LC TROUBLESHOOTING

Sample Adsorption in Liquid Chromatography Injection Valves

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When fixed-volume injections produce variable peak sizes, sample adsorption may be the cause.

Overfilling the sample loop to maximize injection precision is a common practice in liquid chromatography (LC). Most of the time, as long as the loop is overfilled by several loop volumes, the injection precision is satisfactory. This month we look at three cases in which precision was not necessarily improved by overfilling the loop. In one case, in fact, better precision was obtained when the loop was only partially filled.

We first present a case study from a reader's laboratory (1), then we cover the essentials of two papers dealing with the same problem. Other workers have reported similar problems (2–6). These sources allow us to draw some conclusions about the best approach to take if maximum precision is desired in LC assays.

PRECISION PROBLEMS — A CASE STUDY

Recently, an LC procedure successfully used for years in a pharmaceutical company was transferred to another of the firm's laboratories. The procedure is an internal-standard method that simultaneously quantitates an antihistamine and two parabens, using a third paraben as the internal standard. The procedure is performed on a bonded-phenyl column using an ion-pairing reagent to resolve the antihistamine from the parabens. All the analytes are detected at a single UV wavelength.

When the procedure was attempted in the other laboratory, replicate standard injections yielded excellent precision of <0.5% relative standard deviation (RSD) for the two parabens. However, precision for the antihistamine detected in the same standard injections was consistently >3.0% RSD.

Because precision was acceptable for the parabens, the commonly encountered injector problems were eliminated as possible causes. To minimize chromatographic problems when using ion pairing, sample dilution with mobile phase has been recommended (2). In this assay, the antihistamine was resolved using an ion-pairing reagent. Because the standard solutions were diluted in water, the laboratory workers felt that the sample diluent might be part of the problem. A new standard was prepared and diluted with mobile phase, and replicate injections were performed. Precision of <0.5% RSD was then obtained for the antihistamine as well as for the two parabens.

An unacceptable solution: Although this change fixed the problem, it did not explain how the original laboratory had been able to perform the assay for years using water as the diluent. Furthermore, because stability studies had been performed using water as the diluent, changing to a mobile phase diluent would require revalidation studies. Thus, although a fix was available, the change could not be implemented easily.

At the suggestion of the original laboratory, numerous standard injections were performed to ensure that the column was "activated" before quantitative analysis. However, after 50 standard injections no improvement in precision was obtained. The analysis was further performed on several other columns and instruments with the same results — excellent precision for the parabens and unacceptable precision for the antihistamine. Differences in chemicals and reagents between the two laboratories were also ruled out as potential explanations.

The original laboratory was then asked to perform the assay using standards diluted in water and in mobile phase. When the analysis was completed, the researchers found virtually no difference between the two diluents, and excellent precision (<0.5% RSD) resulted for all three analytes using either diluent. In fact, they found that replicate injections of the standard diluted in water actually yielded better precision than those in which the standard was diluted in mobile phase; thus they recommended making no change.

An exhaustive review of both laboratories' data was performed next to try to find differences. In the original laboratory, the peak-height ratios for the antihistamine standards were the same for each diluent. In the second laboratory, the peak-height ratio compared favorably with the original laboratory's only when the standard was diluted in mobile phase. When the standard was diluted in water, the peak-height ratio was two to three times greater.

While the meaning of this difference was being studied, one of the chemists recalled reading a published report that claimed that water used as a diluent for an ion-pair LC analysis of an antitussive was too weak to prevent sorption of the substrate by the walls of the injection loop (7). The report proposed that the mobile phase be strong enough to prevent this sorption. Because the second laboratory's antihistamine was a salt of a tertiary amine like the antitussive, they felt that the same problem could be occurring.

To test this hypothesis, the analysis was repeated using an instrument that had a variable-volume autosampler. (Up to this point, all analyses in the second laboratory had been performed using instruments with fixed-loop autosamplers.) In the first attempt, replicate injections of standard diluted in water yielded peak-height ratios comparable with those diluted in mobile phase and yielded a precision of <0.5% RSD. To further confirm this effect, the autosampler was removed from the instrument in which the 50 standard injections had just been made so that manual injections could be performed. The loop was manually filled using a 10-µL syringe (no overfill), and replicate injections of standard diluted in water yielded a precision of <1.40% RSD (one-half of the previous level).

