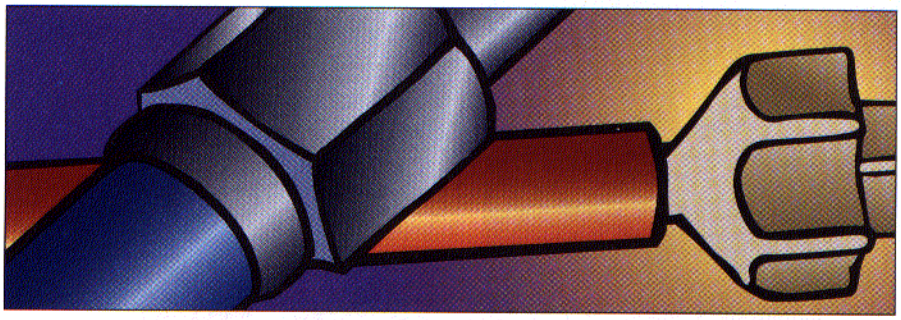
LC Troubleshooting



Late Elution and Carryover Peaks — A Case Study

Jin Y. Huang, Travis Culley, and John W. Dolan

What's the source of an extra peak in the second injection?

Imagine that you have spent days or weeks developing a liquid chromatography (LC) method. Or perhaps someone else's method is set up and ready to run. A blank run looks okay. So does the first sample run, but . . . oh, no! There's an extra peak in the chromatogram for the second sample. All experienced chromatographers encounter this problem at one time or another. Often these extra peaks are caused by the late elution of a sample component from a previous injection or carryover in the system. This month's "LC Troubleshooting" column discusses these problems and presents some tips to help you avoid them in your work.

LATE ELUTION

Late elution refers to peaks that come off the column after the normal run time. This situation can occur with isocratic or gradient runs and in any mode of chromatography. The example used here is for the analysis of a small (molecular weight less than 300) pharmaceutical compound using gradient elution. The mobile phase was a mixture of HPLC-grade methanol and ammonium acetate (pH 4.0). Solvent A was 10% methanol and solvent B was 70% methanol. We operated a 75 mm × 4.6 mm, 3.5-μm d_p C18 column at 30 °C with a 1.5-mL/min flow rate and UV-absorbance detection at 277 nm. The standard run called for a 100-μL injection of sample and a gradient of 0–60% B in 9 min followed by a 1-min hold.

Initial work with the method showed good results, but Figure 1 illustrates the results of a slight change in the sample pretreatment. Injection of a plasma blank at the beginning of the day generated the expected baseline of Figure 1a. However, the next injection, which was the lowest level of the standard curve (Figure 1b), showed a huge peak appearing close to the 7-min retention time of the analyte. This interfering peak was so large that only the highest level of the standard curve showed a distinct analyte peak (arrow, Figure 1c) as a shoulder on the back of the interference.

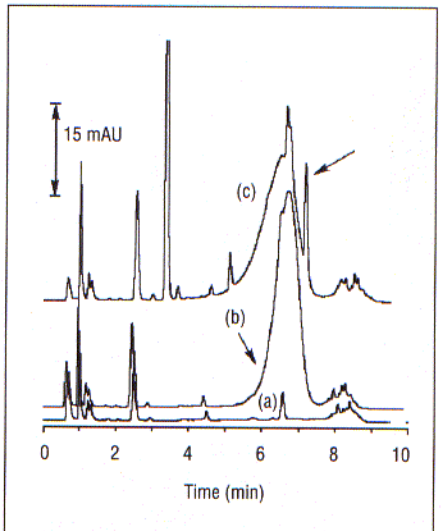


FIGURE 1: Chromatograms generated from injections of (a) a plasma blank, (b) a low-level standard, and (c) a high-level standard. See text for details.

What caused this extra peak? The fact that the peak did not appear in the first run but is present in each following run suggests that it is caused by late elution. If the peak was caused by an interference in each sample, we should have seen it in the first run as well. An easy way to determine the source is to either let the run continue after its normal stop time or to run a water blank as a sample.

Figure 2a shows the resulting chromatogram when we injected water following the high-level standard of Figure 1c. The large peak persists. If the peak were a carryover peak, its size should have been much smaller, but in this case it is essentially unchanged. The next injection (Figure 2b) was a plasma blank analogous to that of Figure 1a. No interfering peak is present.

Let's review the data. An initial plasma blank shows no interference peak, but the low-level standard following a blank shows the interference. Each subsequent sample shows the interference, including an injection of water following a high-level plasma standard. The sample after the water injection, however, shows no interference peak. All this information supports the hypothesis that the peak was caused by elution in the sample following a plasma sample — a late elution from the plasma.

