



LC

## Troubleshooting

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**Determining the cause  
of peak broadening  
isn't always simple.**

# Extracolumn Effects

**T**his month's "LC Troubleshooting" centers on a problem encountered in our laboratory that we initially attributed to extracolumn effects. However, a closer examination of the experimental data reduced our confidence in such a simple explanation. Several factors can contribute to a chromatogram's appearance, and they are not easily deconvoluted without extensive experimentation. As often happens in our laboratory, after we solved this problem a pressing deadline prevented us from systematically isolating the problem's cause. So speculation will have to be sufficient.

### The Setup

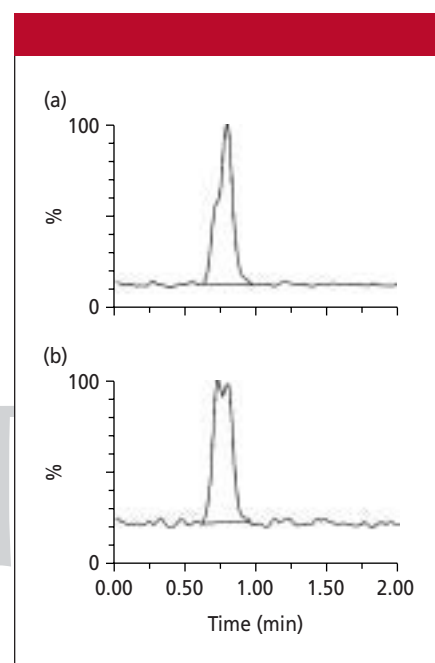
The separation was a liquid chromatography (LC) assay of a very polar drug in plasma that we were developing in our laboratory. The 50 mm × 2.1 mm, 5-μm  $d_p$  column was packed with a C18 phase compatible with 100% water, and it was operated at 35 °C. The mobile phase was 1% formic acid in water at a flow rate of 0.4 mL/min. We used LC with tandem mass spectrometry as the detection method (LC-MS-MS). We reconstituted the extracted plasma sample in mobile phase and injected 10-μL amounts.

### The Problem

Initial development work with standards resulted in single-peak chromatograms with retention times of approximately 0.80 min. When we injected plasma extracts, however, we obtained chromatograms with a shoulder on the leading edge of the peak, such as the one shown in Figure 1a. Initially, we thought the column had failed or had become contaminated with plasma proteins. Partial splitting of a peak often can be attributed to a partially blocked frit at the column inlet or to a void in the column.

We were using a 0.5-μm porosity in-line frit upstream from the column and observed no pressure increase, so we suspected that plasma proteins accumulating on the column might be the most likely cause. We achieved the same results with a new column.

After we had confirmed the integrity of the column, we had to look elsewhere for the problem source. With LC-UV methods, chromatographers might immediately suspect a closely eluted peak that was only slightly resolved from the main parent peak. With LC-MS-MS, however, the specificity of the parent-to-daughter ion transition would require that the partially resolved peak had both a similar retention time and exactly the same molecular weight fragmentation to appear in the MS-MS chromatogram. In the early days of LC-MS-MS assays, chromatographers thought that this specificity was sufficient and that little or no chromatographic separation was needed. As millions of real samples were analyzed and workers came to better understand the technology, they realized that background peaks from plasma or other sample matrices were not as rare as they had previously believed. This observation has generated renewed interest in having a good chromatographic separation as part of an LC-MS-MS method. Because of



**Figure 1:** Chromatograms for LC analysis of (a) 25 ng/mL and (b) 10 ng/mL of drug (second peak) in plasma. Temperature: 35 °C. See text for details.

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specificity, baseline resolution between peaks usually is not required, but distinct peaks are necessary in most cases.

