Troubleshooting

Scaling Gradient LC Methods to LC–MS

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Converting methods from LC to LC–MS doesn't have to be difficult.

In the past few years, analytical chemists have placed increasing emphasis on the use of a mass spectrometer as a liquid chromatography (LC) detector. The combined liquid chromatography–mass spectrometry process commonly is abbreviated as LC–MS, or if multiple MS stages are involved such as with a triple quadrupole mass spectrometer, as LC–MS–MS. The growing popularity of LC–MS has resulted in more workers using the technique, and conventional LC methods often must be converted to LC–MS methods. This month's "LC Troubleshooting" will focus on some problems — and their solutions — that may occur when converting conventional LC methods to LC–MS.

We also direct readers to the end of the column for some feedback and additional information regarding corrosion of stainless steel, which was described in two previous installments of "LC Troubleshooting" (1,2).

Background

When the hyphenated LC–MS technique was first applied, mass spectrometers were at a developmental stage that required an MS expert to operate the system. Today, LC–MS systems are simple and robust, and more chromatographers are using MS in routine work. MS is a separation technique in which chemicals are separated by molecular weight, and high-resolution MS units can distinguish between molecules differing by a fraction of a dalton.

Some workers argued, particularly in the early years, that the LC system was merely a sample preparation unit for the mass spectrometer and that the separation was of little importance as long as the compounds of interest were retained beyond the garbage at the solvent front. We sometimes refer to this approach as *LC–MS* in which LC takes a minor role. At the other extreme are the dyed-in-the-wool chromatographers who accomplish all the important work in the LC column and use the mass spectrometer only because none of the other LC detectors will work with the sample. These workers are using *LC–MS,* in which the LC system is the most important piece.

As workers gain more LC–MS experience, they agree that most practical applications of LC–MS rely heavily on the power that each technique brings to the experiment. Peaks with poor chromatographic resolution can be problematic, especially if they are closely related or one is at trace levels. Sometimes if an interference is coeluted with the peak of interest, ionization can be suppressed, resulting in misleading data.

The Target Method

We currently are developing a method to analyze several drugs and their metabolites in phenotyping studies. In practice, the drugs are dosed and the levels of the drug and metabolite are measured in plasma or urine. The relative levels of specific metabolites can be used to determine if the subject is a fast metabolizer or slow metabolizer for different enzyme systems. This information can be useful in designing test protocols for new drug development. This method is an excellent example to illustrate a relatively straightforward conversion of an LC method to an LC–MS one.

Our laboratory is typical of many analytical laboratories in that it is much easier to get time on an LC system than on an LC–MS system. In addition to availability, LC–MS–MS systems typically cost 5–10 times as much as an LC system with a UV detector. We have taken an approach that saves us time and money in the LC–MS method development process. We begin by developing the initial separation on a conventional LC system, then move the method to the LC–MS system only when we are confident that the separation development is complete. This process allows us to maximize the use of our LC–MS systems and takes better advantage of our LC expertise.

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LC Troubleshooting Editor

Starting Out

We started out using the general guidelines for gradient scouting runs outlined in an earlier series of articles (3–8). Because the method is destined for LC–MS, we chose to use a volatile buffer — trifluoroacetic acid — instead of phosphate. Our first runs with water, acetonitrile, and trifluoroacetic acid buffer generated coeluted peaks that could not be separated under any set of conditions. Changing to water, methanol, and acetate solved this problem and yielded some separation of all peaks. We ran two full-range gradient runs (10- and 30-min gradients of 5–100% B solvent) on a 150 mm × 4.6 mm, 5-µm d_p C18 column operated at 1.5 mL/min and 30 °C. We used DryLab separation modeling software (LC Resources Inc., Walnut Creek, California) to optimize the separation. All figures in this "LC Troubleshooting" column are simulations obtained using this software. The software determined that the chromatogram of Figure 1 was the best gradient time for a full-range gradient. This separation looks good, but it contains wasted time because the first peak doesn't come out for approximately 7 min, and the last peak is eluted at roughly 13 min, but the gradient continues for 35 min.

