LC Troubleshooting

Understanding Split Peaks

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Is it one peak or two?

Peak splitting in a liquid chromatography (LC) separation results from several problems. This month's "LC Troubleshooting" looks at several possible causes of peak splitting and presents some guidelines for isolating the source of peak splitting. Because some cases of peak splitting can be indicative of method problems that should be corrected, it is important to isolate the problem source carefully, if at all possible, so you can make method changes and avoid or delay future problems. In the end, your method will be more rugged.

One Peak or Two?

Often, the first question analysts ask when they observe split peaks is "is this one distorted peak or are there actually two compounds that are partially resolved?"

We encountered this situation with the problem discussed in last month's "LC Troubleshooting" (1). Figure 1 shows portions of the chromatograms that were of concern. In Figure 1a, a shoulder on the front of the main peak became a second peak (Figure 1b) when we reduced the amount of sample. Changing the mass of injected sample is a simple way to help elucidate the characteristics of a chromatogram. If the shoulder were a distortion of a single peak, it would be more likely to change in proportion to the injection size than to resolve into a second peak, as seen in Figure 1. After changing the injection mass, we could take logical steps to change the chromatographic conditions and find conditions that provide acceptable resolution of two different compounds.

How Many Peaks Are Affected?

In the example above, the chromatogram comprised what was assumed to be a single peak in an LC–tandem mass spectrometry (LC–MS–MS) run. For most methods, however, more than one peak exists in the chromatogram. These peaks can be either multiple sample components that the LC method has separated in an LC–UV run or a sample and an internal standard peak in an LC–MS–MS run. In either case, when chromatographers know that more than one peak is present, they can gain additional information about the nature of the problem by examining all peaks in the run.

The examples in Figure 2 illustrate this situation. When every peak in the chromatogram is distorted in a similar way — either by severe tailing as in Figure 2a or by splitting as in Figure 2b — it indicates that the problem relates to the sample before separation occurs. The most common causes of such distorted peaks for the entire chromatogram are a blocked frit or a void at the head of the column.

Figure 3 shows one possible explanation for how a blocked frit could distort peaks. In these sketches, a cross-section of the column shows the frit at the head of the column. When the frit is working properly (Figure 3a), the mobile phase flows through freely in more-or-less parallel paths. A sample introduced under these conditions would be distributed evenly across the top of the column and would be eluted through the column in the normal manner. As Figure 3b shows, the frit can become

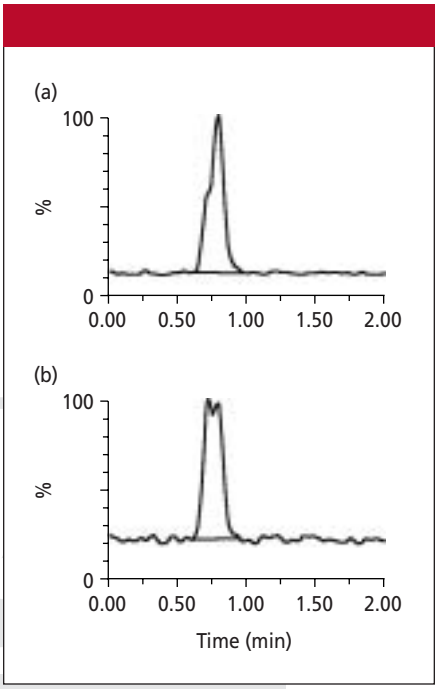

Figure 1: Split peaks due to the presence of a second component. (a) 25 ng/mL and (b) 10 ng/mL of drug (second peak) in plasma.

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partially blocked by a bit of particulate matter from a pump seal or by a poorly filtered sample. This blockage will disturb the flow profile at the head of the column and enable some injected molecules to reach the column after another portion of the sample. This distorted profile occurs before the sample is chromatographically separated, so the distortion will follow each sample band through the column and result in a similar appearance for every peak (see Figure 2).

Another cause of peak distortion at the head of the column is a void in the packing material. In some cases, a void appears as a settled packing bed; in others, it looks like a wormhole in the packing. In these cases, some molecules could travel through the first portion of the column faster than others as they sweep down the channel. Again, because the distortion takes place before chromatographic separation, it affects every peak in a similar way.

Correcting the Problem

Chromatographers have two standard ways to fix a blocked frit. One is to reverse-flush the column, and the other is to replace the frit; each method will fix the problem roughly one-third of the time. Workers can hope that reverse flushing will displace the contaminants from the frit and enable normal column performance. Although columns come with an arrow that indicates flow direction, nearly every silica-based column can be used in either direction without damage. (Check with the column manufacturer if you are uncertain.) Simply disconnect the column and reverse it. Leave the new outlet end disconnected and flush 20–30 mL of mobile phase through the column to displace any loose particulate matter before connecting the column to the detector. It is acceptable to leave the column in the reverse direction for use.

