or other changes in the laboratory routine (for example, a new dishwasher). Once you have eliminated these obvious causes, you need to change the operating environment step by step until you find the source of the problem. I would start by eliminating the sample as the possible source of the problem. Make an injection of mobile phase alone. If the problem goes away, you have discovered that you are introducing the problem with the sample (see discussion below). If the problem persists, however, the injection device is suspect: An autosampler can become contaminated and introduce interferences into the system if the needle-washing system becomes blocked or contaminated. You can verify this by bypassing the autosampler and making a manual injection; if the problem disappears, thoroughly clean or replace the autosampler parts involved in cleaning the needle. (Consult the operators' manual for proper cleaning procedures for your specific instrument.) If this corrects the problem, you should institute a regular cleaning procedure to prevent its recurrence.

If the problem disappears when you inject only mobile phase, the source is probably the mobile phase or the sample. If no obvious changes in the sample have occurred, replace the mobile phase (as you did). When you switch to a new mobile phase, be sure to use solvents from a different lot in order to eliminate a problem lot of solvent. Don't forget to make fresh buffer with another batch of buffer salt. Before you introduce the new mobile phase, it is a good idea to flush the system thoroughly with the strong solvent of the mobile phase (acetonitrile in your case). This will strip strongly retained material from the column and flush out the rest of the system as well. If the problem disappears with the new mobile phase (it didn't in your case), you may or may not want to change one mobile phase component at a time to isolate the specific source. If the problem persists, you might try changing the column, but I don't think your results will change.

If you still have a problem when you use fresh mobile phase components, you must be introducing a contaminant with your sample. First, check to be sure that the sample itself has not changed. Is it from the same source? Has it been obtained, stored, transported, and handled using the same protocol as before? Next, check for contamination during sample pretreatment. The best way to do this is to work backwards from the injection step, injecting samples of the reagent blank. You will see no extra peak until you back up to the contaminating step. For example, let's take a sample that is dissolved, extracted, evaporated, reconstituted, and filtered before injection. Inject the final injection solvent alone, then solvent that has been filtered, then a blank of clean solvent that is evaporated, reconstituted, and filtered. Continue this until you find the problem step. Besides considering potentially contaminated solvents, filters, and other reagents, don't forget to consider glassware washing procedures, the blow-down gas used for evaporation, the vacuum source, and other less common sources of contamination.

Although it is tempting to change the mobile phase and the column simultaneously and to use all new sample pretreatment reagents in an effort to correct the problem, try to avoid this "shotgun" approach. You may solve the problem, but you will have learned little to prevent it from happening again or to solve it more quickly the next time it occurs.

COLUMN PROBLEMS?

Q: My method uses a 25-cm C18 column (with a guard column) and a 35:65 methanol/water mobile phase at 1 mL/min. I inject 50 µL of sample dissolved in 40:60 acetonitrile/water; the peak elutes at ~3.5 min. The column on which I developed the method worked fine, but after I got a new column (same vendor, same part number), another one of the peaks interfered with my sample peak. How can I be assured of choosing a supplier so that I don't have these column-to-column variations?

JWD: Before we blame column-to-column variation as the source of your problem, we should consider a number of other variables in your separation that are less-than-ideal. Let's look at each of these to see what we can learn about developing a rugged separation

that will not change when a new column is installed. The key points are summarized to the right.

First, consider the sample injection. It is a good practice to use the the mobile phase solvents as the injection solvent. Thus, you should inject an acetonitrile–water sample solvent rather than methanol–water to avoid any unwanted changes in selectivity (relative peak separation) caused by injecting dissimilar solvents. It is not always possible to use the same solvents, but your goal should be to design the sample preparation steps so that the sample ends up dissolved in mobile phase.

The second concern is a combination of injection solvent strength and sample size. Your injection solvent should be no stronger than the mobile phase; if this is not possible, you should limit the injection volume to <20 μL. Because acetonitrile is a stronger solvent than methanol, you will need to use a volume percent of acetonitrile in the injection solvent that is lower than the volume percent of methanol in the mobile phase. Otherwise, as in your case, using an injection solvent stronger than the mobile phase (40% acetonitrile is stronger than 35% methanol) can cause peak broadening, peak splitting, and loss of resolution. If you have to use acetonitrile, your choices are either to inject a smaller volume (<20 μL) or to dilute the injection solvent so that it is weaker than the mobile phase. Often you can dilute the sample and inject a larger volume so that the mass of injected sample stays constant. In the present case, you probably could dilute the sample 3:1 with water and inject 150 μL to obtain a better-quality separation. The overall guidelines are to use <20 μL of a solvent stronger than the mobile phase or to use a solvent weaker than 50% of the mobile phase strength if injections of ≥50 μL are to be made.

