LC Troubleshooting

**Nan S. Wilson,
Ryan Morrison, and
John W. Dolan**

Gradient baselines don't always behave as desired.

Buffers and Baselines

Drifting baselines are common with gradient elution liquid chromatography (LC) separations. Drift is influenced by the mobile phase's organic solvents, the detection wavelength, and the presence of additives. This month's "LC Troubleshooting" focuses on the baseline characteristics of several common LC buffers and methods to minimize drift.

When comparing figures in this column, readers should note the scale of the *y* axis, because it is not the same in all figures. For example, the absorbance scales for Figures 1 and 2 differ by 10-fold. Also, don't place too much emphasis on the peaks in the various runs — we chose the chromatograms from our files to illustrate buffer and solvent drift, and we made no special efforts to remove extraneous peaks. Extra peaks in a gradient run often are the result of contaminants in the buffer or water, and they can be a challenge to eliminate. An earlier "LC Troubleshooting" column has an example of the process to isolate extraneous peaks (1).

Phosphate — An Old Friend

Phosphate buffers have long been a favorite of chromatographers. Some of phosphate's endearing qualities include its low cost, high purity, convenient preparation, useful pH ranges, and good chromatographic behavior. Figure 1 shows blank gradients for a 10 mM phosphate buffer with methanol as the organic solvent. At a low wavelength of 215 nm, the baseline drift is minimal, and a flat baseline is produced at 254 nm. We observe drift when the UV absorbance of the starting solvent does not match that of the ending solvent; however, as long as the drift is small enough to allow peaks to stay on scale and provide accurate integration, it can be ignored. Workers should be careful about the solubility of buffer salts in the organic solvent, especially when using phosphate buffers. For this reason, our laboratory personnel avoid using phosphate buffers at concentrations greater than approximately 10 mM combined with organic concentrations greater than approximately 80%. Methanol is less problematic in this regard than acetonitrile.

It is best to maintain constant buffer concentration during the run with equimolar buffer in both the A and B solvents. However, many workers take the easy way out and run with buffer in the A bottle and organic solvent in the B bottle, as is the case for Figure 1. Although this causes a gradient in buffer strength, it seldom is a problem if the buffer concentration is greater than 10 mM, organic concentrations are less than approximately 80%, and the buffer is used within its normal buffering range. For reproducible separations, it is important to specify in the LC method how the buffer is prepared and the formulation of the A and B solvents.

Figure 2 illustrates the effect of a B solvent with higher absorbance than the A solvent. In this case, the same phosphate buffer as in Figure 1 was used with tetrahydrofuran instead of methanol as the organic solvent. As Figure 2 shows, tetrahydrofuran causes the baseline to drift to two absorbance units (2 AU) at 215 nm. This drift makes tetrahydrofuran unusable as an organic solvent for low-wavelength gradients. Although many modern LC detectors are linear to an upper limit of 2 AU, remember that the 2-AU linear range includes any background. For the present example, 50% tetrahydrofuran generates a 1-AU background, so only 1 AU is available as the maximum peak height to maintain detector linearity. Most older UV detectors and many older data systems are
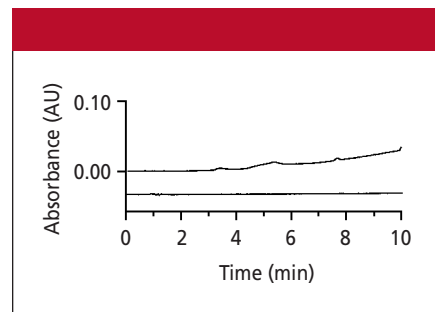

Figure 1: Baselines obtained using phosphate–methanol gradients. Solvent A: 10 mM potassium phosphate (pH 2.8); solvent B: methanol; gradient: 5–80% solvent B in 10 min. Detection wavelength: 215 nm (upper trace), 254 nm (lower trace).

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limited to a 1-AU maximum detector signal, which further limits the usefulness of tetrahydrofuran as a solvent. At higher wavelengths, such as the 254-nm run shown in Figure 2, tetrahydrofuran is satisfactory, and it may be the solvent of choice because of its separation characteristics.

Trifluoroacetic Acid — Another Favorite

An alternative buffer for low-pH work is trifluoroacetic acid. Trifluoroacetic acid is a favorite buffer for the separation of biomolecules. Tetrahydrofuran–acetonitrile gradients, such as those shown in Figure 3, represent standard conditions for biomolecule separations. The volatility of trifluoroacetic acid makes it compatible with mass spectrometry (MS) detection or suitable for sample recovery after mobile-phase evaporation. Because of the ease of formulation

Figure 2: Baselines obtained using phosphate–tetrahydrofuran gradients. Solvent A: 10 mM potassium phosphate (pH 2.8); solvent B: tetrahydrofuran; gradient: 5–80% solvent B in 10 min. Detection wavelength: 215 nm (upper trace), 254 nm (lower trace).