We can trim the beginning and end of the gradient with the aid of a few simple calculations. First, find the mobile-phase compositions that elute the first and last peaks. Use equation 1 for this calculation.

$$\%B_{\mathrm{elution}} = (t_g - t_0 - t_d)(\mathrm{rate}) + \%B_{\mathrm{initial}} \quad [1]$$

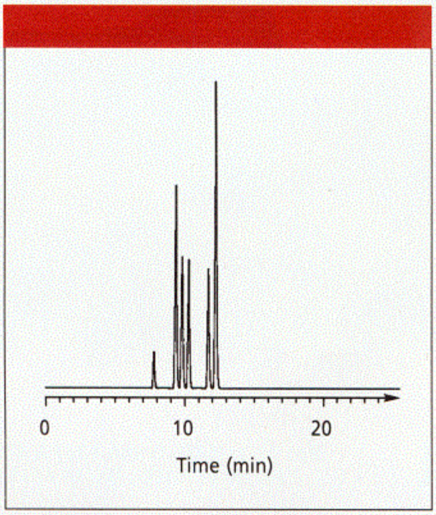

Figure 1: Separation of a synthetic mixture of drugs on a 150 mm × 4.6 mm, 5-µm d_p C18 column operated at 1.5 mL/min and 30 °C. The dwell volume was 2.3 mL, and the gradient was 5–100% methanol–acetate buffer in 35 min.

where $\%B_{\mathrm{elution}}$ is the percentage of B solvent at the end of the column when the peak is eluted; t_g is the gradient retention time; t_0 is the column dead time of V_m/F, where V_m is the column volume and F is the flow rate; t_d is the system dead time of V_D/F, where V_D is the system dwell volume; rate is the gradient rate of $(\%B_{\mathrm{final}} - \%B_{\mathrm{initial}})/t_G$, where t_G is the gradient time; and $\%B_{\mathrm{initial}}$ is the percentage of B solvent at the beginning of the gradient.

For the present case, t_g is 7 and 13 min for the first and last peaks, t_0 is 1.0 min, t_d is 1.5 min for our LC system, and the gradient rate is $(100 - 5)/35 \approx 2.7\%/\mathrm{min}$. So the first peak is eluted at approximately 17% B solvent and the last peak at approximately 33% B solvent. According to the method development guidelines (3–7), we should start the gradient 5–10% before the first peak is eluted, but in the present case, there is a large separation between the first two peaks, so we can get away with 15% B as a starting solvent concentration. Rounding the elution strength of the last peak for convenience, we have a new gradient range of 15–35% B solvent. To maintain the same selectivity in gradient elution, the slope must stay the same. Therefore, the new gradient time should be $(35\%–15\%)/2.7\%/\mathrm{min} \approx 7$ min. Because we had the modeling software available for help, we checked our results and found that we could get a little better separation with a 10-min gradient. Thus by adjusting the gradient range and keeping the slope constant, we obtained an almost identical separation in less than one-third the time (Figure 2).

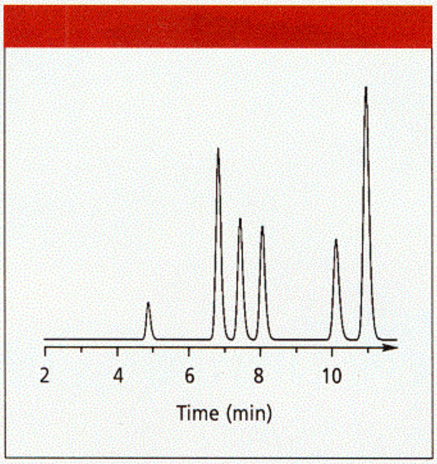

Figure 2: Separation of a synthetic mixture of drugs using a 15–35% B gradient in 10 min. Other conditions were the same as in Figure 1.

Move to the LC–MS

At this stage of method development, we have obtained a good separation with an LC–MS-compatible mobile phase, so we are ready to move to the LC–MS system. When gradient elution is used, most workers would like to have LC–MS methods with cycle times of less than 10 min for optimum use of this expensive instrument. Thus, the separation in Figure 2 is too long, probably amounting to 15–20 min by the time the gradient is regenerated and the column equilibrated. As mentioned above, LC–MS methods generally do not require as much resolution as conventional LC methods. In our case, the first three peaks are isobaric; that is, they have the same molecular weight. For this reason, we want to have sufficient resolution so we can distinguish them from each other, but the baseline resolution in Figure 2 is not required. Because we expect all three peaks in every sample, a separation of 0.1 min should be sufficient to get a good mass spectrum.

The simplest way to decrease the total cycle time is to change to a smaller column. Scaling to a smaller column is fairly simple, just keep in mind the following relationship:

$$\mathrm{constant} = t_G F / V_m \quad [2]$$

This relationship assumes that the gradient range is unchanged. (Equation 2 also works for adjusting flow rate when the column size is changed in isocratic separations, just ignore t_G.) Converting to a 30 mm × 4.6 mm column would require a fivefold adjustment of the gradient time to 2.0 min to compensate for a fivefold decrease in column volume (150 mm/30 mm).