Next, let's look at the mobile phase. When selecting the mobile phase, you should adjust its strength so that your peaks of interest elute with a capacity factor (k') between about 1 and 20 (or ideally, between 2 and 10). This will place your sample bands in a region that is most likely to give adequate resolution. Recall that capacity factor is calculated from the equation

$$k' = (t_R - t_0)/t_0 \qquad [1]$$

where t_R and t_0 are the retention time and column dead time, respectively. The dead time is measured as the first disturbance in the baseline; or, we can estimate it from the observation that 4.6-mm i.d. columns have ~1 mL of dead volume for every 10 cm in length. Therefore, the dead volume is ~2.5 mL for your column; at 1 mL/min this makes $t_0 \approx 2.5$ min. So $k' = (3.5 \text{ min} - 2.5 \text{ min})/2.5 \text{ min} = 0.4$. When $k' < 1$, not only is the resolution likely to be poor, but the probability of interferences with unretained "garbage" at the solvent front is increased. So you should use a weaker mobile phase in order to increase the k' of the sample band. In general, we can estimate that k' will change by a factor of 2 to 3 with a 10% change in mobile phase strength; this would mean that you could increase k' to ~1 by dropping the methanol content of your mobile phase to 25%. If you want to adjust your mobile phase in a more systematic manner, use the resolution map technique discussed in reference 2.

You also may want to consider the use of mobile phase additives to improve peak shape and to minimize the impact of column-to-column variations. Buffers are used to control sample ionization; acids will help suppress tailing of acidic compounds; and triethylamine will help suppress tailing of basic compounds. These additives also can be used in combination. Using mobile phase additives will help to compensate for small differences in bonded-phase coverage of the silica packing material among different manufacturing lots of columns. Guidelines for selecting the proper additive as well as for developing LC methods can be found in reference 3.

Your decision to use a guard column is a good one; one should be used in nearly all cases to help prolong the life of the analytical column. Be sure that the guard column packing matches the analytical column packing as closely as possible; some manufacturers offer guard columns that match their analytical columns. In other cases, you may choose a third-party vendor's guard column, but make sure you use the same type of bonded phase (C18 in the present case).

Once you have made the needed adjustments in your method (or when you are developing a new method), you should ensure that the method is robust. Small and expected changes in the operating environment should not make the method unusable, but if they do, you should know how to adjust the conditions to correct the problem. This is called ruggedness testing. What happens if the mobile phase is varied by ±1% in composition? Over what range is the method linear? Does the method still work when you use a column from another lot? If you aren't controlling the column temperature, what happens if the room temperature changes? If you check the expected variations and errors in the method before you start to use the method for routine samples, you will know how to adjust the conditions if changes occur.

In summary, you need to adjust the mobile phase so that you have a higher potential for a successful separation, choose injection conditions compatible with the mobile phase, and test the method for ruggedness. Only when these steps are finished will you have enough information to determine confidently whether or not you have a column problem.

Goals for a Rugged LC Method

Injection solvent:
- —ideally, use mobile phase
- —use sample solvent that is no stronger than mobile phase
- —inject <20 μL unless solvent is <50% of mobile phase strength

Capacity factor:
- —adjust mobile phase strength so that $1 < k' < 20$

Mobile phase:
- —use additives to control ionization and peak shape

Column selection:
- —use a matched guard column to prolong column life

Ruggedness testing:
- —check column, mobile phase, and other variables before starting routine use of the method

REFERENCES

(1) J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems* (The Humana Press, Clifton, New Jersey, 1989), chapter 15.
(2) J.W. Dolan, *LC•GC* **6,** 1052–1056 (1988).
(3) L.R. Snyder, J.L. Glajch, and J.J. Kirkland, *Practical HPLC Method Development* (Wiley–Interscience, John Wiley & Sons, New York, 1988).

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Lafayette, California, USA, and is a member of the Editorial Advisory Board of LC•GC.

Bulletin

Regnier receives ACS award. Professor Fred E. Regnier of Purdue University received the American Chemical Society's Award in Chromatography at the ACS national meeting in Dallas on April 10. Regnier was honored for his achievements in the field of biochemical chromatography, including his development of new column packings and his contribution to the fundamental understanding of the macromolecular retention process. Colleagues recognize him as a pioneer in the use of high performance liquid chromatography for analyzing proteins and nucleic acid. He also was noted for his interaction with the general scientific community and for his contributions in education. He is a member of the Editorial Advisory Board of *LC•GC*.