Frit replacement was a common procedure in the past, but we are unaware of any laboratories that still regularly replace frits. The demise of frit replacement has several reasons. First, most columns used to come with a replacement frit, but this is no longer the case, so extra frits are not readily available when they are needed. Second, current packing technology often results in column packing being under tension in the column. Packing material can extrude from the column when a frit is removed, and the column would be irreversibly damaged. Third, with the widespread use of in-line precolumn filters, frit blockage is encountered much less often today than it was before filters were standard.

As a practical guideline, if column reversal does not correct the peak distortion problem, it is advisable to discard the column and replace it with a new one. With today's columns, filling a void is neither practical nor effective. If column voiding is a regular problem, use a guard column, a less aggressive mobile phase, a more stable column, better sample cleanup, or some combination of the above.

Peak splitting can be indicative of method problems that should be corrected, so it is important to isolate the problem source.

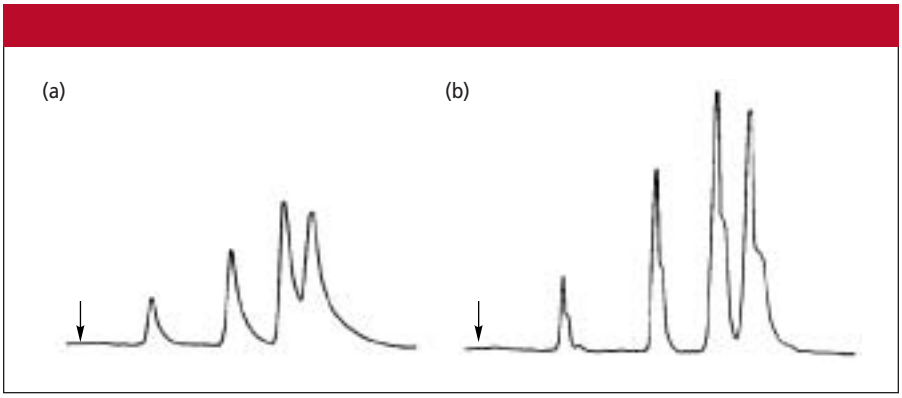

Figure 2: Peak distortion — (a) tailing and (b) splitting — affecting all peaks in the chromatogram in a similar way. See text for details.

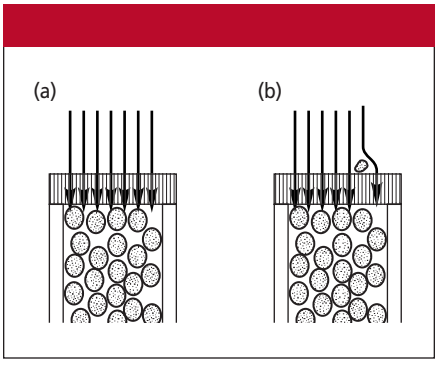

Figure 3: Schematics showing introduction of sample at the column inlet (a) under normal conditions and (b) with a partially blocked frit.

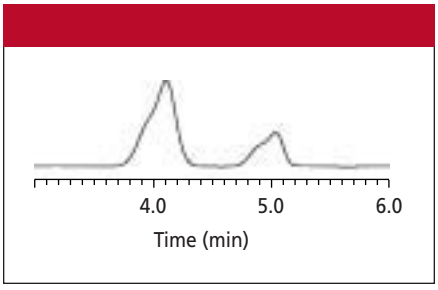

Figure 4: Partial chromatogram showing similarly distorted peak fronts for each peak. See text for details.

Case in Point

Let's take a look at a peak distortion problem we recently encountered in our laboratory. We first noted the problem when examining LC–UV chromatograms, such as the one in Figure 4, during a sample purity test. The peak of interest had a retention time of approximately 4 min; the peak at 5 min was an unknown compound that was not present in every sample. The similarly distorted front edge on both peaks suggested that the problem originated at the head of the column before separation occurred. Based upon the discussion above, a blocked frit or column void could be the problem source.

A close examination of the method highlighted another possible problem source. The isocratic method is performed using a 150 mm × 4.6 mm, 5-µm d_p C18 column with 78% acetonitrile–buffer mobile phase and flow rate of 1.5 mL/min. A 10-µL sample was injected in 100% acetonitrile as the sample solvent. Although 10 µL of 100% acetonitrile should not cause peak distortion problems under these conditions, any time 100% strong solvent is used for injection the potential for peak distortion exists.

We approached the problem in a stepwise manner. Figure 5 shows enlarged portions of the chromatograms resulting from the changes. Figure 5a shows the peak of interest with the problem distortion observed in Figure 4. The easiest change

was to inject the sample with a weaker injection solvent. Figure 5b shows the same sample mass injected in 50% acetonitrile rather than 100%. The peak shape did not change, which confirms the suspicion that a small injection of strong solvent was not causing the observed distortion.

