TROUBLESHOOTING

Gradient Elution Separation Problems, Part II

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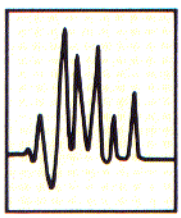

Last month's "Troubleshooting" column discussed problems sometimes encountered when liquid chromatography (LC) separations are performed in the gradient elution mode (1). It covered the basic principles of gradient elution liquid chromatography and discussed the ways in which the gradient range, gradient time, flow rate, and column volume are interrelated such that we can predict the changes in separation when one or more of those parameters are varied. It was also pointed out that many of the "problems" observed with gradient elution liquid chromatography are not problems at all but are the expected outcome of changes in experimental conditions. This month's column continues the discussion of gradient elution problems.

TABLE I: TROUBLESHOOTING GRADIENT ELUTION PROBLEMS

Symptom	Solution
Bands bunched at front of chromatogram with poor resolution	Decrease % B at beginning of gradient
Poor resolution in middle of chromatogram	Increase k', N, and/or α (see reference 1); remember that k' varies with gradient time, flow rate, and column length
Retention times early in chromatogram are not reproducible	Increase regeneration time between gradient runs; inject samples at regular intervals
Baseline drift	Usually caused by different UV absorbance of A- and B-solvents; add a nonretained UV-absorber to the less-absorbing solvent to equalize absorbances (see text); also, can be detector-related (try a different model of detector)
Artifactual peaks in blank gradient	Caused by impurities in solvents or mobile phase additives; purify mobile phase components or use a better grade of reagents
Solvent demixing	Avoid use of silica columns

COLUMN REGENERATION

In liquid chromatography — under either isocratic or gradient conditions — the column must be equilibrated with the mobile phase before the first sample is injected. When samples are chromatographed isocratically, the column is in equilibrium with the same mobile phase composition throughout the run, so the next sample can be injected as soon as the last band from the previous sample has eluted. This is not the case, however, with gradient elution.

When a gradient run is completed, the column contains a stronger mobile phase than was used at the beginning of the run. Before the next sample is injected, the column must be reequilibrated with the starting mobile phase. Generally, this can be achieved by flowing 15 column volumes of the starting mobile phase through the column. Some workers prefer to run a "reverse gradient" (that is, from *strong* to *weak* solvent) for equilibration. For example, if the original gradient was 5–95% acetonitrile (ACN), a gradient from 95% to 5% ACN would be used in regeneration. There is no inherent advantage in using a reverse gradient rather than returning directly to the initial conditions.

One should also understand that the important factor in column regeneration is the *volume* of mobile phase used. Thus, if a 15 cm × 4.6 mm column is used (≅ 1.5 mL dead volume), 1.5 mL × 15 column volumes = 22.5 mL of mobile phase should be used for regeneration. It doesn't matter if the pump is run for 7 min at 3 mL/min or 22 min at 1 mL/min — the result is the same. Once the regeneration time is complete, the LC system should be run for a couple of minutes under initial gradient conditions to ensure that the system has stabilized (indicated by a steady baseline).

If the column is not flushed sufficiently between gradient runs, problems can arise in run-to-run reproducibility. The usual problem is that retention times for early peaks in the chromatogram vary from one run to the next. When this is observed, it means that more washing of the column by the starting mobile phase is required between each run. This can be achieved by increasing the regeneration time or increasing the flow rate during equilibration. Alternatively, if the time between sample injections is kept constant, retention variation will be less, even when the column is not completely equilibrated. In some cases, complete regeneration of the column for gradient elution would require a prohibitive amount of time between runs.

OTHER GRADIENT ELUTION PROBLEMS

For the most part, problems observed in gradient elution are the same as those observed in isocratic elution. Therefore, if your problem is not summarized in Table I, simply treat it as if it had arisen during an isocratic separation. Problems specific to gradient elution include drifting baselines, artifactual peaks, and solvent demixing.

Drifting baseline: A drifting baseline during gradient elution is common. An example of this can be seen in Figure 4a of last month's column (1). In that case, the baseline rose during the gradient as a result of the greater absorbance of the B-solvent (for example, ACN when water is the A-solvent). This problem is magnified by the use of lower UV wavelengths for detection and is also more serious for those detectors that do not filter out second-order wavelengths produced by

Erratum: Please note that in the May 1987 issue of *LC•GC* (Volume 5, Number 5), on page 388, in "Troubleshooting," the third sentence in the fourth paragraph should read, "An increase in the flow rate for a gradient separation, however, reduces the resolution, the band widths, and the retention times, and, as shown in equation 1, causes k' to be higher."

