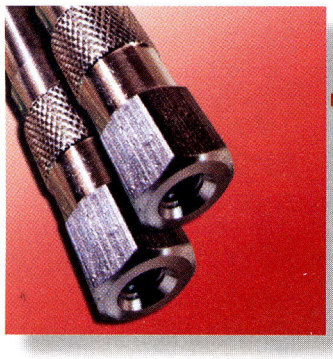

LC TROUBLESHOOTING

Too Little or Too Much

What should you do when a peak is too small or too large?

John W. Dolan
LC Troubleshooting Editor

The topics for this month's "LC Troubleshooting" column come from the attendees of some of the method-development and troubleshooting classes that I have taught recently. One of these problems involves a liquid chromatography (LC) method in which an occasional sample gives a peak that is too small. The second case is one in which the analyte peak appears when a drug-free placebo sample is run. Although the topics are interesting in themselves, they also provide practical examples of the application of some of the general troubleshooting techniques discussed in last month's "LC Troubleshooting" (1).

Too Little

The first case involves a method for the analysis of a drug extracted from a tablet. After running the system-suitability test, a duplicate injection of the reference standard is made. Then five samples are run, each injected in duplicate. The sequence of two standard injections and ten sample injections is continued, until all the samples are analyzed, ending with duplicate injections of the standard. The problem is that occasionally — perhaps 1 sample in 20 — one of the sample injections has an area that is several percent lower than its duplicate, exceeding the allowed variability of the method. When the sample is reinjected in duplicate, invariably both results match the larger of the two previous attempts at analysis. The question is what has caused this and what corrective action can be taken.

The cause and solution are not immediately obvious to me, but there are several possible causes, and some additional experiments might help to clarify the source. I suspect that the odd injection is the result of injecting a smaller volume of sample, so my isolation experiments would focus on that.

Blocked needle: Is a piece of the vial septum partially blocking the sample needle now and then? Some septum types can be more susceptible to "coring" than others. One popular type of septum has a PTFE film on the bottom and a polymer seal on top. If the needle cuts a piece of the seal out, it could be drawn into the needle and cause problems. A change to a presplit septum or simply a PTFE-film septum might be a solution if this were the problem. Sometimes damage to the sample needle will cause the tip to get roughened or sharp so that it cuts the septum instead of tearing or puncturing it; a needle replacement should eliminate this as a problem source.

Particulate matter in the sample could cause problems similar to drawing a piece of septum into the sample needle. Inspect the sample for particulates to see if this could be a problem. Each sample could be filtered or centrifuged before injection to ensure that no suspended particles remain. Other sources of particulate matter are the mobile phase (not likely) or particles released from worn pump seals. If either of these is suspected, correct the problem at its source. Addition of a 0.5-µm porosity in-line filter just upstream from the autosampler also would serve to trap any particles from the pump or mobile phase before they caused problems in the autosampler.

Bubbles: If a bubble were drawn into the needle instead of or along with sample, the sample volume would be low, giving results consistent with those

observed. I would make sure that the needle and connecting tubing to the syringe mechanism was thoroughly purged and did not contain bubbles. Most autosamplers have an adjustable syringe speed to accommodate viscous samples. With viscous samples, a fill rate that is too fast can create bubbles in the syringe, which will compromise precision. If excessive sample viscosity is observed and this is suspected, a change in the syringe fill rate might help.

Look at historical data: It would be useful to inspect historical data to determine the real frequency of failure and any patterns that might exist. For example, are failures only associated with samples or also with the reference standards? Do the failures occur at any particular place in the sequence, such as one of the last two samples of each five-sample group? Is the amount of error constant, or does it vary from one failure to the next. Are the errors really unique or are they just the extremes of error about a mean value? This could be determined by plotting a distribution of all sample results to see if it forms a Gaussian distribution of error about a mean, as expected. Are the errors just those samples that exceed the acceptance criteria, but fit the distribution? If this is the case, there might be nothing wrong with the equipment, but instead the method might have too much variability for its intended purpose.

Additional studies: If no solutions are obtained from the above equipment changes or data review, it will be useful to make additional studies in which the number of variables is reduced. I would make a large sample volume, perhaps by combining several sample extractions into a single vial. This removes any sample-to-sample variability. Make an extended series of injections from the same sample vial. For example, perhaps 50 injections could be made in an overnight run. Does this sequence result in any failures? If so, is there a pattern or frequency that can be used to help track the source of the problem? It might also be useful to make a similar series of injections from a single vial of reference standard and make the same examination of the data to determine if the problem is associated with the sample or standard. If both sample types have the problem, the source is likely in the equipment. If only one has the problem, the source might be related to the sample type.

