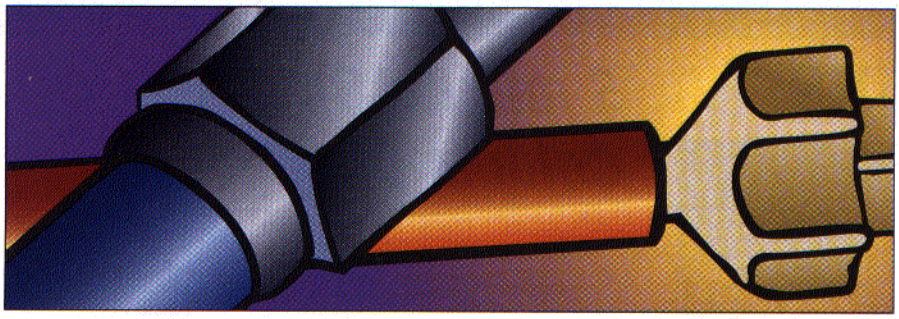
LC Troubleshooting



Poor Peak Shape: A Case Study

Gianfranco Bruno

Peak shape problems don't always have a single solution.

My laboratory routinely uses liquid chromatography (LC) to analyze urine samples for total hydroxyproline. My co-workers and I recently encountered a problem with peak shape. In each case, we analyzed a sample that contained an internal standard and the active compound. When problems occurred, they seemed to affect only the active compound, which was the second peak. Although we were able to solve the problem on an ad hoc basis, the original cause remains somewhat ambiguous. In this "LC Troubleshooting" column, I will briefly discuss the specific application and describe the solution and some possible causes in a manner that may provide useful guidance for other chromatographers who encounter similar problems.

This month's "LC Troubleshooting" column presents a problem from a reader's laboratory: only one peak of a sample set showed abnormal peak shape. Although this example is very specific, the general problem type and the approach to solving it are applicable to a wide range of samples. As always, I welcome your feedback and further suggestions. The quickest way to provide feedback to a large number of readers is the "Chromatography Forum" web site (reach it from www.lcgcmag.com). If you haven't visited the "Chromatography Forum" web site yet, you're missing a resource that has been visited by thousands of chromatographers and has created a great opportunity to discuss chromatographic problems with your colleagues.

John W. Dolan
LC Troubleshooting Editor

Hydroxyproline is produced by posttransductional hydroxylation of proline. It is found almost exclusively in the collagens and represents approximately 13% of the amino acid content of the collagen molecule. Hydroxyproline present in biological fluids results from the degradation of various forms of collagen, and it cannot be reutilized in collagen biosynthetic processes. Because half of the total collagen is contained in bones, and this collagen has a quicker turnover in comparison with the connective tissue turnover rate, the excretion of hydroxyproline in urine is considered a sensitive marker of bone resorption.

Hydroxyproline is present in biological fluids in different forms. Roughly 90% of the hydroxyproline released by the breakdown of collagen in the tissue is degraded to the free amino acid that circulates in plasma. Approximately 10% of the hydroxyproline released by the breakdown of collagen circulates in a peptide-bound form and is filtered and excreted in urine without any further metabolism. Urinary hydroxyproline is present as a dialyzable fraction (90%) and a nondialyzable fraction (10%). The nondialyzable fraction comprises high molecular weight peptides derived from immature recent collagen; this fraction reflects the formation of bone matrix. The dialyzable fraction contains a small quantity of the oligopeptides derived from the mature collagen; this fraction reflects the resorption of bone matrix. The dialyzable fraction always is present and for this reason most of the studies indicate that the total urinary hydroxyproline content is a good marker of bone resorption.

EXPERIMENTAL

In my laboratory, workers prepare samples by quantitatively pipetting 1 mL of control sample or patient sample into labeled hydrolysis tubes. We add 100 µL of internal standard to each tube. Sample preparation involves overnight hydrolysis of the clean urine. We add 1 mL of 12 N hydrochloric acid to 1 mL of urine, and the sample undergoes hydrolysis at 95 °C for 16 h. Next, 100 µL of this hydrolysate is neutralized with 0.3 M sodium carbonate, and the pH is adjusted with several drops of 0.5 N sodium hydroxide to pH 8.5–9.0 and checked with pH indicator strips. This solution is called Solution 1.

