Troubleshooting

Reversed-Phase LC in 100% Water

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How can you avoid phase collapse when 5% organic solvent is too strong?

Reversed-phase liquid chromatography (LC) columns with embedded polar stationary phases increasingly are becoming available as manufacturers respond to the application of these columns to problem separations. This month's "LC Troubleshooting" describes a case study in which one of these columns was used to solve a challenging separation problem.

The Problem

A client asked us to help identify a degradant in a formulated drug product. A *United States Pharmacopeia* (*USP*) method for the drug called for the use of a 150 mm × 4.6 mm, 5-µm d_p C8 column operated at a 1.5-mL/min flow rate with a low-pH mobile phase containing octane sulfonate, phosphate buffer, and 15% methanol. These ion-pairing conditions were required to obtain adequate retention of the drug compound.

In extended shelf-life tests, our client discovered the presence of a poorly retained peak with an area of approximately 25% of the parent compound peak under the test conditions. This peak can be seen in Figure 1 in which a chromatogram of the degraded sample is compared with a drug standard using the *USP* method. Using customary procedures, the client had injected standards of many compounds that might have been present in hopes of discovering the identity of the unknown degradant. The retention time of the unknown peak did not correspond to any of the suspected degradant candidates or any precursors or reaction by-products. We were asked to use our liquid chromatography–mass spectrometry (LC–MS) system to determine the structure of the unknown degradant.

The Challenge

The most obvious problem we faced was the presence of an LC–MS-incompatible mobile phase. If additives are used in mobile phases that are fed into a mass spectrometer, they must be volatile and not suppress the ionization of the sample molecules. Ion-pairing reagents, such as the aliphatic sulfonates, are unsuitable for use with LC–MS methods and so are phosphate buffers. Often analysts can convert a method using a nonvolatile buffer to an LC–MS-compatible method simply by substituting low-pH conditions with a volatile buffer or acid such as 0.1% formic acid.

Before we could start converting the *USP* method to an LC–MS-compatible one, we needed standards of the analytes so that we could determine which peak was which. To obtain a standard of the unknown, we collected fractions across the sample peak under the *USP* conditions. Reinjection of a portion of each fraction allowed us to identify a fraction that was sufficiently enriched in the degradant to use as a retention standard. We couldn't feed the collection fraction directly into the mass spectrometer, however, because the solvent contained the original mobile phase.

For our first attempt at converting the method, we selected a highly endcapped C18 column based on the presumption that a less polar column would aid reten-

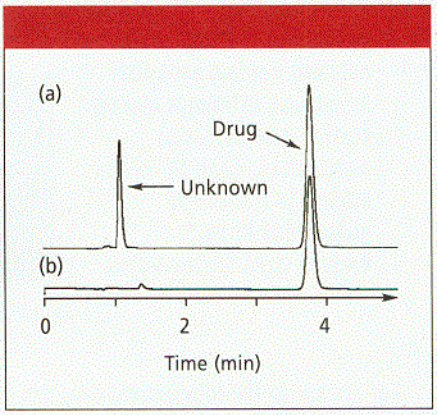

Figure 1: Comparison of (a) a degraded drug product and (b) a drug standard. Column: 150 mm × 4.6 mm, 5-µm d_p C8; mobile phase: octane sulfonate, phosphate, and 15% methanol; flow rate: 1.5 mL/min; detection: UV absorbance.

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tion of the poorly retained degradant peak. We chose a mobile phase of 5% methanol in 0.1% formic acid. A reduced organic solvent concentration should favor increased retention of the polar degradant.

Figure 2 shows chromatograms obtained under these conditions. We expected the drug peak to be poorly retained in the absence of the ion-pairing reagent, but we hoped that the degradant would be separated from the drug. This was not the case. We saw no significant difference between the chromatograms of the drug standard and the degradant, as Figure 2 shows. However, the degradant was slightly retained.

LC–MS Trial Number 1

We would like to have the unknown peak chromatographically separated from the other components in the sample, particularly from the parent drug, for optimal performance of the LC–MS system when identifying an unknown. Because of the ubiquitous impurities at the solvent front, we also desired retention beyond the column void. The results of Figure 2 are marginal, at best. The unknown does not appear to be separated from the drug, and the degradant is barely retained beyond the column dead time (t_0), which is the first peak in Figures 2a and 2b. However, we had the enriched fraction of the degradant and decided to attempt an LC–MS experiment to see if we could see anything useful. We were disappointed — back to the drawing board.

