LC TROUBLESHOOTING

Ion-Pairing Problems

John W. Dolan

Don't upset the (equilibrium) applecart is one key to successful ion-pairing separations.

This month's column discusses two readers' questions about ion-pairing methods. Although the symptoms of each problem are different, the causes appear to be related. This is often the case — more than one symptom can result from a single problem. To further complicate matters, as the second question illustrates, a symptom (split peaks) can be the result of more than one type of problem. By carefully eliminating potential causes, you should be able to clearly identify the cause–symptom relationship and solve the problem.

BASELINE HUMP

Q: I have a baseline problem in a method I recently developed for the assay of a pharmaceutical impurity. As Figure 1 shows, the baseline is flat for a blank injection, except for a couple of small peaks near the void volume. When my sample is injected, however, a baseline hump appears under the major compound. This pattern is repeatable for all sample injections. I tried many things to fix this problem, with no success. The compound is proprietary and has a mass of ~350 Da. I am using a 30-cm phenyl column, and the mobile phase is 75:25 (v/v) 5 mM decanesulfonic acid (pH 4.4)–acetonitrile. How would you recommend curing this problem?

JWD: Your baseline upset is probably the result of an equilibration problem. Two important guidelines for ion-pairing methods such as yours will help you obtain the best results.

First, be sure that the concentration of ion-pair reagent is sufficient. Unless you have reason to, never use less than ~25 mM ion-pair reagent in the mobile phase. Your present mobile phase contains <5 mM ion-pair reagent. Low levels of these additives can result in irreproducible retention times, variation of response with the injected sample mass, and other problems such as those you have seen.

A second type of problem arises if your sample is not injected in mobile phase. This problem occurs because of the nature of ion pairing in the column. Put simply, two types of interactions take place with ion-pairing methods. First, the ion-pair reagent becomes immobilized on the column, forming an in situ ion exchanger. When this occurs, sample molecules are retained as they would be in an ion-exchange column. Second, the reagent pairs with oppositely charged sample molecules, forming an ion pair in solution. This ion pair has more hydrophobic characteristics than the unpaired sample molecules, so it is retained in classical reversed-phase manner. Both of these mechanisms occur, and it should be obvious that in both cases an equilibrium is set up between free ion-pair reagent in the mobile phase and the ion-pair reagent either immobilized on the column or paired in solution. Anything that upsets the equilibrium of the system can cause baseline upsets, retention changes, peak splitting or tailing, and other changes in the chromatogram.

Unless you have reason to, never use less than ~25 mM ion-pair reagent in the mobile phase.

You did not mention the composition of your injection solvent or the injection volume. Injections of more than 5–10 µL of non-mobile-phase solvent can cause some of the equilibrium problems mentioned above (for more details, see the discussion of the following reader's question below).

I have two suggestions: First, inject your sample in your mobile phase. You may need to dilute your sample in a concentrated mobile-phase solution so that the final dilution is equivalent to the concentration of the mobile phase. Second, adjust the mobile-phase ion-pair concentration to 25–50 mM. Changing the ion-pair reagent concentration may require some adjustment of the proportion of acetonitrile to control retention. The first step may fix your problem, but if you want a more stable method, use both steps.

Finally, be aware that ion-pairing chromatography requires longer equilibration times than do standard reversed-phase separations. This means that you should allow at least 20 column volumes — a total of 50 mL of mobile phase in your case — to pass through the column before you inject each day. It is the volume of solvent that is important for equilibration, not the time, so if you can increase the flow rate and keep the pressure within an acceptable range, you can speed the equilibration process.

SPLIT PEAK

Q: I have a method for analyzing three antibiotics in serum. The method has worked well in the past (Figure 2), but since I installed a new column I get an extra peak at the beginning of the chromatogram that interferes with the first peak. I use a 25-cm C8 column and a mobile phase of 28:72 (v/v) acetonitrile–buffer run at 1.5 mL/min. The buffer consists

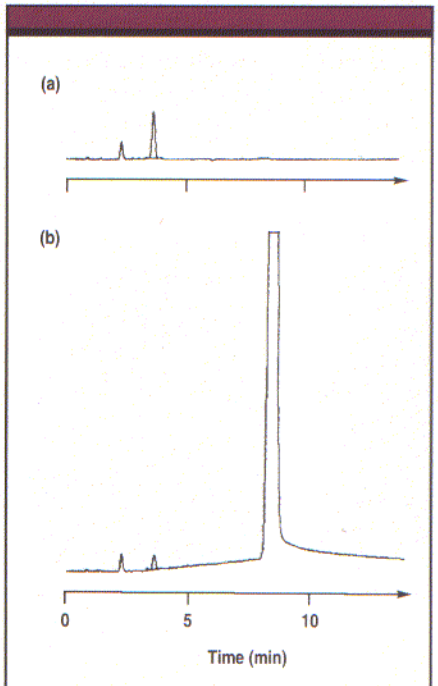

FIGURE 1: Chromatograms illustrating a baseline disturbance, including those generated by (a) blank and (b) sample injections.

of 15 mM phosphate ion and 20 mM tetrabutyl ammonium ion, adjusted to pH 3 with phosphoric acid. My sample preparation involves dilution of the sample in water, acid precipitation, and extraction with ethyl acetate. The ethyl acetate is then back-extracted with a buffer of 20 mM phosphate ion, 80 mM tetrabutyl ammonium ion, and 50 mM phosphoric acid. I inject 30 µL of the final 0.5 mL of extract. Unextracted standards produce normal peaks (Figure 2a).

I checked with the column manufacturer, and the new column is from the same lot as the old one. A second new column yields the same results. Can you help solve this problem?