The next step in the process is subjecting the sample to a double derivatization. The first derivatization is with *o*-phthaldialdehyde (OPA) in acetonitrile to block the primary amino acids. 500 µL of Solution 1 and 200 µL of OPA are vortexed for at least 30 s to create Solution 2. The second derivatization step uses dabsyl chloride (4-dimethylamino-azobenzene-4′-sulfonyl chloride in acetone) at 70 °C for 10 min (200 µL of Solution 2 with 200 µL of dabsyl chloride). After dilution with distilled water, 20 µL of the sample is injected onto a 150 mm × 4.6 mm C18 column and analyzed using UV detection at 471 nm. The mobile phase is 30% acetonitrile with a 0.25 M citrate–phosphate buffer (pH 3.5). This solution is prepared by mixing 330 mL of approximately 0.3 M citric acid with 380 mL of approximately 0.3 M dibasic sodium phosphate. The pH is adjusted to pH 3.5 with phosphoric acid. Finally, 300 mL of acetonitrile is added to the solution. The apparent pH is now pH 4.1.

The laboratory analyzes these samples using a Chromat liquid chromatograph (Bio-Rad, Richmond, California) that is equipped with an autosampler, a step-gradient controller, and a UV–vis photometer as well as a model HP 3394A integrator (Hewlett-Packard Co., Wilmington, Delaware).

Under these conditions, two peaks are prominent in the chromatogram: the internal standard, dabsyl-methyl taurine, at approximately 3.5 min and the analyte, dabsyl-hydroxyproline at approximately 4.5 min.

THE PROBLEM

Figure 1 shows chromatograms obtained from a calibration standard containing only the internal standard and a standard of the analyte (Figure 1a) with the chromatogram obtained from a sample of normal urine spiked with the internal standard (Figure 1b). As the figure shows, although the internal standard peak is narrow and sharp, the dabsyl-hydroxyproline has a significantly broader peak. This excess

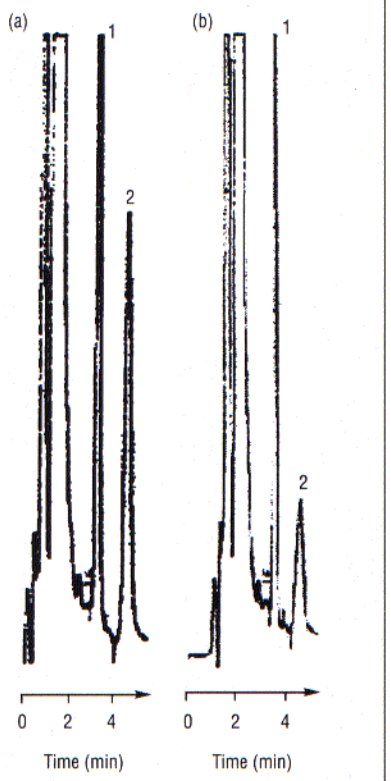


FIGURE 1: Injection of (a) calibration standard and (b) internal standard spiked into a normal urine sample. Sample volume: 20 µL. Other conditions are described in the text. Peaks: 1 = internal standard, 2 = dabsyl-hydroxyproline.

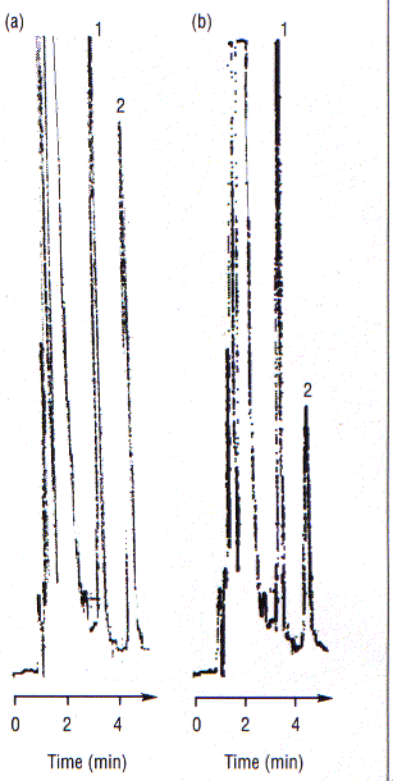


FIGURE 2: Injection of 40 µL of (a) the calibration standard following 1:1 dilution with water and (b) internal standard spiked into normal urine following 1:1 dilution with water. Sample volume: 40 µL. Other conditions are described in the text. Peaks: 1 = internal standard, 2 = dabsyl-hydroxyproline.

peak width is most apparent in Figure 1b for the urine sample.

The magnitude of the excess width of the analyte peak is obvious from the plate number values (N). In the calibration standard, the plate numbers were 8000 and 4700 for the internal standard and analyte, respectively. For the normal urine sample, the plate numbers were 7400 and 2500 for the internal standard and analyte, respectively.