(just add 1 mL/L) and its ability to minimize absorbance differences between the A and B solvents at low wavelengths, 0.1% trifluoroacetic acid can be added to water as the A solvent and acetonitrile as the B solvent.

Figure 3 also illustrates the effect of the system dwell volume and the column volume on the baseline. The combination of these two volumes delays the arrival of the gradient at the detector by almost 3 min in this case, as the baseline step for the 215-nm plot shows. Note that although the step looks large in Figure 3, the actual offset is approximately 0.03 AU. The column dead time can be identified by the baseline disturbance immediately after 1 min.

Acetate for Intermediate pH

Trifluoroacetic acid is useful near pH 2, and phosphate is useful from approximately pH 2 to pH 3.1 and from pH 6.2 to pH 8.2. For intermediate pH values, acetate buffers often are the best choice. These buffers also have the advantage of volatility, so they can be used with LC–MS detection. However, using acetate can be a challenge if chromatographers fail to take proper care during mobile-phase formulation. This problem is illustrated in Figure 4, in which we took the buffer-in-A solvent and organic-in-B solvent approach, as was the case with phosphate in Figures 1 and 2. At 215 nm, ammonium acetate has much greater background absorbance than methanol and results in the severely negative drift in Figure 4.

Negative drift is especially problematic because most data systems cannot process detector outputs less than roughly −100

mV (equivalent to −0.1 AU in this case). Under these restrictions, analysts would observe the baseline dropping to −0.1 AU and then remaining flat, much as in the days of strip chart recorders when the pen would drift to the bottom of the paper. This set of conditions clearly is unacceptable. To compensate for the negative drift, workers can add a UV-absorbing compound to the B solvent so its absorbance matches the A solvent. In the present case, we accomplished this task by adding ammonium acetate to the methanol. For the best performance with ammonium acetate, chromatographers should prepare the A and B solvents so they have the same buffer concentration — 25 mM in this case.

When we used equimolar buffer in both solvents, the drift was greatly reduced, as Figure 5 shows (note the absorbance scale change). We observed a slight positive drift, which is easy for the data system to handle. The magnitude of the drift (approximately 0.2 AU in this case) is small enough so integration will be satisfactory and the chromatograms will be visually pleasing. Note that when working at the higher wavelength of 254 nm, matching the mobile-phase absorbance is unnecessary.

