LC Troubleshooting

When Ideal Isn't Practical

Melissa M. Harrington and John W. Dolan

Sometimes you must sacrifice chromatographic performance to obtain analytical results.

A wide chasm separates chromatographers who work with liquid chromatography (LC) under well-behaved, ideal conditions and those who must develop and use methods for routine application. With the freedom to choose samples, mobile phases, and column conditions, chromatographers can make an LC column perform near its theoretical performance specifications. We often see such well-behaved chromatograms displayed in application notes that come with columns. Most users, however, are somewhat constrained by one or more parameters and, as a result, obtain separations that are very workable but may use only one-half or three-quarters of the separation power available from the LC system. Because these separations are robust under routine operation, workers have little need to improve method performance.

This month's "LC Troubleshooting" installment deals with a situation that is becoming more common with the advent of LC systems coupled with mass spectrometry systems (LC–MS systems), in which speed and miniaturization are necessary because of throughput and instrument requirements. Through the example discussed below, we will show that even if column performance is much less than one-half of its theoretical capabilities, users can find a workable solution — *if* the right conditions can be determined.

The LC of LC–MS

As a contract research organization, our laboratory develops many analytical methods for LC and LC–MS applications. For historical and economic reasons, we own roughly three times as many LC systems as LC–MS systems, so we commonly develop separations on LC systems using conventional detection and then move them to LC–MS systems later. This practice allows us to perform separation optimization using conventional 150 mm × 4.6 mm columns in conjunction with resolution mapping software to obtain optimal performance of the LC portion of LC–MS analysis. The software allows us to scale the column size to fit LC–MS requirements for both isocratic and gradient separations. We can use the LC-only method to support the development of sample preparation methodology, thus further reducing the workload of the expensive LC–MS systems.

Our example is a separation destined for routine analysis of a drug, its metabolite, and an internal standard in plasma. We performed initial development on a 150 mm × 4.6 mm, 5-µm d_p C18 column. For LC–MS, we like to work with 50 mm × 2.1 mm columns. These smaller columns provide a sufficient column plate number, so some separation can occur, and the narrow-bore size both reduces the mobile-phase volume introduced into the mass spectrometer interface and generates narrow peaks for better limits of detection. In Figure 1, the 50 mm × 2.1 mm column operated at 0.3 mL/min has the same mobile-phase linear velocity as a 4.6-mm i.d. column operated at 1.5 mL/min. Because the column length is one-third that of the 150-mm column used in initial development, the column plate number (N) also should be reduced to one-third, but the resolution will diminish only by $N^{0.5}$.

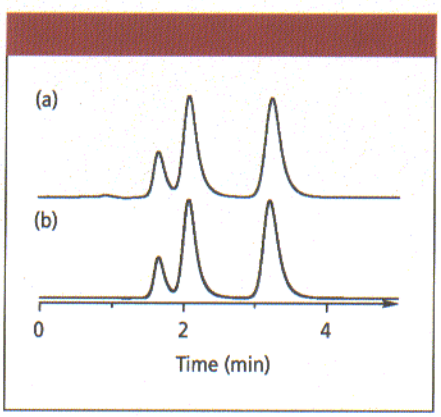

Figure 1: Chromatograms resulting from 50-µL injections of (a) 1-µg/mL and (b) 10-µg/mL aqueous standard samples of a drug, its metabolite, and an internal standard. Column: 50 mm × 2.1 mm, 5-µm d_p C18; mobile phase: 5:95 (v/v) acetonitrile–water with 0.05% heptafluorobutyric acid added; flow rate: 0.3 mL/min; detection: UV absorbance; column temperature: 35 °C. The y axis is arbitrary and should not be used for comparison of runs.

John W. Dolan
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To reduce ion suppression in LC–MS, it is best to elute the first peak of interest with a retention factor (k) of greater than 1. Often, it is beneficial to adjust the mobile phase to obtain a k value of greater than 2 to move the peaks away from the solvent front and simultaneously increase the flow rate to keep the retention times low. These changes help prevent loss of detector signal caused by early eluted materials that suppress the ionization of sample compounds.

Figure 1 shows chromatograms of standards for our example. In these runs, the k values were approximately 3.5, 4.6, and 7.9 for the three major peaks, which is well beyond the ion-suppression region of the chromatograms.

On-column concentration is a technique often used in trace analysis to improve detection limits. In on-column concentration, users inject a large volume of sample in a solvent weaker than the mobile phase. If the sample solvent is sufficiently weak, the technique traps the sample components at the head of the column. Then, after the normal-strength mobile phase follows the injection solvent, the sample begins to move in the expected fashion. This technique has the net effect of allowing the injection of a larger volume of sample than would be possible using mobile phase or a stronger sample solvent. The runs of Figure 1 use an isocratic mobile phase of 5:95 (v/v) acetonitrile–water with 0.05% heptafluorobutyric acid. We chose this weak mobile phase because the polar metabolite was unretained when much more acetonitrile was added to the mobile phase.