PREVENTING LATE ELUTION

The strategy for preventing late elution is to combine time and a sufficiently strong solvent so that the elution occurs before the next run

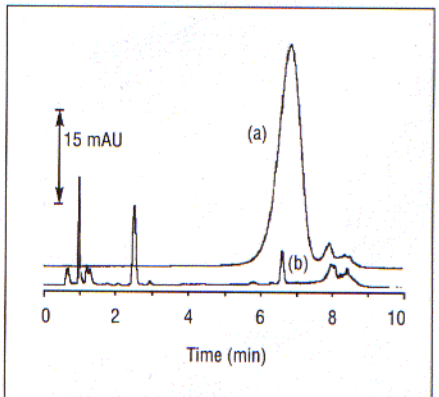


FIGURE 2: Chromatograms generated by injection of (a) a water blank following injection of high-level standard (Figure 1c), and (b) a plasma blank. See text for details.

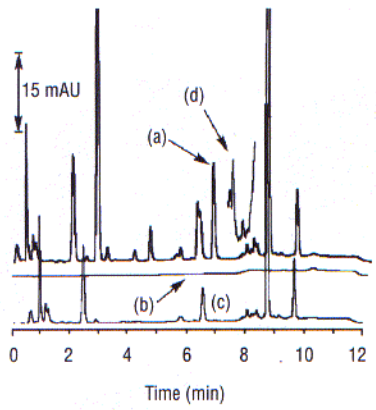


FIGURE 3: Chromatograms obtained following incorporation of strong-solvent flush in a method. Shown are results from injection of (a) a high-level standard (see Figure 1c), (b) a water blank (see Figure 2a), (c) a plasma blank (see Figure 2b), and (d) a low-level standard (see Figure 1b). See text for details.

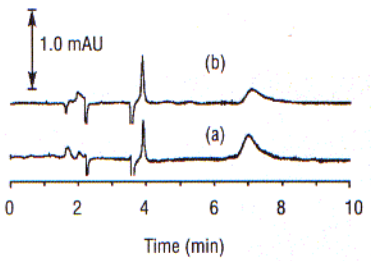


FIGURE 4: Chromatograms obtained after injection of (a) a plasma blank following injection of a 30-µg/mL sample of spiked plasma and (b) a 30-ng/mL standard. See text for details.

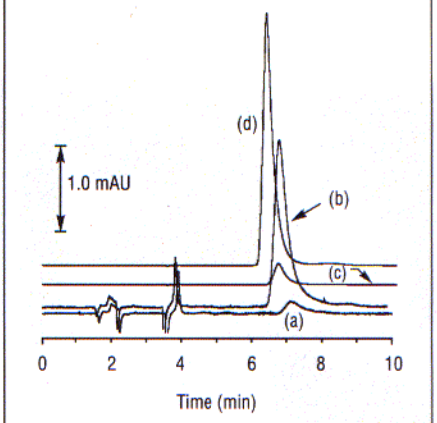


FIGURE 5: Chromatograms showing retention shifts caused by sample overload. Shown are results from sample concentrations of (a) 30 ng/mL, (b) 300 ng/mL, (c) 3 µg/mL, and (d) 30 µg/mL. Figures (a) and (b) use the displayed *y* axis; Figures (c) and (d) are attenuated 100-fold. See text for details.

is made. For gradient runs this process is fairly simple — just add a strong-solvent flush at the end of the gradient or continue the gradient to 100% B for a few minutes. In the present case, we modified the method by adding a step change to 100% B solvent for 3 min as soon as the analyte peak was eluted. Figure 3 shows the results. The high-level standard in Figure 3a, the equivalent to Figure 1c, is free of the interference. The water blank in Figure 3b following the high-level standard shows no interference peaks, as does the plasma blank of Figure 3c. The low-level standard (Figure 3d, 200× amplification, retention offset for clarity) has a peak large enough for use with the present application. Thus, simply washing the column with a stronger solvent between runs removes the interfering peak. In the present method, fewer than 50 samples were analyzed, so the extra run time was not important.

In the case of an isocratic run, the simplest way to avoid late elution is to allow the chromatogram to run until the elution occurs. However, the run time under isocratic conditions may be unacceptably long. Adding a strong-solvent step flush between samples accomplishes the same result as the solvent flush in the gradient example.

Another alternative for avoiding peaks from late elution is to change the sample pretreatment. In the present case, the investment of time to improve the sample pretreatment was not cost-effective considering the small number of samples analyzed.

SAMPLE CARRYOVER

A peak from a late elution originates in a previous sample and can appear even when no sample is injected, such as in the water injections of the previous example. In the previous example, the peak from the late elution appeared in the second run at about the retention time of the analyte, but this occurrence was coincidental — the peak could have been eluted in an area of no interest so that quantification of the analyte would have been possible.