High-pH Buffers

Although phosphate is a useful buffer at pH levels as high as pH 8.2, we recommend avoiding phosphate at levels higher than pH 8 in favor of organic buffers that minimize the dissolution of the silica-based column packing (2). One alternative is Tris (tris[hydroxymethyl]aminomethane), a buffer widely used by biochemists. Some workers have found ammonium bicarbon-
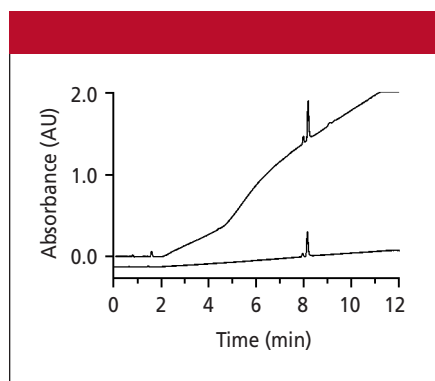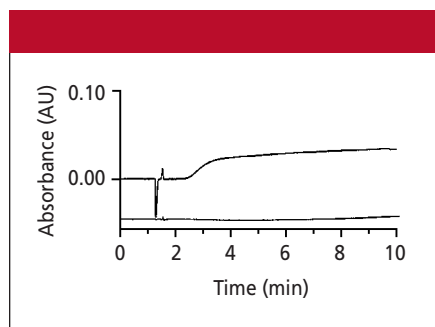
Figure 3: Baselines obtained using trifluoroacetic acid–acetonitrile gradients. Solvent A: 0.1% trifluoroacetic acid in water (pH ≈ 1.9); solvent B: 0.1% trifluoroacetic acid in acetonitrile; gradient: 10–90% solvent B in 10 min. Detection wavelength: 215 nm (upper trace), 254 nm (lower trace).
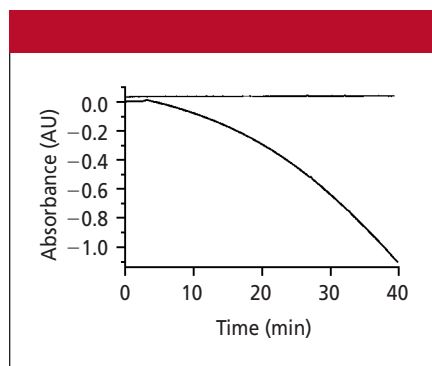
Figure 4: Baselines obtained using ammonium acetate–methanol gradients. Solvent A: 25 mM ammonium acetate (pH 4); solvent B: 80% methanol in water; gradient: 5–100% solvent B in 40 min. Detection wavelength: 254 nm (upper trace), 215 nm (lower trace).
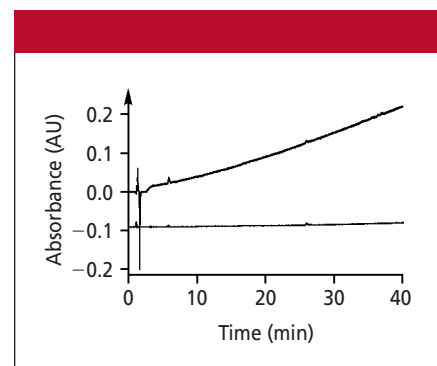
Figure 5: Baselines obtained using equimolar ammonium acetate–methanol gradients. Solvent A: 25 mM ammonium acetate (pH 4) in 5% methanol; solvent B: 25 mM ammonium acetate in 80% methanol; gradient: 0–100% in 40 min. Detection wavelength: 215 nm (upper trace), 254 nm (lower trace).
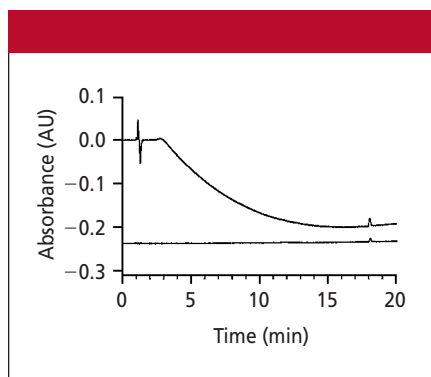
Figure 6: Baselines obtained using Tris–methanol gradients. Solvent A: 50 mM Tris; solvent B: methanol; gradient: 5–80% solvent B in 20 min. Detection wavelength: 215 nm (upper trace), 254 nm (lower trace).
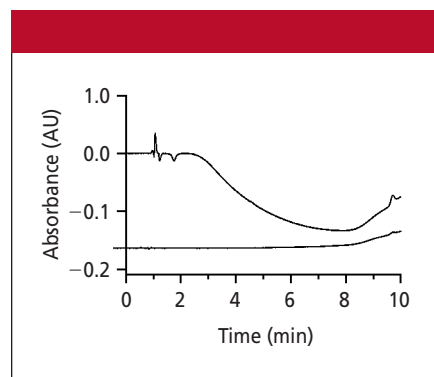

Figure 7: Baselines obtained using ammonium bicarbonate–methanol gradients. Solvent A: ammonium bicarbonate (pH 9); solvent B: methanol; gradient: 5–60% solvent B in 10 min. Detection wavelength: 215 nm (upper trace), 254 nm (lower trace).

ate to be especially useful at high pH levels (3).

Figures 6 and 7 illustrate examples of baselines obtained with these buffers. In both cases, the present negative drift is sufficient to cause problems with data-system performance at low wavelengths. At higher wavelengths, drift is no problem.

Conclusions

Examining gradient baselines for several combinations of buffers and organic solvents is useful to illustrate several common problems and practices in mobile-phase preparation. As a general rule, baseline drift caused by absorbance mismatch between the solvents will be more problematic at lower wavelengths than at higher ones. To avoid undesirable drift, workers may need to add a UV-absorbing compound to one solvent or the other so their absorbance is similar. From a scientific standpoint, analysts will get the best chromatographic behavior and reproducible separations if the A and B solvents have the same concentration of buffer and the organic solvent concentration varies during the gradient. From a practical standpoint, however, workers often can get satisfactory results by placing buffer in only the A solvent.

Although we did not discuss it in this column, using buffers within their effective buffering range — ±1 pH unit from their pK_a value — is important. Outside this range, chromatographers will obtain poor buffering even with high buffer concentrations. The runs described in this column also illustrate the importance of running a blank gradient to determine drift problems and to identify any background peaks in the gradient.

The baseline drift problems discussed in this column will not occur with isocratic separations using the same mobile-phase components. Any baseline offset caused by solvent or buffer absorbance will be hidden when the detector autozeros at the beginning of each run. However, the background absorbance of the mobile phase is still present, so the useful linear range of the detector may be reduced.

References

(1) M.D. Nelson and J.W. Dolan, *LCGC* **16**(11), 992–996 (1998).
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(3) Uwe Neue, personal communication, April 2001.

Ryan Morrison and Nan Wilson are chemists at LC Resources Inc. (McMinnville, Oregon) who have special interests in column characterization and the development of stability-indicating assays for pharmaceutical products.

John W. Dolan

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. 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@LCResources.com.

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