### A Second Peak?

With the peak-splitting problem eliminated, we tried to characterize the shoulder on the main peak more fully. The chromatogram shown in Figure 1a is from a plasma sample spiked with 25 ng/mL of drug. An injection of a plasma sample spiked with 10 ng/mL of the drug resulted in the chromatogram shown in Figure 1b, in which a second peak is clearly present. We concluded that the peak was unrelated to the analyte of interest because it did not change in proportion to the concentration of the analyte. Later, we showed that the problem peak came from the plasma background. The quantification of drugs in plasma is difficult when an interference is present at levels of more than approximately 20% of the peak of interest. This amount would limit our assay to 50 ng/mL or so, and we needed a limit in the 5–10 ng/mL region. It was apparent that we needed to obtain a better separation of the two peaks if we wanted to quantify the drug.

### Extracolumn Effects

Just before we discovered this problem, we had participated in an extended conversation about extracolumn effects, which prompted a close examination of the system for potential extracolumn problems. We discovered a 20 cm  $\times$  0.25 mm stainless steel solvent preheater tube. We eliminated the tube and shortened several pieces of connecting tubing in an effort to reduce extracolumn effects. Figure 2 shows the resulting chromatogram.

Aha! We had discovered the problem source. Or had we? Let's look at all the data and see if they make sense.

By extracolumn effects, we mean peak broadening that can be attributed to factors external to the chromatography column. The most common contributions are connecting tubing volumes that are too large, although injection volume, fittings, and detector cell volumes also can contribute. Mathematically, we describe extracolumn band broadening as

$$V_T^2 = V_C^2 + V_{\text{tub}}^2 + V_{\text{inj}}^2 + V_{\text{det}}^2 \quad [1]$$

where  $V_T$  is the total peak variance;  $V_C$  is the contribution by the column; and  $V_{\text{tub}}$ ,  $V_{\text{inj}}$ , and  $V_{\text{det}}$  are contributions from the tubing and fittings, injector, and detector,

respectively. For convenience, volumes can be used for the variances, but remember to add the squares of the volumes.

In general, extracolumn effects influence early eluted peaks more than later peaks because  $V_C$  increases with retention time in isocratic separations, whereas the remaining contributions are constant. So it seems logical that the extra volume of the preheater tubing would negatively affect a peak with a retention time of less than 1 min. We should be able to verify this outcome with a calculation. Based upon equation 1, we would expect

$$V_{1b}^2 = V_2^2 + V_{\text{preh}}^2 \quad [2]$$

where  $V_{1b}$  is the peak volume of the analyte in Figure 1b,  $V_2$  is the peak volume of the analyte in Figure 2, and  $V_{\text{preh}}$  is the volume of the preheater tubing. We can measure the peak volumes by drawing tangents to the sides of the peaks and measuring the peak width at the baseline. Convert these values into volume by multiplying them by the flow rate. The analyte volume in Figure 2 is 0.13 min  $\times$  0.4 mL/min, or 52  $\mu$ L. The tubing volume is approximately 10  $\mu$ L. The analyte peak volume for Figure 1b should be  $(52^2 + 10^2)^{0.5}$  or 53  $\mu$ L.

Hmm. It is highly unlikely that a 1- $\mu$ L change in peak width would cause the difference observed between Figures 1b and 2. A closer look at the chromatogram shows that the retention times are approximately 10 s longer in Figure 2 than in Figure 1b. The increase in retention time can be attributed to a small reduction in column temperature as a result of removing the preheater tubing. Although the reduction of extracolumn volume might have helped, it was not solely responsible for the observed improvement.

Maybe it is time to step back and look at the separation a little more carefully.

### Chromatographic Performance

It is a good idea to examine a chromatogram to be sure the results are reasonable. One of the first tasks is to measure the retention factor ( $k$ ) to see if retention is in a region that can be expected to yield good chromatography. The current method was a challenge because the compound was so polar that it required 100% water to obtain any retention at all. To calculate the retention factor, we need to calculate or measure the column volume. With LC–MS–MS methods, a peak at the column dead time could be invisible or a diversion valve could divert the solvent front and its associated

polar contaminants from the detector source. Usually it is easier to calculate the void volume from