These large peak widths could have several causes. The three obvious sources are column overloading, an injection solvent that is too strong, and an excessive pH difference between the sample and the mobile phase.

IS IT OVERLOADING?

My first suspicion was that the column had become overloaded. Assuming that the normal sample also included additional undetected compounds, column overloading would account for the fact that both of the peaks were wider in the normal sample than in the calibrator. The easiest way to test for sample overloading is simply to dilute the sample and reinject it. In this case, diluting the derivatized sample 1:1 with water had a dramatic effect — the plate count for both the internal standard and the analyte increased significantly. Interestingly, however, the retention time of the analyte peak did not change significantly.

One of the symptoms of peak shape problems caused by sample mass overloading is a decrease in the retention of the peaks. Although retention shifts are not always observed, constant retention times in the dilution test argues against the overload hypothesis. If column overloading was the source, doubling the volume of diluted sample (same sample mass on column as originally) should recreate the observed peak width problem. However, when we doubled the injection volume of diluted calibrator and sample solutions from 20 µL to 40 µL, we obtained the chromatograms of Figures 2a and 2b. The peak broadening did not return, as confirmed by the resulting column plate numbers. In the calibration standard, the plate numbers were 9100 and 8900 for the internal standard and analyte, respectively. For the normal urine sample, the plate numbers were 10,300 and 6400 for the internal standard and analyte, respectively.

These results cast further doubt on simple overloading as the major cause of the problem.

HOW ABOUT THE INJECTION SOLVENT?

A second common cause of peak shape problems is using a dissolution solvent that is significantly stronger than the chromatographic mobile phase. This strong solvent prevents proper equilibration of the sample between the mobile phase and the column packing and causes the injection to be smeared across a significant part of the column length, resulting in misshapen and abnormally wide peaks.

Some have suggested that diluting the samples with water decreased the effective strength of the injected samples, even when the injection volume was doubled. We consider this conclusion to be relatively unlikely for two reasons: First, the problem was much more severe for the second peak than for the first; a difference in solvent strength should affect both peaks similarly or it should affect the weakly retained first peak more than the second peak. Second, the sample solvent was almost completely aqueous even before the dilution, so it should be substantially weaker than the 30% acetonitrile mobile phase in any case.

A pH EFFECT?

With the first two solutions rejected, the third possible problem source seemed to be the most likely. Significant differences in pH between the sample and the mobile phase can cause misshapen, excessively wide peaks. This effect is most likely to occur when the mobile-phase buffer have insufficient capacity to control the pH shift caused by the sample injection.

Although the mobile-phase buffer was somewhat concentrated (250 mM), the evidence indicates that it may have been insufficient to overcome the large pH difference between the sample (pH 8.5) and the mobile phase (pH 3.5). To confirm this hypothesis, we attempted to adjust the pH of the sample to match that of the mobile phase. Unfortunately, the sample was not stable at this pH because of hydrolysis of the dabsyl amino acid complexes.

We were able to adjust the sample pH to pH 7.5. After this adjustment, the shape of the analyte peak improved significantly, with the efficiency increasing to 11,700 plates in this experiment. The width of the first peak, however, remained constant. These results tend to reinforce the hypothesis that the excess peak width problem was the result of the mismatch in pH between the injected samples and the mobile phase.

But why did the insufficient buffer capacity affect only the second peak? We speculate that the internal standard and the dabsyl-hydroxyproline have different pK_as. If the pH of the mobile phase was within approximately two pH units of the analyte's pK_a, yet outside that range for the internal standard, we would expect a greater pH sensitivity for the analyte, as observed.

CONCLUSIONS

From the experiments my co-workers and I performed, it appears that the mismatched pH of the mobile phase and injection solvent primarily was responsible for the peak shape problems we observed. As a matter of practicality, however, diluting the samples with water and increasing the injection volume to compensate for the dilution is much more convenient than adjusting the pH. This example illustrates that the causes of LC problems are not always immediately apparent.

In LC a good rule to follow is to disturb the equilibrium as little as possible. Although this rule should not be pushed too far (to minimize the equilibrium upset absolutely, you must never inject a sample), it does mean that chromatographers generally will obtain the best results when the sample is dissolved in the mobile phase. If this is not feasible, the injection solvent should match the mobile phase in solvent strength, buffer concentration, and pH as closely as possible. If the injection solvent is not matched exactly, it should be weaker than the mobile phase in both solvent strength and buffer concentration, if possible.

Sometimes, such as the present example, even this approach is unfeasible. Under these circumstances, chromatographers can minimize potential problems by limiting the injection volume or, as in this case, diluting the sample. As is true for so many other LC problems, the present solution was a practical compromise.

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