In summary, they discovered empirically that water diluent was too weak a solvent to prevent sorption of the antihistamine by the walls of the injector loop. However, the mobile phase was strong enough to prevent this sorption from occurring, and when the injector was placed in the inject position, the mobile phase desorbed all the antihistamine from the walls. This effect was confirmed by injecting mobile phase and water after injections of standard. Other studies confirmed that the amount of antihistamine injected onto the column actually depended on the volume of loop overfill that the autosampler performed.

When the original investigators were notified of the results, they realized that the autosamplers in their laboratory were variable-volume injectors. Thus, they had never seen the problem because their autosamplers did not overfill the injection loop. In the second laboratory, the problem was disseminated to all

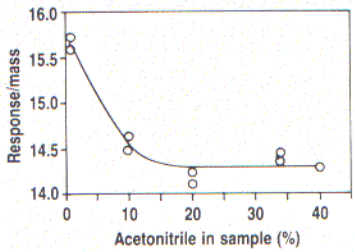

FIGURE 1: Area response per mass of analyte injected as a function of the injection-solvent strength. (Reprinted with permission from reference 8.)

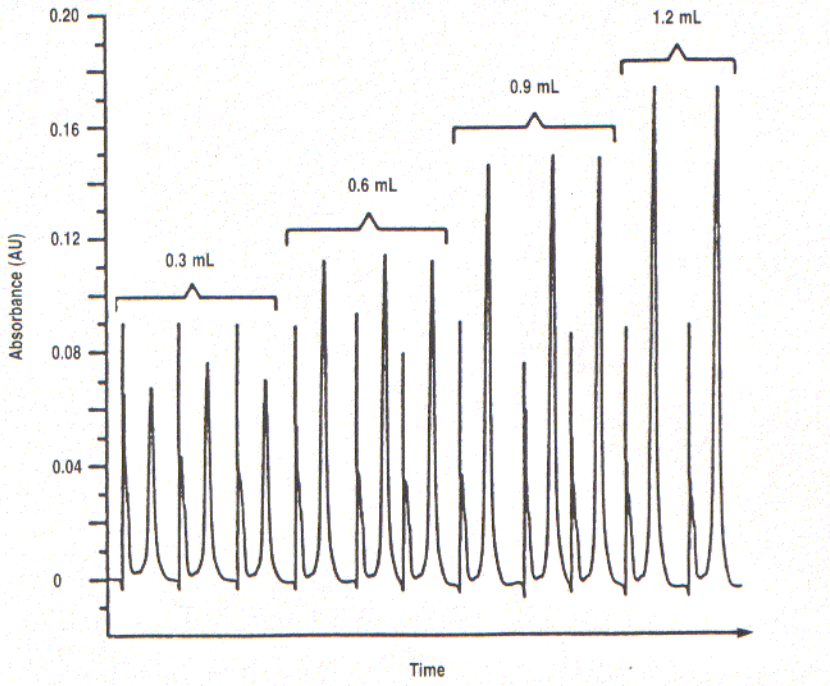

FIGURE 2: Chromatograms showing the effect of overfill volume when a surfactant is injected using a 100-μL sample loop. (Reprinted with permission from reference 9.)

analytical areas, and the use of variable-volume injectors was highly recommended.

MORE EVIDENCE

Another group observed similar problems when analyzing an anxiolytic agent (containing imine, amide, and oxadiazole functionalities) using a reversed-phase system with a C8 column and a mobile phase of 35% acetonitrile–water with an amine modifier (8). The samples were taken from an aqueous dissolution medium and injected directly. When standards were prepared in 35% acetonitrile, a bias of 8–10% for the drug levels was observed. This bias was not observed when the standards were prepared in 1% acetonitrile, indicating that a lower mass of standard must have been injected when 35% acetonitrile was used as the diluent.

The degree of overfill during loop filling affected the assay response in some cases but not in others.

This hypothesis led the workers to prepare the standard in several concentrations of acetonitrile to determine the response vs. diluent curve shown in Figure 1. In these experiments, a 20-μL sample loop was overfilled with 200 μL of sample before injection. At this level of overfill, maximum precision should have been obtained because the loop was thoroughly flushed (9). As shown in Figure 1, when a diluent concentration < 20% acetonitrile was used, an increase in the apparent response per mass of solute injected was observed. Further experiments showed that this variation in response was not observed if a variable-volume injector was used. The authors theorized that the adsorption problem was caused by the polymeric surface of the valve-rotor seal adsorbing the sample.

