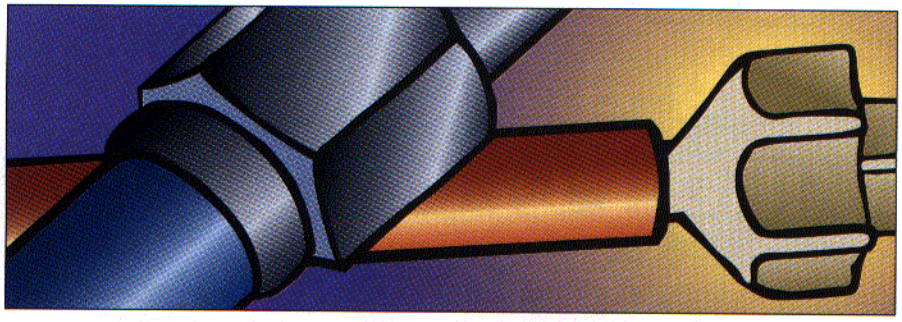
LC Troubleshooting



Extracolumn Band Broadening from Injection and Peak Transfer

John W. Dolan

Chromatographers can use band-broadening calculations as a tool for better separations.

Extracolumn band broadening in liquid chromatography (LC) is a very real but often overlooked problem that can compromise LC separations. This phenomenon can be especially problematic when changing a method from a conventional 15 or 25 cm × 4.6 mm, 5-μm d_p column to one of the shorter, narrower, and smaller-particle-size columns such as the 1.5 cm × 2.1 mm, 3.5-μm d_p column used for the LC–MS example discussed in January's "LC Troubleshooting" column (1). I attribute much of LC's success as a separation technique to the fact that most separations are performed on larger, user-tolerant columns. For example, although a 25-cm-long, 5-μm d_p column may demonstrate a plate number (N) greater than 20,000 under ideal test conditions, a large number of practical separations require N to be much less than 10,000. For these separations, a significant loss in the column performance can go unnoticed and often is unimportant.

However, when the same separation is performed on a 7.5-cm-long, 3.5-μm d_p column with 7500 plates, very little loss in performance can be tolerated before the separation becomes impossible. I feel that chromatographers were fortunate that the technique was developed with the larger, fault-tolerant columns. If the foundation for LC were built upon smaller columns, the added demands for more careful operation would have been extremely discouraging and would have reduced the success of this separation technique.

So where does this problem of excessive band broadening originate? Although problems are more commonly encountered with short, small-particle columns, the key factor is the peak volume, not the column size. Band-broadening problems also can appear as narrow peaks eluted early from larger columns. The process is treated statistically by summing the variance contributions of the factors contributing to the overall peak variance:

$$\sigma^2_{total} = \sigma^2_{column} + \sigma^2_{extracolumn} \quad [1]$$

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where the variance (σ^2) for the total process is determined by adding the variance in the column and the variance from processes outside the column. In turn, these extracolumn contributions break down into other parts of the LC process that contribute to band broadening, such as injection volume, tubing volume, detector volume, and detector response characteristics. The variance, of course, is just the square of the standard deviation (σ) for a peak. The standard deviation can be determined by measuring the peak width. When tangents are drawn to the sides of a peak, the width at baseline is 4σ; the half-height width is 2.35σ. For the present discussion, I prefer half-height widths because they are easier to measure, can be determined accurately when baseline resolution between peaks does not exist, and are less influenced by peak tailing. (You can use a factor of 1.7 to convert half-height widths or volumes to baseline values.) Finally, as long as the same units are used throughout, equation 1 works for peak widths measured in any convenient unit of distance, volume, or time. For the present discussion, I will use volume (V) measured in milliliters, so equation 1 converts to

$$V^2_{total} = V^2_{column} + V^2_{extracolumn} \quad [2]$$

INJECTION VOLUME

One application of band-broadening calculations is the determination of the maximum injection volume that can be used before a peak is unacceptably broadened. If the sample is injected into mobile phase, two extremes can exist. If the sample volume is very small (for example, 1 μL) and the peak volume is 500 μL (width of 30 s at a flow rate of 1 mL/min), it is obvious that the injection volume will have very little influence on the resulting peak volume. However, a 1-mL injection in the same system is broader than the normal peak, so the peak will be broadened unacceptably. One common rule of thumb is that chromatographers can use an injection volume of as much as 15% of the peak volume with immunity from practical problems when using mobile phase as the injection solvent.

Let's see how this situation works. For the same 500-μL peak (V_{column}), this example would allow an injection volume of 75 μL (0.15 × 500 μL), so equation 2 would yield

$$V^2_{total} = (505\ \mu L)^2 = (500\ \mu L)^2 + (75\ \mu L)^2$$

Thus the 75-μL injection in this case broadens the peak by only 1%. But how close are these numbers to reality? A typical separation

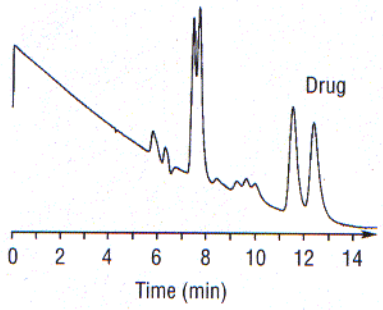

FIGURE 1: Separation of an enantiomeric pair of chiral drugs on a 25 cm × 4.6 mm, 5-µm d_p chiral cellulose column (Chiracel OD-R, Chiral Technologies, Inc., Exton, Pennsylvania) operated at a flow rate of 1.5 mL/min with a 36% acetonitrile mobile phase.