Injecting 50 µL of standard in water generated peaks with a column plate number of approximately 1000. This plate number seems quite low, especially if you

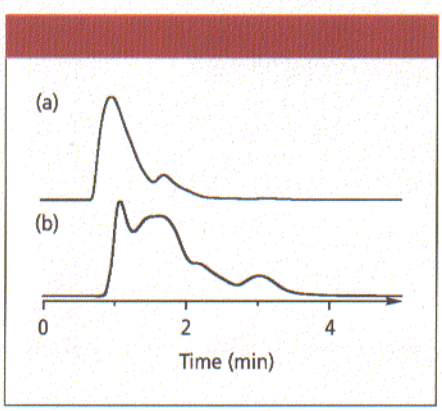

Figure 2: Chromatograms resulting from injections of (a) 50 µL of reconstituted extract of a 10-µg/mL sample spiked into plasma and (b) 50 µL of reconstituted extract of a 10-µg/mL aqueous sample. Other conditions were the same as in Figure 1.

are used to 150-mm columns that generate 8000–10,000 plates. Based on experience with 150-mm columns, this 50-mm column should generate approximately 3000 plates for a real sample, so $N \approx 1000$ is lower than expected. This reduced plate number has several potential sources. The injection volume is large for the column diameter, roughly equivalent to injecting 250 µL on a 4.6-mm i.d. column. The difference in solvent strength between the sample (100% water) and mobile phase (5% acetonitrile) is small, so on-column concentration may be marginal. Finally, the short, small-diameter column is more susceptible to extracolumn band broadening. In the present case, we made no special efforts to reduce extracolumn volume, so this factor will have some influence on the reduced plate number. However, in spite of this reduced performance, the resolution (R_s) between peaks is more than adequate for LC–MS applications. The resolution of the first two peaks is approximately 1.3; analysts can obtain satisfactory LC–MS performance if R_s is greater than 1.0.

At this stage, the separation shown in Figure 1 appeared to be satisfactory to support sample preparation experiments. So let's see what happened when we introduced samples.

Enter the Sample

Preparation of biological samples usually follows one of three approaches. Liquid–liquid extraction generates very clean samples, and this technique is having a resurgence in popularity because of the increased reliability of the LC–MS operation with clean samples. Unfortunately, this technique was not applicable for our sample because of the sample's high polarity. Chromatography is a powerful method to clean up samples. On-line techniques such as column switching can be used, but the most popular chromatographic cleanup technique is solid-phase extraction (SPE). We initially screened SPE cartridges and elution conditions for our sample and found that the metabolite was especially problematic — either it didn't stick to the cartridge during sample loading or it stuck so tightly that we couldn't release it during the elution phase. Further efforts may be justified, but we chose to look at the third sample preparation approach. Variously called precipitate-and-inject or crash-and-shoot, the name derives from the addition of organic solvent to plasma, which causes protein precipitation. The sample is vortexed to ensure mixing and then centrifuged to remove most of the plasma protein. The supernatant is either directly injected or evaporated to dryness and reconstituted in a more favorable injection solvent. The technique is simple and avoids some of the sample loss issues of liquid–liquid and solid-phase extraction, but the resulting sample is relatively dirty. Considerable protein remains, so analysts should expect shorter LC column lifetimes with the precipitate-and-inject sample preparation technique. Also, more endogenous peaks from the plasma will appear in the background, but this problem is mitigated by using MS as a highly selective detector.

In our case, we chose a recipe that we've found useful for other applications: combine 200 µL of plasma with 1.4 mL of methanol that contains 1% formic acid. After centrifugation, we removed an aliquot of the supernatant, evaporated it to dryness, and reconstituted the residue in water. Figure 2a shows a chromatogram of a plasma extract. Something is definitely wrong! All the peaks move to lower retention times, peak tailing and broadening increases, and resolution is lost. When these symptoms appear, we know to suspect overload.

Overload can result from three processes. Volume overload can occur when the sample volume is too large relative to the peak volume. Mass overload can happen when the sample mass is greater than the sample capacity of the column. Matrix overload can occur when sample matrix components block or mask the column surface and change its retention characteristics. All three of these processes are possible with our present sample.

The influence of the sample matrix is easy to check. We used an aqueous standard instead of plasma during the extraction. This change produced the chromatogram of Figure 2b. The separation is improved compared with the plasma extract of Figure 2a, but it still is not satisfactory. It appears that the sample matrix contributed to the problem, but it doesn't appear to be the only factor.

Reduce Sample Mass

A simple technique to test for sample mass overload is to reduce the mass on the column and observe the change in the chromatogram. If column overload were a problem, we would expect retention times to increase and peaks to sharpen. The two chromatograms of Figure 1 differ 10-fold in mass on the column. The two have no practical difference in their chromatography, so it doesn't appear that overload is

a problem at this mass on the column. To double-check, we consider the rule of thumb that states that chromatographers can load approximately 1 µg of compound per centimeter of column length for a 4.6-mm i.d. column. Thus, we can load 10–20 µg on a 150 mm × 4.6 mm column. The fivefold reduction in cross-sectional area and threefold reduction in length for the 50 mm × 2.1 mm column means that approximately 1 µg should be tolerated by the smaller column. The injection in Figure 1b is 50 µL of a 10 µg/mL solution or approximately 0.5 µg on the column, which is well within the rule of thumb.