STILL ANOTHER STUDY

Other workers observed that the degree of overfill during loop filling affected the response for their assay in some cases but not in others (10). Their primary assay was for the determination of a nonylphenol ethoxylate surfactant in an aqueous process stream. They used a 100-μL loop on the injector and a C18 column with an 85% acetonitrile–water mobile phase. They observed that when the surfactant was diluted in water, the degree of loop overfill influenced the height of the chromatographic peaks (Figure 2). However, when samples of acenaphthene or vanillin (prepared in 5% methanol) were used, no such dependence of response on the degree of overfill was observed. These observations led the workers to suspect that the sample loop was adsorbing the surfactant but not the other compounds. To further test this adsorption hypothesis, the sample loop was filled at different rates by varying the velocity at which the sample passed through the loop.

The results of this study are shown in Figure 3. These and other experiments support the hypothesis that sample is being adsorbed from solution by the internal parts of the valve. Quantitation was satisfactory when the sample loop was loaded with a 0.6-mL constant excess of sample.

THE COMMON THREAD

What does all this mean? The common thread of the results from these three laboratories is that sample components can be adsorbed by the internal surfaces of a sample injector. This adsorption is more of a problem with some compounds than with others. The problem can be overcome by using a strong solvent or by carefully controlling the volume of sample that flows through the sample loop.

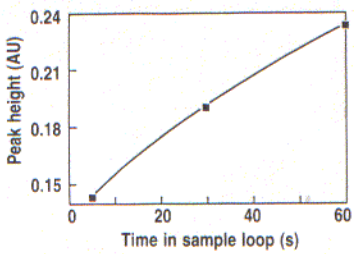

FIGURE 3: Effect of residence time in the sample loop on the peak height of a surfactant sample. (Reprinted with permission from reference 9.)

The evidence above does not indicate whether the stainless steel loop or the polymeric rotor seal was the source of the problem. Further discussion with the first group of workers (1), however, resolved this question. When they replaced the stainless steel loop with a PEEK (polyether ether ketone) loop, the problem disappeared. This result indicates that the stainless steel surfaces were the problem. The observed differences between fixed- and variable-volume loop injectors appear to result from adsorption phenomena as well.

With the use of variable-volume loops, the sample only partially fills the loop; therefore, all the sample is contained in the loop and the

entire sample is injected. With fixed-volume loops used in the overfill mode, sample components can be adsorbed by the surfaces from the sample that passes through the loop to waste. Thus, a higher concentration of sample exists in the loop than in the incoming sample matrix. When the loop contents are injected, the (stronger) mobile phase desorbs the sample and a larger-than-expected peak is observed. If this injection is a standard, normally behaving samples will appear to have a lower concentration than expected. Because the degree of sample adsorption is related to both the volume of sample introduced (Figure 2) and the speed of loop filling (Figure 3), a decrease in precision is reasonable under these circumstances.

Is all this surprising? In some ways it is, because we have been taught that stainless steel is "inert" in LC. However, in many cases — especially in the assay of biological materials — sample loss or degradation has been observed when stainless steel tubing is used. After all, stainless steel tubing is just a very selective capillary column of low efficiency; and the result is that certain chemicals interact strongly with the surface and others do not. This is a chromatographic phenomenon, so these interactions are reduced or eliminated when stronger solvents are used. We are practicing chemistry, not magic, so simple chemical adjustments to the system can be used to correct the problem.

Although few of us will encounter this problem, we should file it away in our memory archives for future reference. These examples should also prompt us to be a little more aware of some of the variables that can influence sample injection. The common rules are that the loop should be overfilled by at least threefold when filled-loop injection is used and that the injection solvent should be no stronger than the mobile phase (2).

These rules can be expanded somewhat for even better results. First, use the mobile phase as the injection solvent if possible; if you need to dilute the sample for larger injection volumes, check the system for adsorption problems. Second, use a constant volume of sample for loop overfill, so that even when adsorption problems exist, they will be constant.

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Bulletin

Report projects electrophoresis market growth. A market report from Theta (Middlefield, Connecticut) predicts that the demand for electrophoresis equipment will increase 12% annually through 1994. According to "Biotechnology/Biomedical Separation and Purification Equipment," sales of HPLC systems in 1989 totaled $115 million. For more information, contact Phyllis Klaben, Theta Corporation, Theta Building, Middlefield, CT 06455, tel. (203) 349-1054.