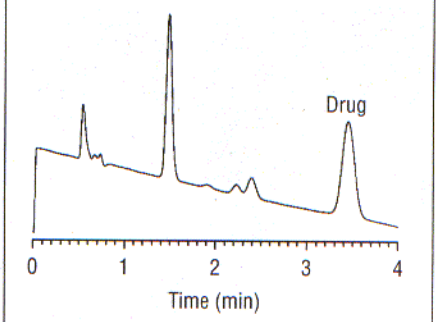

FIGURE 2: Achiral separation of the drug enantiomers of Figure 1 on a 7.5 cm × 4.6 mm, 5-µm d_p cyano column (Stablebond SB-CN, Mac-Mod Analytical, Inc., Chadds Ford, Pennsylvania) under the same conditions as in Figure 1.

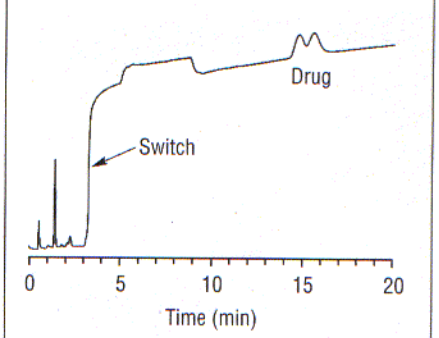

FIGURE 3: Column-switching separation of standards in which the drug peak from the initial achiral separation shown in Figure 2 is transferred to the chiral column used in Figure 1.

on a 15 cm × 4.6 mm, 5-µm d_p column might generate approximately 8000 plates. For a peak with a retention factor of 2 (retention time of 4.5 min at 1.5 mL/min), the peak volume would be nearly 200 µL, allowing an injection volume of approximately 30 µL with no untoward effects for this analysis. However, if the same separation were tried on a smaller column, the demands would increase. For example, the same separation obtained on a 10 cm × 2.0 mm, 3-µm d_p column would yield a peak volume scaled by the square of the column diameter and directly proportional to the length, nearly eightfold. This result means that an injection volume of less than 4 µL would be necessary for similar performance — a considerably more demanding condition. (This discussion considers only the injection volume. Other factors, such as transfer tubing volume and the detector cell volume, also contribute to extracolumn band broadening.)

So where does this problem of excessive band broadening originate? The key factor is the peak volume, not the column size.

COLUMN SWITCHING

Next, let's look at the utility of the band-broadening calculations of equations 1 and 2 for a practical problem encountered in my laboratory. In this particular case, the workers in the laboratory needed to perform a chiral separation of a proprietary drug extracted from plasma. Because the chiral column was three times as expensive as a typical reversed-phase column and much less tolerant to contamination, they chose to use column switching to provide additional cleanup before injection on the chiral column. A cyano column generated the initial separation, and the band containing the two enantiomers was switched onto a chiral cellulose column for the chiral separation.

Figure 1 shows the conditions that yielded a satisfactory chiral separation for the standards using the cellulose column alone. The enantiomeric pair of peaks at approximately 12 min is nearly baseline separated. This resolution is adequate for the present application. Figure 2 shows the same mobile-phase conditions for the achiral separation on a 7.5-cm cyano column. The drug peak at approximately 3.5 min is well separated from other known drug components in the sample. However, the separation deteriorated when the peak from the cyano column was switched onto the cellulose column (Figure 3). Resolution (R_s) dropped from an acceptable value of approximately 1.2 to 0.8 — marginally adequate for the present application. Figure 4 shows results from an analysis of a real sample in plasma where further deterioration of the separation occurred.

The dramatic drop in resolution when comparing the chiral column alone (Figure 1) to the coupled system (Figure 3) raised concerns about whether excessive band broadening was occurring. For example, if poor plumbing or excessive switching-valve volumes were the problem source, it might be possible to modify the LC hardware to improve the separation. It was simple to measure the peak broadening on each column and compare the theoretical combined peak width with the experimental one. I used half-height widths because it was impossible to measure baseline peak widths accurately for Figure 3. The half-height width for the drug peak on the cyano column (Figure 2) was 0.244 mL. (I measured the widths from expanded chromatograms with an error of less than ±5 µL.) The average width for the two enantiomers on the chiral column alone (Figure 1) was 0.173 mL and 0.301 mL for the switched system (Figure 3).