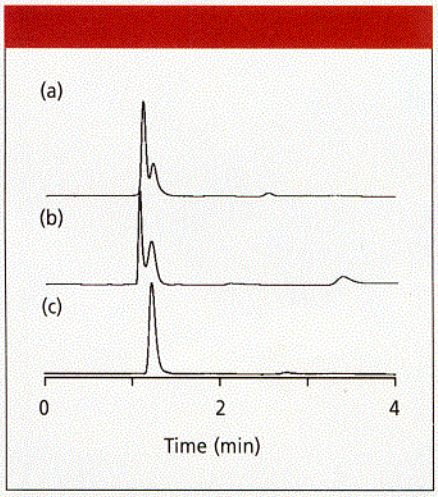

Figure 2: Comparison of (a) a degraded drug product, (b) a drug standard, and (c) an enriched fraction of unknown degradant. Column: 150 mm × 4.6 mm, 5-µm d_p C18; mobile phase: 0.1% formic acid and 5% methanol; flow rate: 1.5 mL/min; detection: UV absorbance.

Avoiding Phase Collapse

One way to increase reversed-phase retention is to decrease the organic solvent content of the mobile phase. However, at levels of less than roughly 5% organic solvent, we would be concerned about collapse of the column's stationary phase. This phenomenon was discussed in detail in an earlier "LC Troubleshooting" column (1).

Under normal conditions (>5% organic solvent), the stationary phase can be thought of as C_{18} chains attached to the silica surface and extended in a brush-like conformation, similar to the sketch of Figure 3a. Under these conditions, sample and solvent molecules have access to the stationary phase, so reproducible chromatographic behavior should be observable. If the organic solvent content of the mobile phase is too low, the stationary phase tends to collapse on itself into a lower-energy conformation, similar to a shag rug as illustrated in Figure 3b. As was discussed in reference 1, this collapse can lead to abnormal chromatographic behavior and generally undesirable results. The mobile-phase organic solvent concentration at which collapse occurs varies depending on the column packing, mobile-phase solvent, temperature, and other variables, but approximately 2–3% organic solvent is the general lower limit of operation to avoid phase collapse. Phase collapse is the reason most workers avoid mobile-phase organic solvent concentrations of less than approximately 5%.

In the last several years, a new bonded-phase family called *embedded polar phases* has become increasingly popular. These phases sometimes are called amide or carbamate phases, but the generic term embedded polar phases seems to be the preferred term for these columns. Some of the more widely known brands are Zorbax Bonus RP (Agilent Technologies, Wilmington, Delaware), Discovery RP-AmideC16 (Supelco, Bellefonte, Pennsylvania), and Symmetry Shield and YMC ODS-AQ (Waters Corp., Milford, Massachusetts). More manufacturers are offering these phases, so you should check with your favorite column supplier for its products.

Although each brand of column has a unique design, the basic structure of embedded polar phases involves the incorporation of a nitrogen-containing side-chain near the silica end of a C8 or C18 bonded phase. By placing this basic functional group deep in the bonded phase, unique selectivity is generated, and some people have speculated that the bonded phase or silica surface may be stabilized. Another benefit of embedded polar phases is that they do not undergo phase collapse in 100% water. Chromatographers now have a C8- or C18-like phase that can be used in the absence of organic solvents.

We decided to try an embedded polar phase column for our problem because it would allow us to use 100% water and ideally retain the degradant.

Retention, At Last

We selected a 150 mm × 4.6 mm C18-type embedded polar phase for our next experiments. Figure 4 shows the resulting chromatograms. The unknown peak is well retained beyond the solvent front. At 1.0 mL/min, t_0 is approximately 1.5 min in Figure 4, whereas t_0 is roughly 1.0 min at 1.5 mL/min in Figures 1 and 2. When comparing the degraded sample (Figure 4a)

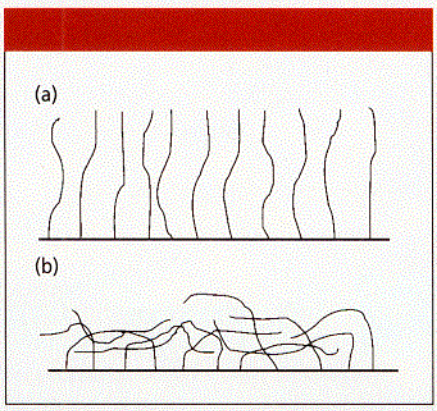

Figure 3: Diagrams comparing bonded-phase orientations, including (a) an extended or brush configuration when solvated with mobile phase, and (b) a collapsed bonded phase after using a too-weak mobile phase.