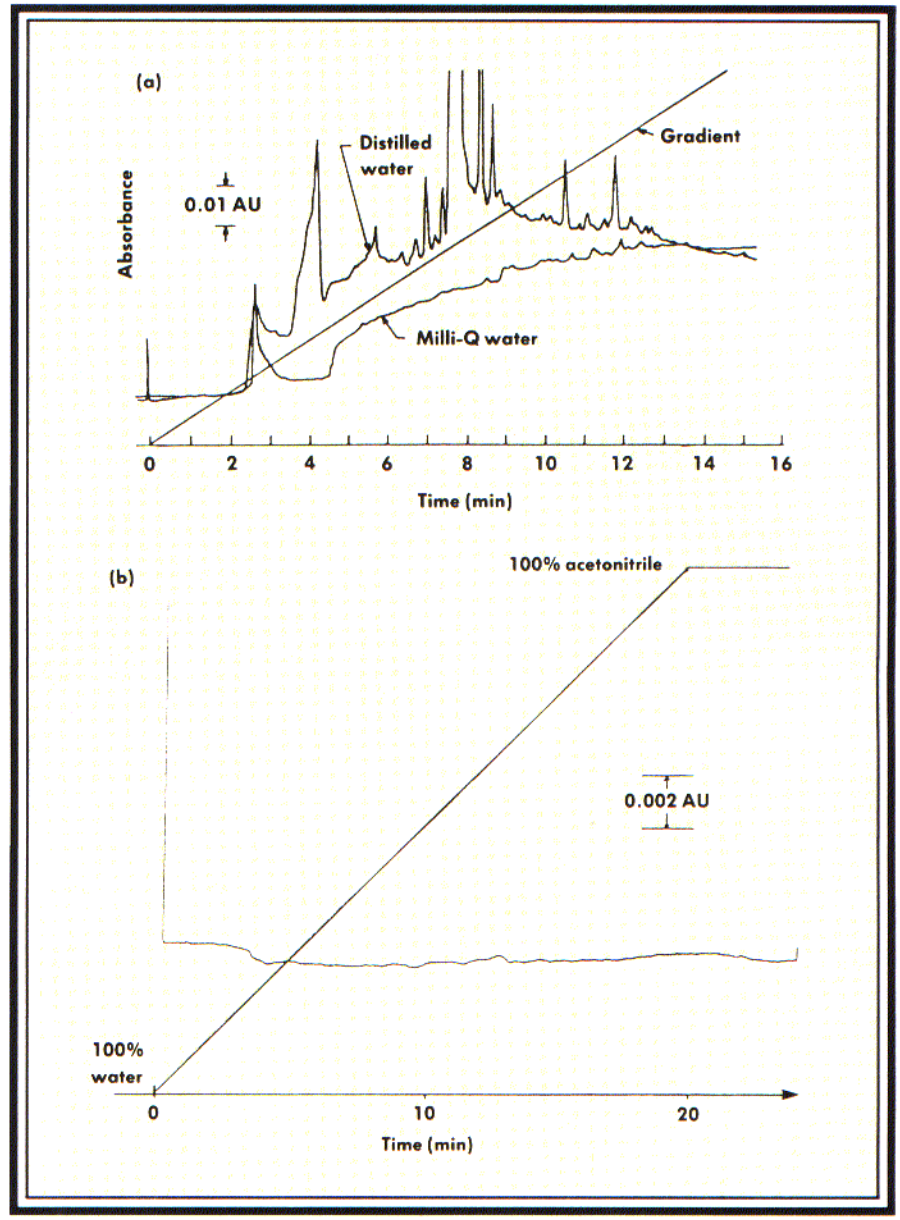

FIGURE 1: Blank gradients run with water (A-solvent)/ACN (B-solvent). Detection: UV 254 nm. (a) Blank gradients with distilled water (upper trace) and with HPLC-grade water prepared in the laboratory (lower trace); (b) blank gradient with commercially prepared HPLC-grade water. (a) From reference 4; (b) from reference 5, with permission.

diffraction gratings. One solution to the problem is to add a UV-absorbing compound to either the A- or B-solvent to equalize the absorbance of both solvents. However, the UV-absorbing additive must not be retained under the conditions of the gradient, or it will separate, creating a major baseline upset. Berry discussed this problem in detail and proposed adding nitrous oxide gas to the A-solvent (water) in reversed-phase gradients (2). A more convenient alternative for 185–200 nm detection is to use nitric acid or nitrate in small concentrations (3). At higher detection wavelengths, salts such as bromate or periodate that contain heavier elements can be used.