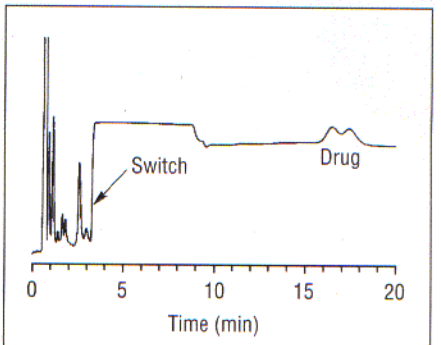

FIGURE 4: Column-switching separation of a plasma extract containing the chiral drug. Conditions were the same as in Figure 3.

Thus I calculated

$$V^2_{\text{expected}} = (0.299\ \text{mL})^2 = (0.244\ \text{mL})^2 + (0.173\ \text{mL})^2$$

So within experimental error, the calculated and actual peak widths for the column-switching system are identical (0.301 mL ≈ 0.299 mL). This calculation shows that the 70% increase in peak width (0.299/0.173) is the result of injecting a broad peak from the first column to the second. No observable band broadening occurs during the transfer of the peak from the achiral system to the chiral one.

One way to improve the separation of Figures 3 and 4 would be to reduce the volume of the peak that is switched onto the chiral column. This procedure is analogous to reducing the injection volume for a normal separation as discussed in the previous section. If an increase of 20% in peak width were acceptable (0.210/0.173), this injection-volume reduction could be accomplished by transferring half the peak volume from the cyano column to the cellulose column:

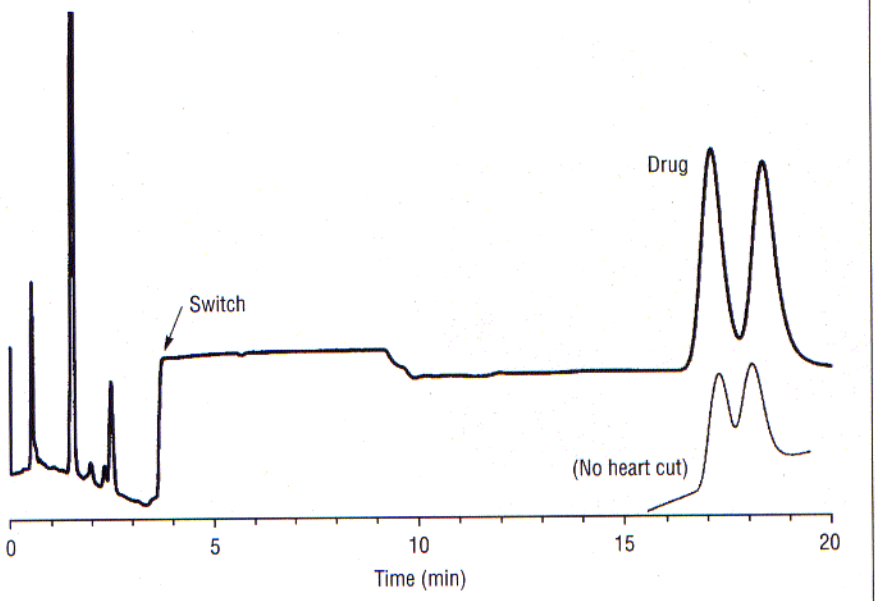

FIGURE 5: Column-switching separation of the chiral drug in plasma using a heart-cut transfer from the first column to reduce the peak volume. Inset shows the peaks of interest from Figure 3.

$$V^2_{\text{acceptable}} = (0.210\ \text{mL})^2 = (0.120\ \text{mL})^2 + (0.173\ \text{mL})^2$$

Although a twofold reduction in the transferred volume seems drastic, it is less significant than might be expected. For example, the 244-µL peak volume at half-height (2.35σ width) is equivalent to 415 µL at baseline (4σ width). Similarly the desired 120-µL half-height volume is equivalent to 205 µL at baseline. For a Gaussian peak, the 4σ width (±2σ) accounts for 96% of the peak area. Reducing the transfer volume twofold by selecting only the center 2σ portion of the peak (±1σ) still will account for 68% of the area. In terms of sensitivity and limits of detection, the peak height is more important than peak area. For the chiral column, any reduction in peak width will translate into increased peak height. Thus the reduction in peak volume and peak width by 30% from 0.299 mL to 0.210 mL should result in a 30% increase in peak height, which would compensate for the 30% reduction in the mass of sample transferred from the cyano column.

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In theory, it appears that reducing the transferred volume by a factor of two should improve the chiral resolution without a major compromise in sensitivity. Figure 5 shows the improved resolution resulting from this heart-cut transfer technique. The inset of Figure 5 shows just the peaks of interest from Figure 3. The heart-cut transfer does indeed restore the desired resolution for this separation.

CONCLUSION

Calculation of chromatographic parameters may be inconvenient and seem esoteric with little practical application, but the present discussion shows two examples where band-broadening calculations can yield significant insight into separation problems. The determination of maximum injection volumes for an acceptable degree of peak broadening can be obtained by measuring the peak width and completing a simple calculation. For the more complex case of a column-switching application, calculating band-broadening contributions helped us gain an understanding of the separation process and guided us to an effective solution to a practical problem.

REFERENCE

(1) J.W. Dolan, *LC•GC* **16**(1), 16–19 (1998).

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