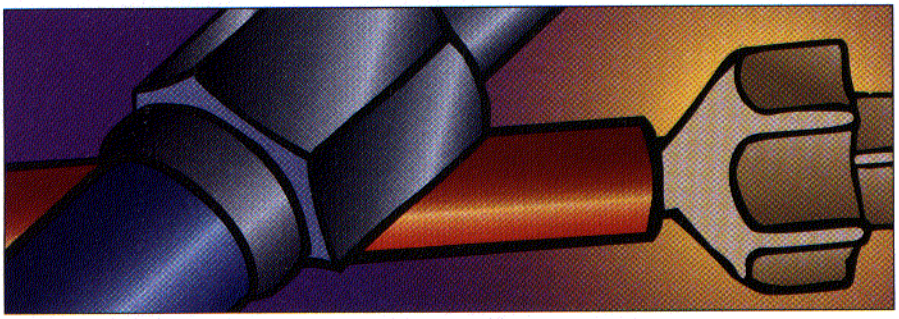
LC Troubleshooting



How Much Is Too Much?

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Just how much sample can you load onto a liquid chromatography column?

In my laboratory, analysts sometimes develop stability-indicating liquid chromatography (LC) assays for pharmaceutical compounds in which they are challenged to simultaneously quantify parent drug peaks and minor impurities or degradants at levels equivalent to 0.05% of the parent peak. At other times, my staff may use a short, narrow-bore column on an LC–mass spectrometry system. In both cases, the analysts often want the maximum detector response so that they can lower the detection limits. This need to maximize response leads to an important question: How much sample can be loaded onto the column without compromising the method's performance? This month's "LC Troubleshooting" column addresses three aspects of this question — sample mass, sample volume, and injection-solvent strength.

One problem related to the amount of sample placed on an LC column is overload. Two types of overload are of particular concern: mass overload and detector overload. Let's look at each of these types in turn.

MASS OVERLOAD

In the common chromatography modes in which retention depends on chemical interactions between the sample and the stationary phase, the stationary phase (column packing) has a finite number of active sites available for sample interaction. This situation is true for the reversed-phase, normal-phase, and ion-exchange separations that account for most LC applications. This limited number of active sites determines the number of sample molecules that a column can interact with at a time. Mass overload occurs when the number of sample molecules exceeds the number of active sites.

One approach that I like to use is to visualize the column as a series of 1-L beakers lined up in a row. If a small sample, say 250 mL, is loaded onto the column, all of the sample fits in the first beaker. To move the sample down the column, I pick up the first beaker and pour it into the second, then the second into the third, and so forth until the sample reaches the end of the column. Under these conditions, the sample arrives at the end of the column in a narrow band — still fitting in one beaker. Now consider the same scenario with a 5-L sample. The sample won't fit completely in the first beaker, so I have to put part of it in the second, third, fourth, and fifth beakers before I can load it all onto the column. Now to move the sample down the column, I have to pick up the first beaker and leap-frog over the next four until I reach the first available empty beaker, number six. Number two goes to seven, and so forth. The net result is that the sample's center of mass moves more quickly through the column and the band is broader than in the small-sample case.

Although LC columns are not made of beakers, an analogous phenomenon occurs with interactions between sample molecules and active sites on the column. When active sites are interacting with a molecule, another molecule cannot interact there, so the second molecule must travel downstream until it finds a free active site. When more sample molecules are present than available active sites, mass overload occurs. Under these conditions, analysts observe broader bands and shorter retention times, just like with the beaker column. Peaks under overload conditions approach a characteristic right-triangle shape, with a steep front and angular tail.

The solution to the mass overload problem is quite simple — put less sample on the column. If mass overload is suspected, one simple test is empirical. Just reduce the sample mass 10-fold by injecting a smaller volume or a more dilute sample. If retention times increase and peaks become narrower, overload was occurring. Reduce the sample size further until you observe no further changes in retention or peak shape. Now you've found nonoverload conditions.