$$V_m \approx 0.1L \quad [3]$$

or

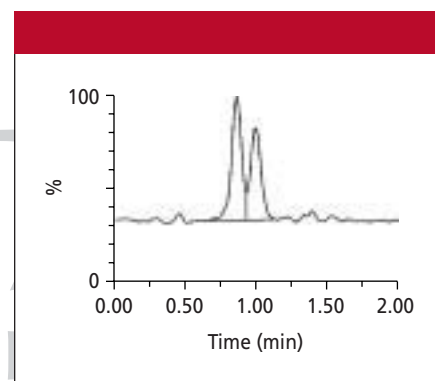
$$V_m \approx 0.5d_c^2L \quad [4]$$

where  $V_m$  is the column volume in milliliters,  $L$  is the column length in centimeters, and  $d_c$  is the inner diameter in centimeters. Equation 3 is used for 4.6-mm i.d. columns; equation 4 is used for columns of all other inner diameters. In the present case, the 50 mm  $\times$  2.1 mm (5 cm  $\times$  0.21 cm) column has a dead volume of approximately 110  $\mu$ L or, at 0.4 mL/min, a dead time of approximately 0.3 min. This determination allows calculation of the retention factor as

$$k = (t_R - t_0)/t_0 \quad [5]$$

where  $t_R$  and  $t_0$  are the retention and dead times, respectively. For Figure 1,  $t_R$  is approximately 0.80, so  $k$  is approximately 1.7. Generally if  $k$  is between 1 and 20, then chromatographers can obtain acceptable chromatographic performance. With LC–MS–MS methods for biological samples, it is a good idea to have  $k$  greater than 3 to minimize problems caused by coelution of ion-suppressing peaks in the background. In the present case, we felt lucky to have a  $k$  of 1.7 because the compound of interest was very polar and difficult to retain.

Another chromatographic measurement that is good to check is the column plate number ( $N$ ). Manufacturers often quote plate numbers in the range of 80,000



**Figure 2:** LC separation of 10 ng/mL of drug in plasma with no mobile-phase preheater tubing. Temperature: 35 °C. Other conditions were the same as in Figure 1.

plates/m for 5- $\mu$ m  $d_p$  packing materials. This value translates to 4000 plates for our 50-mm column. Because manufacturers' tests are performed under ideal rather than real-world conditions, a more realistic estimate of  $N$  can be determined by

$$N \approx 3000L/d_p \quad [6]$$

For our 50-mm (5 cm) column,  $N$  is approximately  $3000 \times 5 \div 5$  or 3000. A measurement of  $N$  from Figure 2 provides a plate number of approximately 800 — certainly much less than 3000. In our experience, the LC–MS–MS interface degrades column performance, and the 2.1-mm i.d. column is much more susceptible to extracolumn effects. Remember, though, that resolution increases in proportion to the square root of the plate number. Even if we could double the plate number, the peaks of the chromatogram shown in Figure 2, with a resolution ( $R_s$ ) of approximately 1.0, still would not be baseline-resolved.

### Temperature Effects

With the removal of the column mobile-phase preheater tubing, we wanted to be sure that cold mobile phase entering the column wasn't causing a problem. If a temperature gradient is present along the column, band distortion can occur. This situation usually is not a problem at column temperatures below 40 °C, but we decided

to check just to be safe. When we reduced the column temperature to 25 °C, we obtained the chromatograms shown in Figure 3. Comparing Figure 2 and Figure 3 shows that retention has increased by approximately 0.2 min or 20%. This amount is in accordance with the general rule that retention increases approximately 1–3% for each 1 °C reduction in temperature. Here, a 10 °C change yielded a 20% change in retention, or 2%/°C.

The relationship between resolution and retention factor is such that at small  $k$  values, small increases in  $k$  can make dramatic increases in resolution. The lower temperature of the runs shown in Figure 3 caused an increase in average  $k$  for the two peaks, from a  $k$  of 2.0 to a  $k$  of 2.6, which was a 30% increase or a 50% increase from the runs shown in Figure 1. This change, coupled with an apparent increase in selectivity ( $\alpha$ ) with the lower temperature, makes the conditions of Figure 3 preferable for the assay.