The next case we'll discuss is carryover from a previous injection. Carryover and late elution often are confused. Remember that carryover is the elution of *the same compound* in a following injection, usually as a result of the compound sticking to something in the injection system. For the carryover example, we'll be considering the analysis of a porphyrin-like compound (molecular weight approximately 1000) using a 150 mm × 4.6 mm, 5-µm d_p C18 column operated at a 1.0-mL/min flow rate and a temperature of 40 °C. We used an isocratic mobile phase of 10:90 (v/v) methanol–0.2% trifluoroacetic acid. We used an autosampler to inject a volume of 150 µL.

Figure 4 clearly illustrates the problem. Following the injection of a 30-µg/mL sample of spiked plasma, we ran a plasma blank. Figure 4a depicts the resulting chromatogram. Figure 4b shows the results from an injection of a 30-ng/mL sample. The peak of Figure 4a represents approximately 50 ng/mL or a carryover of approximately 0.17% (50/30,000). Another injection of the plasma blank showed no peak at the retention time of interest (7 min).

The late-eluted compound considered earlier generated a peak about the same size regardless of the concentration of the analyte. In contrast, a carryover peak is proportional to the peak in the previous run. Thus, analysts are most concerned with carryover peaks from a quantitative standpoint when a high-level sample or standard is followed by a low-level sample or a blank.

Carryover usually results from one of two phenomena in the autosampler. If the autosampler is not plumbed properly, small unswept volumes may be present, acting as reservoirs to hold a small volume of sample. Each subsequent injection washes some of this sample into the system, creating the carryover peak. The best way to avoid unswept volumes is to ensure that all fittings are made properly with tubing fully seated before tightening the nut and ferrule and that factory-recommended flow paths are used. Ensure that all drain lines are clear so that waste sample does not back up into the injection flow path.

Carryover also can occur if the sample sticks to the tubing or other surfaces in the injection valve or autosampler. When adsorbed sample is displaced, a peak can result. To avoid sample adsorption in the autosampler, use a wash solvent sufficiently strong to displace the sample. Sometimes it is necessary to exchange stainless steel tubing for polyetheretherketone (PEEK), but this problem is uncommon. A related source of carryover is a sample that is nearly insoluble in the mobile phase. In this case, sample can precipitate in the system, only to dissolve slowly in subsequent injections. Again, this occurrence is rare.

Usually analysts can correct carryover problems by combining increased flushing volumes with use of better flushing solvents. In the present case, a stronger autosampler flush solvent and a larger flush volume corrected the problem.

SAMPLE OVERLOAD

When sample carryover occurs, the retention time of the carryover peak should be the same as the normal sample retention time. Sometimes sample overload problems can confuse interpretation of the results, as illustrated by the injections of Figure 5 and the corresponding data of Table I. The data indicate that this analyte overloads the column at low levels. This situation is illustrated by progressively

shorter retention times with successively larger injection masses. Although retention is shorter for larger concentrations of analyte, the peak area is proportional to mass, so analysts can plot a linear calibration curve. A more detailed discussion of carryover effects can be found in a previous "LC Troubleshooting" installment (1).

TABLE I: Concentrations and Retention Times for Sample Overload Example

Figure	Concentration (ng/mL)	Retention Time (min)	Area
4b, 5a	30	7.09	6,911
5b	300	6.88	51,849
5c	3,000	6.68	542,798
5d	30,000	6.35	5,446,629
4a	blank	7.02	11,674

In the present example, the carryover peak (Figure 4a) had an approximately 10% longer retention time than the preceding high-level standard (see Table I), which might lead to misidentification of the peak based on retention alone. For proper identification based on retention, chromatographers should compare the carryover peak with a standard of similar size (see for example Figure 4b or 5a).

CONCLUSIONS

An unexpected peak in a chromatogram can result from a late-eluted compound or carryover from a previous sample or standard. To check for late elution, simply allow the chromatogram to make an extended run — the peak should be eluted eventually. If the peak is a carryover peak, it will not appear at a later retention time using the extended run test. Another way to check for a carryover peak is to flush the column with strong solvent between runs. This flush should remove late elution but is unlikely to correct a carryover problem. Another clue to late elution is that the problem peak tends to be about the same size in all runs, whereas a carryover peak is proportional to the size of the corresponding peak in the previous run. Because of this proportionality, carryover peaks rarely appear in two subsequent blank injections.

Carryover and late elution often are confused. Remember that carryover is the elution of the same compound in a following injection.

Although late elution and carryover peaks cannot be avoided in all cases, identification of the problem source should be straightforward if the ideas discussed here are incorporated in the problem isolation strategy.

REFERENCE

(1) N.S. Wilson, J.R. Kern, and J.W. Dolan, *LC•GC* **16**(5), 442–446 (1998).

Jin Huang is a chemist, and Travis Culley is a staff chemist at LC Resources' laboratory in McMinnville, Oregon.

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, and a member of LC·GC's editorial advisory board. Direct correspondence about this column to "LC Troubleshooting," LC·GC, 859 Willamette Street, Eugene, OR 97401, e-mail John.Dolan@LCResources.com.