Figure 3 shows a separation under these conditions. Resolution is poorer than that of Figure 2, but the three isobaric peaks are sufficiently resolved from each other; the third and fourth peaks will be resolved by the mass spectrometer. The retention time for the last peak is approximately 2 min, so an injection cycle time of 4–5 min should be possible.

Some workers prefer narrow-bore columns with LC–MS analyses because these columns can generate narrower peaks, which means better sensitivity (taller peaks) for the same injection mass or smaller injections for the same peak size when compared with conventional 4.6-mm i.d. columns. The change to a narrower column requires adjustment of the flow rate, because the column volume changes (equation 2). The volume changes with the square of the area, roughly fivefold, so a flow rate of 0.3 mL/min should be used with a 2.1-mm i.d. column.

Figure 4 shows a chromatogram generated under these conditions. The separation is slightly changed, but this result is an artifact of a slight change in the gradient caused by the dwell volume. Note that the retention times have increased by approximately 0.5 min, which also is due to dwell volume. It is interesting to note that for the present separation, other system factors — most likely extracolumn effects — prevent us from seeing any improvement in peak width with the smaller bore column.

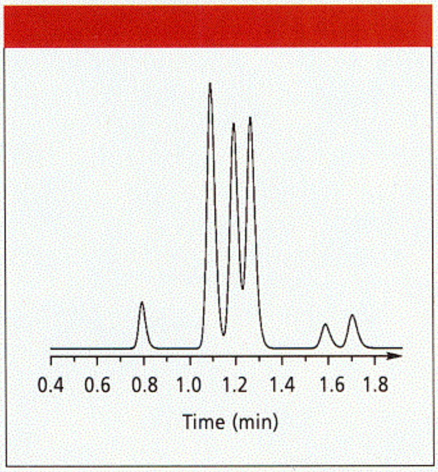

Figure 3: Separation of a synthetic mixture of drugs using a 30 mm × 4.6 mm, 3-µm d_p column at a 1.5-mL/min flow rate and with a 0.1-mL dwell volume. Gradient: 15–35% B in 2.0 min. Other conditions were the same as in Figure 1.

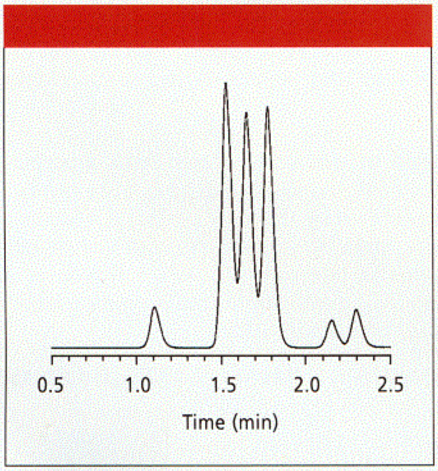

Figure 4: Separation of a synthetic mixture of drugs using a 30 mm × 2.1 mm column at a flow rate of 0.3 mL/min. Other conditions were the same as in Figure 3.

The Importance of Dwell Volume

Figures 3 and 4 illustrate that dwell volume can produce unexpected changes in the separation with gradient elution. Dwell volume is the system volume from the point at which the solvents are mixed to where they reach the head of the column. This volume is sometimes referred to as the gradient lag, and it effectively inserts an isocratic hold at the beginning of the gradient. Conventional LC systems have dwell volumes in the 0.5–5 mL range; our LC systems have dwell volumes of 2.3 mL. Although a 2.3-mL dwell volume results in a delay of 1.5 min at a 1.5 mL/min flow rate, it creates relatively small changes in retention with 150 mm × 4.6 mm columns and run times of 10–20 min. However, when the column volume is reduced, large dwell volumes can cause significant increases in run times and changes in the expected separation. For this reason, analysts generally should try to reduce the dwell volume to a practical minimum when using small diameter columns.

When using the mass spectrometer, we reduce the dwell volume of our conventional LC systems by installing an aftermarket micromixer instead of the standard one. This small mixer and other system plumbing amount to roughly 0.1 mL of dwell volume in our LC–MS systems. Examination of Figures 3 and 4 make it clear that the change in retention for the last peak by approximately 0.4 min is due primarily to the time it takes to wash out the mixer. Thus the dwell time is negligible with the 4.6-mm i.d. column (0.1 mL/1.5 mL/min), whereas 20 s are required for the gradient to reach the column at 0.3 mL/min (0.1 mL/0.3 mL/min), thus shifting the retention times in the run of Figure 4.