Artifactual peaks: Any UV-absorbing impurities in the mobile phase components can separate during the gradient, causing the appearance of peaks that do not correspond to sample bands. Figure 1 shows gradient runs carried out without injecting sample ("blank" gradients). In the upper trace of the two superimposed chromatograms of Figure 1a, ordinary distilled water was used in a water/ACN (A-solvent/B-solvent) gradient. This chromatogram contains many peaks representing impurities in the water. When the same source of water is used for isocratic runs, the spurious bands don't appear because the mobile phase strength is constant. With gradient elution, on the other hand, under *weak* (initial) mobile phase conditions, these bands build up on the column. Then, when the mobile phase strength increases, the bands elute as if they were sample bands.

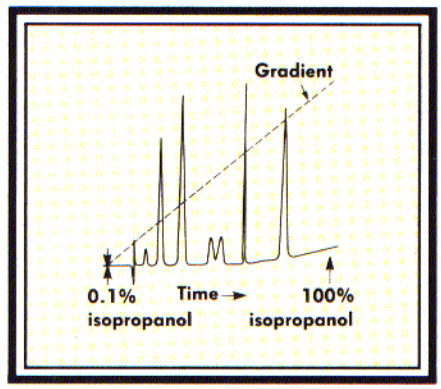

FIGURE 2: Effect of solvent demixing on a gradient separation. Hexane–isopropanol gradient with a silica column. (Reprinted from reference 4, with permission.)

A good way to verify that spurious bands are from the mobile phase is to run two blank gradients with different equilibration times between runs. For example, after running a blank gradient, reequilibrate the column with a known volume of initial mobile phase (say, 15 column volumes), then make the first experimental blank run. After that run, use a larger equilibration volume (for example, 60 column volumes) before the second experimental blank run is made. The longer equilibration time should produce proportionally larger bands (in this case four times larger) if the bands are originating from the A-solvent.

To avoid problems with extra peaks that originate from the water used in the mobile phase, use better quality water. The lower trace in Figure 1a was run with HPLC-grade water prepared with an in-lab purification system. Most of the extra peaks have been removed by the purification scheme. Techniques for commercially preparing HPLC-grade water have advanced to such a degree that essentially no baseline drift caused by water impurities can be observed during a blank gradient run (Figure 1b). This blank gradient is quite acceptable.

Artifactual peaks in gradient elution can arise whenever the various mobile phase components are inadequately purified. Even if HPLC-grade solvents are used, peaks can appear when detection wavelengths of less than 220 nm are used and the detector is set for maximum sensitivity. A "bad" lot of HPLC-grade solvent can, of course, magnify the problem. For this reason, it is a good idea to run a blank gradient before injecting any sample. If artifactual peaks are seen, then different lots of solvent or mobile phase additives can be used in an effort to reduce the problem.

When reversed-phase gradient elution is used for samples that require low-UV detection, problems often are encountered with the quality of both the water and the organic solvent. Milli-Q–grade water (Millipore Corp., Bedford, Massachusetts) (or equivalent) can be further purified by irradiation with UV light. ACN is the only organic solvent that can be used for detection at 210 nm or lower, but HPLC-grade ACN forms UV-absorbing impurities as it ages (2). Consequently, for low-UV use (particularly at high sensitivity) the ACN must be either "fresh" or purified with an alumina precolumn (2).

Solvent demixing: Solvent demixing can arise in gradient elution with silica columns. If the B-solvent is very polar (for example, propanol) and the A-solvent is very nonpolar (for example, hexane), the B-solvent will be taken up by the column during the initial part of the gradient, followed by a "breakthrough" of the B-solvent later in the gradient. The result is a sudden loss of resolution at that point in the chromatogram. This is illustrated in Figure 2 for the conditions just described. It can be seen that the next-to-last band is much narrower than the bands on either side. This peak elutes at the point at which the B-solvent (isopropanol) has broken through (after solvent demixing); because it elutes under stronger solvent conditions, the band is narrower. The solution to the problem of solvent demixing is to use a column that has less tendency to sorb the B-solvent. Generally, this problem does not occur with bonded-phase columns.

SUMMARY

Only a few problems are specific to gradient elution liquid chromatography, and they are summarized in Table I. The remaining problems encountered with the technique fall into two categories. First, there are problems such as column degradation and band broadening, which are common to all LC separation modes. These problems should be approached in a similar manner in all cases. Second, there are the problems discussed last month (1); they result more from a poor understanding of the gradient elution separation process than from anything else. These problems can be best addressed by using equation 1 of reference 1 as a guide to determine the effect of changes in the various LC operating parameters.

If you have experienced other gradient elution problems that you would like to see discussed in detail, please submit them to "Troubleshooting," c/o The Editor, *LC•GC*, P.O. Box 10460, Eugene, OR 97440.

REFERENCES

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Portions of this column were taken from *Troubleshooting HPLC Systems*, J.W. Dolan and L.R. Snyder, in preparation for Humana Press.

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