The next-easiest step was to reverse-flush the column. We didn't have high hopes for this fix, because we used a 0.5-µm porosity in-line filter upstream from the column, and it should have trapped any particulate matter before it arrived at the column frit. However, the test was easy. We reversed the column and ran 20–30 mL of mobile phase through in the reverse direction before connecting it to the detector and hoped that any particulate matter on the inlet frit would be displaced. The resulting chromatogram of Figure 5c shows no improvement, as we suspected, which gave credence to the assumption that a blocked frit was not the problem source.

Our next suspected problem source was a column void, so we replaced the column with a new one. The new column produced a chromatogram (Figure 5d) with significantly improved peak shape. The original column certainly was bad, and the change is consistent with a column void, but something still appears to be wrong because the peak still fronts a little bit.

By the time we conducted this last test, it was the end of the day, so we left further investigations for another day. When we found time to revisit the problem, several days had passed. In the meantime, the original column and mobile phase had been discarded. The first step was to prepare a new batch of mobile phase and inject the same sample.

The results of Figure 5e were surprising. The peak is considerably narrower and tails slightly, as is normal for this compound. It would be nice to go back and check this mobile phase on the original column or to switch back to the old mobile phase on the new column to gain a further understanding of the problem, but unfortunately, these options are impossible because the old mobile phase and column had been discarded.

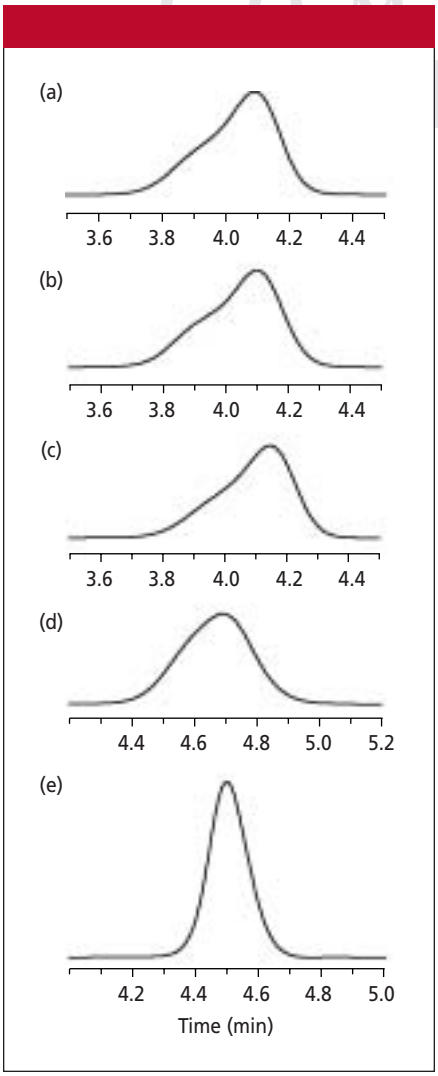

Figure 5: Expanded view of the first peak of Figure 4 with various changes: (a) initial conditions with a 10-µL injection of sample in 100% acetonitrile; (b) same as (a), but with 50% acetonitrile injection solvent; (c) same as (b), but with the column reversed; (d) new column with original mobile phase; and (e) same as (d), but with fresh mobile phase on another day. See text for discussion.

Conclusions

Although the experimental results do not fit into a neat package, as we might have hoped, we can use the examples above to help guide us when peak shape problems occur.

- Determine whether the peak distortion problem is related to all of the peaks or just some peaks in a run. When all peaks are affected in a similar manner, it is likely that the problem occurs at the head of the column, and frit blockage, column voids, and injection problems are the most likely sources. When only one peak is affected, the problem more likely is related to the separation itself. Try changing the mobile phase, temperature, or column type to improve the separation.
- Check the easy fixes first. Inject a smaller or larger volume of sample in a weaker or stronger solvent if you suspect injection problems. Reverse-flush the column to see if it helps. Changing a frit seldom is worth the trouble with today's columns. The exception might be expensive specialty columns, such as chiral columns, which can cost several times as much as conventional reversed-phase columns.

In some cases, a void appears as a settled packing bed; in others, it looks like a wormhole in the packing.

- Replace the column. Replace the guard column. Replace the in-line frit. Replace the mobile phase. Replacement can be the fastest and most certain way to fix a problem. By replacing components in a stepwise manner, you're more likely to find the root cause of the problem, but if speed is of the essence, replacing everything at once could be the best method.
- After the problem is corrected, take measures to minimize the chances that the same problem will recur or to ensure that it can be solved more quickly in the future. Using an in-line filter, replacing the mobile phase more frequently, counting the number of injections, or using some other trick will improve future operation.

Reference

(1) R.M. Minikis and J.W. Dolan, *LCGC* **21**(11), 1050–1054 (2003).

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