### Conclusions

The conditions for the runs shown in Figure 3 provide much more satisfactory results than the corresponding chromatograms shown in Figure 1. The overlap between the two peaks is minimal, so obtaining a lower limit of quantification of 5 ng/mL should be possible.

What can we learn from this case study? When using short, narrow-bore columns, such as the 50 mm  $\times$  2.1 mm column we used, analysts need to be careful with plumbing connections so that extracolumn contributions are minimal. It was inconclusive that extracolumn effects were an important factor in the present example, but we did discover a piece of 0.25-mm i.d. tubing that should have been nowhere near an LC–MS system. The tubing was a leftover from when the column oven was used with an LC–UV system with a 150 mm  $\times$  4.6 mm or a 250 mm  $\times$  4.6 mm column, in which it would have been unnoticeable.

We learned that it is a good idea to check for reasonable chromatographic behavior. The retention factor for the analyte in our assay was less than that normally desired for LC–LC–LC–MS–MS work, but we had to tolerate it because of the polar nature of the compound. Plate numbers generally will be lower with an MS detector than with a UV detector because of the increased volume of the interface, but analysts still should check to be sure that plate numbers are acceptable.

Column temperature appears to have been a critical factor for the separation we have discussed in this "LC Troubleshooting" column. This statement underlines the importance of controlling temperature and checking its influence on each method. The observed shift in retention after the preheater tube was removed illustrates how sensitive our separation was to temperature.

In troubleshooting, the *Rule of One* states that just one thing should be changed at a time when isolating a problem source. Sometimes more than one thing is changed, either intentionally or unintentionally. Most of us work under deadlines, and any problems usually result in pushing against those deadlines. The result often is that we do not have the luxury to go back and determine exactly which system change fixed the problem. Such is the case with the example in this discussion — was it the mobile phase, extracolumn effects, temperature, or a combination that resulted in an acceptable separation? We'll probably never know.

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### John W. Dolan

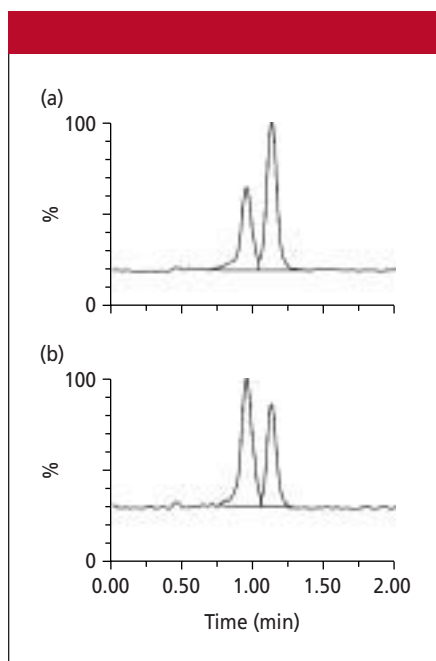
"LC Troubleshooting" editor John W. Dolan is vice-president of BASi Northwest Laboratory of McMinnville, Oregon; a training consultant for Rheodyne LLC, the LC Resources Training Group, of Walnut Creek, California; and a member of LCGC's editorial advisory board. Direct correspondence about this column to "LC Troubleshooting," LCGC, 859 Willamette Street, Eugene, OR 97401, e-mail John.Dolan@Bioanalytical.com.

For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at <http://www.chromforum.com>.



### Erratum

The September "LC Troubleshooting" column ("Honoring Readers," LCGC 21[9] 888–892 [2003]) contained an error on page 890. The statement that a 50% loss in plate number reduces resolution by approximately 7% is incorrect. The reduction should be 30%.



**Figure 3:** LC separation of (a) 25 ng/mL and (b) 10 ng/mL of drug in plasma. Temperature: 25 °C. Other conditions were the same as in Figure 1.