If we were to extract less sample, both the mass on the column of sample compounds and the mass of the matrix would be reduced. The same effect can be tested simply by diluting an extracted sample before injection. Figure 3a shows the resulting chromatogram when we diluted the extracted plasma sample 10-fold with water before injection. We observed a significant improvement in the chromatogram when compared with Figure 2a.

It appears that matrix overload is a problem. We performed the same experiment with the aqueous sample of Figure 2b and obtained the chromatogram of Figure 3b. This separation (Figure 3b) is suitable for LC–MS and compares favorably with the runs of Figure 1. An additional reduction in the plasma volume might make the method usable if we could reach the detection limits.

Inject Less Volume

As long as the injection volume is less than approximately 15% of the peak volume, chromatographers should be able to inject sample in mobile phase without a negative impact on the chromatogram. By examining the runs of Figure 1, we found that the peak widths are approximately 0.5 min (by drawing tangents to the peaks and measuring the baseline width). Thus, the peak volume is 0.5 min × 0.3 mL/min or roughly 150 µL. A 50-µL injection is much greater than 15% of the peak volume, so we can expect band spreading caused by volume overload. This volume overload is a likely cause of the low plate numbers observed in Figure 1. Injection volume overload also is a contributing factor to the problems observed in Figures 2 and 3, because they also used a 50-µL injection volume.

In an effort to separate the injection volume effects, we reinjected the samples of Figure 2 with 5-µL instead of 50-µL injections. As expected, the chromatograms of Figure 4 are an improvement over their counterparts in Figure 2. These results confirm that injection volume was a significant factor in the observed problems.

Conclusions

A final contributing factor for poor chromatographic performance is likely. After observing the precipitation, evaporation, and reconstitution steps, we noticed that the sample did not evaporate to complete dryness. A small drop of aqueous residue remained after the evaporation step. This residue probably contained some residual methanol, so the injection solvent wasn't 100% water in any case. This aqueous residue may be the reason for the discrepancy between the chromatograms of Figures 3b and 4b when compared with the aqueous standards of Figure 1.

This example illustrates that a relatively foolproof technique such as precipitate-and-inject won't apply in every situation. We saw that volume, mass, and matrix overload all contributed to reducing chromatographic performance in the method. Although we were willing to accept column performance significantly below that normally expected, in this situation, marginal was not good enough for the application. Although the column performance of Figure 1 was well below what we knew was possible, it was acceptable with aqueous standards. When translated to real samples, however, the separation deteriorated beyond recovery. The next step would be to change sample pretreatment techniques, change the chromatographic conditions, or change both.

Our knowledge of basic chromatographic principles allowed us to compare method performance with expected parameters, so we could thoroughly examine the shortcomings of the method. Column plate number, retention factor, mass, volume, and matrix overload all were considered.

Whether your separation problems are as challenging as this one or not, it is important to ask how the method is performing relative to its ideal case. Armed with answers to your questions, you may be able to make significant method improvements in terms of throughput, detection limits, reduced variability, and improved reliability.

Melissa M. Harrington is a chemist at LC Resources Inc. (McMinnville, Oregon) who specializes in LC–MS–MS analysis of pharmaceuticals in biological matrices.

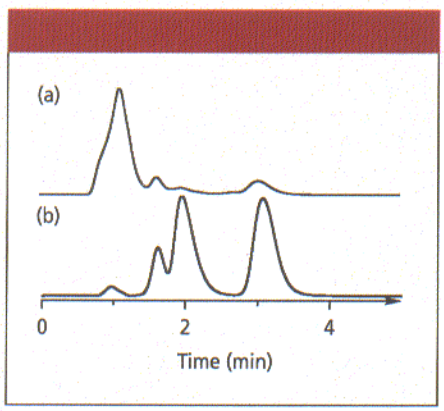

Figure 3: Chromatograms resulting from injections of (a) 50 µL of the sample of Figure 2a diluted 10-fold with water before injection and (b) 50 µL of the sample of Figure 2b treated as in Figure 3a. Other conditions were the same as in Figure 1.

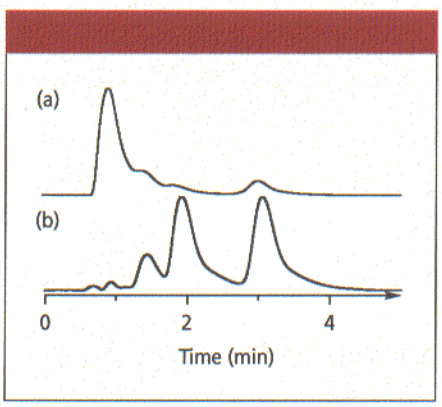

Figure 4: Chromatograms resulting from injections of (a) 5 µL of the sample of Figure 2a and (b) 5 µL of the sample of Figure 2b. Other conditions were the same as in Figure 1.

John W. Dolan

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