Figure 5 illustrates the importance of dwell volume's contribution to run time. This figure shows the same two separations of Figures 3 and 4, but for a system with 1.0-mL dwell volume instead of a 0.1-mL dwell volume. The small-bore column has almost twice the run time, which is entirely due to the delay of the gradient reaching the column (1.0 mL/0.3 mL/min ≈ 3 min). When using short or small-diameter columns, dwell volumes need to be minimized for best results.

Conclusions

We have covered some of the important aspects of converting conventional LC methods to LC–MS methods. We find it cost- and time-effective to do initial method development on a conventional LC system and then move to the LC–MS system. Scaling the method for smaller columns is simple if you remember that the gradient slope and the linear velocity of the solvent should be constant. Dwell volume, which can be a minor annoyance with conventional LC systems, becomes very important for high-throughput separations using small-volume columns.

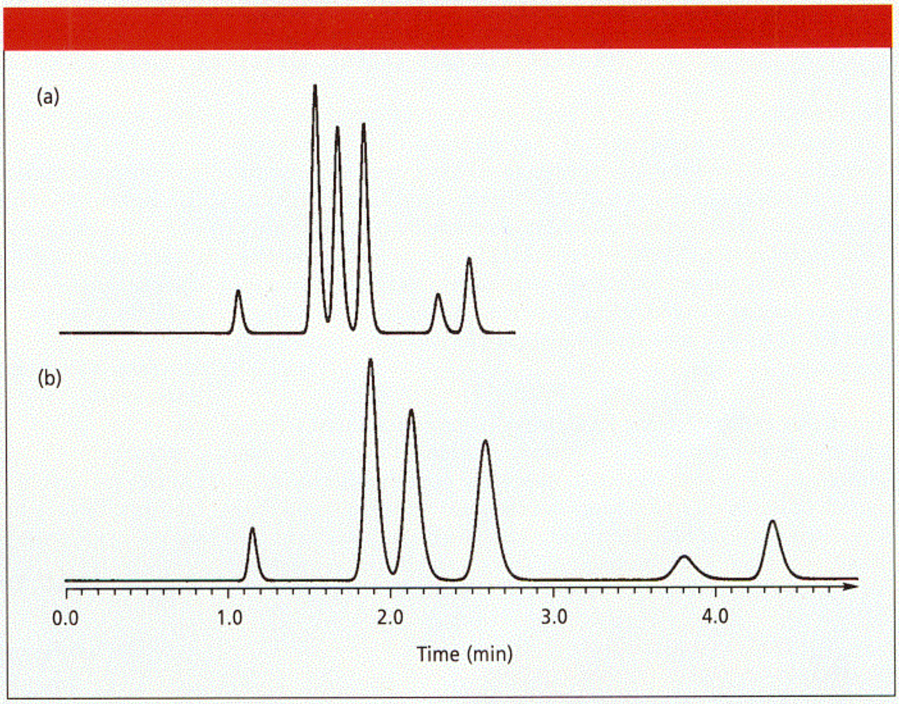

Figure 5: Separations showing the effect of dwell volume with small columns. Shown are chromatograms generated using (a) the same conditions as in Figure 3 but with 1.0-mL dwell volume and (b) the same conditions as in Figure 4 but with a 1.0-mL dwell volume.

More on Stainless Steel Corrosion

Two recent "LC Troubleshooting" columns covered some aspects of stainless steel corrosion and passivation of stainless steel in LC systems (1,2). We've received some reader feedback in the editor's electronic mailbox and would like to share it here.

First, some readers were a little confused about the concentration of nitric acid to use in passivation. All of the references were to dilution of concentrated nitric acid, generally labeled 70% HNO_3. Thus, a 1:1 dilution of acid with water will give a concentration of approximately 7.5 M.

One reader wrote to remind others to make sure to check the LC system for leaks before flushing with nitric acid. If you have a small leak at the rear of the piston seals, the acid can attack metal parts and lead to expensive repairs.

Finally, another reader reported nitric acid cleaning of an LC system that used a stream splitter to send the column effluent to two different detectors. After cleaning, the split ratio changed, and the reader suspected that the nickel tubing in the splitter had corroded. According to reference 9, nickel does not dissolve under the 50% nitric acid flushing conditions. Two other possibilities exist. The simplest cause could be the dissolution or displacement of a physical blockage in one of the pieces of tubing after the splitter, which changed the back pressure and thus the split ratio. The other possibility is more serious. Sometimes splitters or other tubing connections are made by silver soldering the connections together. Nitric acid readily dissolves silver solder, generating a green solution, so analysts must be careful to avoid this combination.

As mentioned in the original columns, always check the owners manuals before flushing any system with nitric acid — it may contain incompatible components.

References

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