Autosampler performance test: At some point it may be useful to check the autosampler performance independent of the method. Use a well-behaved sample for this test. For example, put a C18 column on the system and make a 2-µg/mL sample of anthracene. Set the flow rate at 1–2 mL/min and the UV detector at 250 nm. An injection of 10 µL in an 80:20 (v/v) methanol–water mobile phase should give a peak with a retention factor k of approximately 4–5 (4–9 min retention time on a 150 mm × 4.6 mm column). Under these conditions, inject $n = 6 \times 10$ µL. The relative standard deviation of peak area should pass the autosampler specifications — I would expect to see a maximum of 1% RSD. Many autosamplers will perform with 0.3–0.5% RSD under these conditions. If this is part of your periodic system check suite, compare it to historical results. If the error is larger than 1% RSD, the autosampler should be serviced. If the system will not perform adequately under ideal conditions, it cannot be expected to perform adequately for sample analysis.

This discussion should lead to discovery of a failure pattern and source of error. Once this is found, corrective action can be taken to resolve the problem. It might be appropriate to include periodic checks or preventive maintenance to avoid a repeat of the problem in the future.

Too Much

The second problem also had its source in a method to determine drug concentrations in a drug tablet extract. The method for analysis of the drug tablet appeared to work adequately, but when an extract of the placebo tablets — those containing all components except the drug — was injected, a tiny peak with the same retention time as the drug appeared. Each component of the placebo was then analyzed independently, and it was discovered that the problem occurred only when an injection of a polymer excipient was made. The question related to what was causing this problem and if it could be eliminated. Or if it could not be eliminated, was it justifiable to subtract the background peak from the reported drug concentration?

It seems to me that one of two possibilities exist. One is that the polymer might be contaminated with drug or might contain another substance that is coeluted with the drug. Alternatively, the presence of the polymer might be releasing drug that is bound to the system and causing it to be eluted in the polymer injection. Let's see how we might isolate the problem.

Contaminated excipient: The first case is the easiest to examine. If it is available, I would move to another LC system that had not been used with this particular analysis and install a new column and fresh mobile-phase components. If a second LC system is not available, thoroughly clean the system, replace the solvent reservoirs, and install a new column. Repeat the analysis of the polymer sample and see if the interfering peak appears. If it is present, it must be coming from the polymer sample. If the peak is absent, the polymer is free of contamination and the second option should be pursued. If the peak is present, I would make up a new polymer sample, hopefully from a new batch of polymer. Take special care to ensure that all glassware, pH meters, and other apparatus that contact the sample during preparation are extra clean, so they are not a source of contamination. If the new sample is clean, the original was contaminated and should not be used.

If the peak persists, it must be determined if the peak really is the drug or another compound that co-elutes. This will require additional analytical work such as running the sample under different conditions (for example, an alternate method, different column type, different mobile phase, or different analytical technique). If an LC–mass spectrometry (MS) system is available, it might be possible to compare the mass spectrum of the problem peak with the drug to determine if the two are the same or are different. If the peak is another compound, it must be treated as any other impurity from a regulatory standpoint, including separation from the drug peak during analysis. If the drug is always present in the polymer (a

highly unlikely case), a drug-free source of polymer must be found.

Drug bound to system: Once the polymer sample is shown to be free of the drug, it is most likely that the polymer is somehow releasing bound drug from the LC system. The location of the drug would have to be from the point it is picked up from the sample vial to the head of the column. If drug were bound within the column and were released, it would have a different retention time than injected drug. Simple carryover from drug that is physically trapped in poorly flushed fittings is unlikely, because I would suspect that the extra peak would appear when polymer-free blanks were made, too. I would examine the autosampler flushing procedure, making changes in the flush solvents that might increase the solubility of the drug, such as a change in pH or organic solvent. If the problem persists, a change in the internal surfaces might be necessary. I would start by replacing the sample loop and connecting tubing with another material. For example, stainless steel can be replaced with PEEK (poly ether ether ketone) or titanium tubing. If this is unsuccessful, the valve rotor might need to be replaced with another material. Many injection valve manufacturers offer alternate rotor-seal materials. An in-line filter or guard column could also contribute to the problem, so these items could be removed from the system to see if any difference was observed. Hopefully, one of these changes will locate the source of the problem and allow it to be eliminated.

Conclusions

The two case studies covered this month illustrate some of the perplexing problems that occur during analysis of samples by LC. There is really nothing special that sets these problems apart from any other problem. We just have to make logical experiments to help isolate the problem source. I rely heavily on the divide-and-conquer technique discussed in last month's "LC Troubleshooting" (1) to eliminate possible causes of the problem until I am left with just one.

References

(1) J.W. Dolan, *LCGC North America* **27**(12) 1040 (2009).

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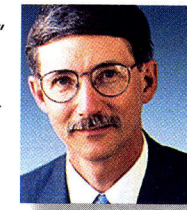

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