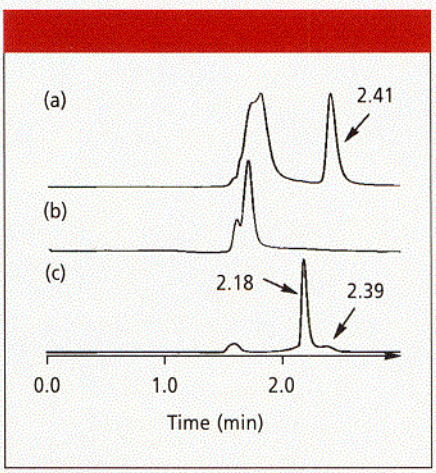

Figure 4: Comparison of (a) a degraded drug product, (b) a drug standard, and (c) an enriched fraction of unknown degradant. Column: 150 mm × 4.6 mm, 5-µm d_p embedded polar phase; mobile phase: 100% water; flow rate: 1.0 mL/min; detection: UV absorbance.

with the drug standard (Figure 4b), we see an extra peak eluted at 2.41 min. The collected fraction (Figure 4c) contains a major peak at 2.18 min and a minor one at 2.39 min. We succeeded in meeting the two requirements stated earlier for a reasonable attempt at LC–MS analysis. First, we separated the unknown from the drug peak, and, second, the unknown peak was retained sufficiently beyond the solvent front to avoid interference from unretained materials.

Comparison of the degraded sample (Figure 4a) and the collected fraction (Figure 4c) raised some questions as to whether the correct material was collected for the enriched degradant standard. From examination of the chromatograms, it appears that the small 2.39-min peak in the collected fraction corresponds to the major unknown peak at 2.41 min in the degraded sample.

How could this be? Several possibilities exist. One possibility is that we picked the wrong fraction for the enriched degradant. We reexamined the chromatograms of the analysis of the collected fractions (not shown), and, although minor peaks were present, the proper fraction was identified as containing the highest concentration of the unknown. So it didn't seem that we had selected the wrong fraction. Another possibility is that the injection of a large amount of sample and sample matrix in the degraded sample shifts the retention of the degradant in the degraded sample chromatogram (Figure 4a). We could test this possibility by injecting smaller volumes of the sample and seeing if the retention changed. An alternate test of this hypothesis would be to mix the degraded sample with the fraction and reinject it to determine if one or two peaks appeared.

A reinjection of the co-mixed sample contained a single peak at 2.24 min with a small trailing peak. This retention time is between that observed for the two samples injected individually, so the problem appears to be a retention shift, not the presence of two different compounds.

However, we did not need to worry much about this problem with our tools at hand. With the LC–MS system, we could inject the degraded sample and examine the peak at 2.41 min in Figure 4a and compare this peak with the 2.18-min peak of the collected fraction. By using MS–MS experiments, we can compare the fragmentation patterns of the unknown peak in each sample and determine with a reasonable amount of confidence whether the peaks are the same compound.

Conclusions

The case study presented in this month's "LC Troubleshooting" illustrates some of the challenges in converting an existing method to an LC–MS-compatible method. In this example, merely selecting an LC–MS-compatible mobile phase was insufficient. Without some retention, the power of a triple-quadrupole mass spectrometer was insufficient for structural elucidation of the unknown degradant. By using an embedded polar phase column, we were able to retain the degradant sufficiently to support the LC–MS–MS experiments. We also observed that when chromatographic conditions change, surprises sometimes appear and what appeared initially to be a simple problem could be more complex than anticipated.

Reference

(1) R.G. Wolcott and J.W. Dolan, *LCGC* **17**(4) 316–321 (1999).

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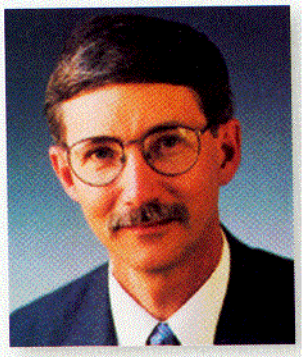

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