JWD: I suspect that this problem is similar to the one reported above. Your final injection solvent is not matched to the mobile phase, so some upset of the mobile-phase equilibrium is expected. With the relatively large injection volume, the solvent effect can be magnified. Furthermore, ethyl acetate has a sufficiently high solubility in water that you may be injecting too much of this strong solvent residue. Peak splitting of early peaks is a common result of injecting too much strong solvent (see reference 1 for another example). Examining the retention times provides further evidence of excessively strong injection solvent. All three peaks are eluted earlier in Figure 2b than in Figure 2a, suggesting that the strong injection solvent may be washing the peaks along the column before it is diluted by the mobile phase. The other common causes of peak splitting — a column void or a blocked frit — will result in split peaks throughout the chromatogram rather than just in the early peaks, so these causes can be eliminated from consideration.

I suggest two tests to check this theory. First, inject 5 µL of sample instead of 30 µL, which should reduce the effect of the strong solvent and give better peak shape than the larger volume injection. This may not be feasible for the final method because of sensitivity considerations, but the small injection should help to pinpoint the problem source.

Peak splitting of early peaks is a common result of injecting too much strong solvent.

The second test is to inject 30 µL of sample that has been diluted 1:1 with mobile phase. This test should also reduce the amount of strong solvent injected and remove or reduce the peak splitting.

(The reader performed the above experiments, and both tests resulted in a chromatogram with a single peak where the double peak had been previously.)

Your tests plus the observation that unextracted standards yield normal peaks indicate that the problem is probably excess ethyl acetate in the final injection. To correct this problem, you will need to modify the method.

Using the 5-µL injection, if you can get by with it, would be the easiest modification. You could try larger injections until you observe the peak splitting again. To be safe, I would limit the injection volume to about half the minimum volume at which peak splitting occurred. You may be able to adjust the detection wavelength or make some other adjustment in the method to compensate for some of the sensitivity loss that results from using the smaller injection.

Injecting a larger volume of dilute sample is another simple modification of the method that may be satisfactory. Because you need to match your injection solvent to the mobile phase, you cannot use on-column concentration to compensate for large injection volumes of dilute sample. When the mobile phase is used as a sample solvent, a rule of thumb is that you can inject as much as 10% of the volume of the first peak of interest without noticeable degradation of the chromatogram. From Figure 2a, I estimate that the first peak is ~0.6 min wide. At 1.5 mL/min, the peak volume is ~900 µL, indicating that injection volumes of as much as ~90 µL should be acceptable. My tendency is to be conservative on these guidelines, so I would try diluting the final sample with mobile phase by a factor of two and injecting 50–60 µL. This dilution should provide about the same response and may overcome the problems associated with an excessively strong sample solvent.

Another alternative is to rework the extraction procedure to reduce or eliminate the ethyl acetate residue in the final sample. This approach might involve using another solvent, adding an evaporation step, using more rigorous phase separation, or some other modification. This is clearly the least desirable alternative because it is a lot of work and may require revalidation of the extraction steps.

Finally, follow the guidelines discussed in the answer to the previous reader's question. Your phosphate buffer and tetrabutyl ammonium ion-pair reagent are both below the recommended 25 mM minimum. Although the final sample diluent contains all the components of the buffer portion of the mobile phase, the proportion of ingredients is incorrect for making the injection solvent match the mobile phase. One easy way to correct this problem is to make your sample diluent twice the concentration of the mobile phase in all components. When you dilute your extracted sample 1:1 with this diluent, the concentration of the injection solvent should be close enough to that of the mobile phase to be free of problems.

The unanswered question is, why does the peak-splitting problem show up with some columns and not others of nominally the same packing material? As is the case in the example of reference 1, your method is probably right on the edge of reliability. Some small change in the environment or chromatographic conditions can be enough to push the method into a region of unreliability. Changes in column temperature, laboratory humidity, the source of reagents, or other seemingly insignificant differences may be the cause of the problem. It is very difficult to pinpoint the cause-and-effect relationships for such problems, so it is best to design methods in which these problems are unlikely to occur. Even then, it is advisable to stress the method during validation to determine which factors are likely to cause problems.

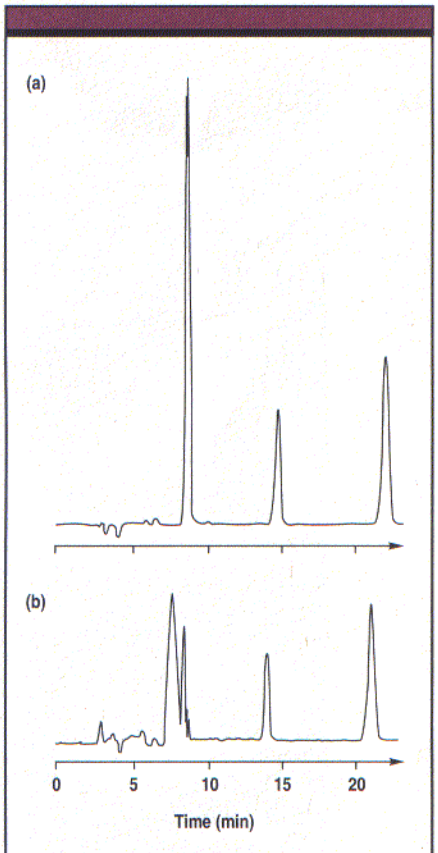

FIGURE 2: Chromatograms from the analysis of three antibiotics, including those generated using (a) good and (b) bad injection conditions.

REFERENCE

(1) J.W. Dolan, *LC•GC* **10**(2), 84–86 (1992).

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