Should you expect to always operate under nonoverload conditions? Often limiting the method to nonoverload operation is impractical, especially when the method must determine large and minor peaks simultaneously. Fortunately peak area does not change during overload, so an assay can perform quite well when the upper end of the standard curve requires operation under overload conditions. This aspect of overload was covered in an earlier "LC Troubleshooting" column (1).

How much sample can be placed onto a column before overload occurs? Most reversed-phase columns will handle 1–10 μg of sample per gram of packing material as a first approximation. A 4.6-mm i.d. column contains approximately 1 g of packing for every 10 cm of length. Thus, a 150 mm × 4.6 mm column should be able to handle as much as 20–25 μg of sample. Because the column capacity varies with surface area and other column characteristics, I recommend performing the empirical overload test described above before you try to operate near these limits. Remember that sample capacity is related to the mass of sample per mass of packing material; therefore, narrower columns will overload sooner than their conventional counterparts. For example, a 2-mm i.d. column will contain one-fifth as much packing material as the same length 4.6-mm i.d. column packed with the same packing. So it is possible that the narrower, sharper peaks obtained with a narrow-bore column will be compromised by peak broadening and subsequent loss of resolution if mass overload occurs.

DETECTOR OVERLOAD

Another aspect of overload relates to the UV-absorbance detectors most analysts routinely use with LC methods. In general, UV-absorbance detector response is linear to approximately 1 absorbance unit (AU). Some manufacturers make detectors that are linear beyond this range, but the combination of detector performance and data system linear range make it wise to adjust the injected mass so the peak does not exceed 1 AU. The actual sample mass that produces 1 AU will vary widely depending on the spectral characteristics of the sample, the wavelength used, and the peak width. So although the peak-area response

remains linear under column overload conditions, peak-area response will be nonlinear if the detector is overloaded. An analyst can restore the detector response to the linear range by reducing the sample mass injected onto the column or detuning the detector by selecting a wavelength at which the detector does not respond as well to the sample compound. Finally, remember that different types of detectors have different response characteristics, so overload and nonlinearity may be more severe problems with one type of detector.

SAMPLE VOLUME

There is a limit to how large a sample volume can be injected. Most chromatographers prefer to inject samples using mobile phase as the sample solvent because it is convenient and unwanted interactions and disturbed equilibria are less likely. If the injected volume is too large, broad sample peaks can result. In the extreme, consider a 3-mL sample injection onto a 150 mm × 4.6 mm column. The column volume is approximately 1.5 mL, which means that approximately half of the injection solvent will have passed through the column before the injection process is complete. Obviously this injection is too large, but what is the limit?

One guideline for injection is to keep the sample volume for an isocratic separation less than approximately 15% of the peak volume when injecting a sample prepared in mobile phase. A 150 mm × 4.6 mm, 5-µm d_p reversed-phase column should generate approximately 10,000 theoretical plates in a typical application. The peak width can be calculated by rearranging the standard plate number equation

$$N = 16\,(t_R/w)^2 \qquad [1]$$

and substituting 10,000 for N

$$w = t_R/25 \qquad [2]$$

where N is the plate number, t_R is the retention time in minutes, and w is the baseline peak width in minutes. So an analyte eluted at 5 min will generate a peak 0.2-min wide. If the flow rate is 1.5 mL/min, the peak volume will be 300 µL. Using the 15% guideline, analysts could inject approximately 50 µL of sample without worrying about noticeable band broadening. As with any generalized rule of thumb, it is best to check the prediction experimentally. To be safe, I like to restrict the injection volume to no more than approximately half of what can be tolerated. In the present case, I would inject 100-, 50-, and 25-µL samples and observe the chromatograms. If I observed unacceptable peak broadening and loss of resolution at 100 µL but not at 50 µL, the 50-µL injection should be acceptable.

SOLVENT STRENGTH

Although usually it is preferable to inject the sample using the mobile phase as the injection solvent, sometimes this practice is impossible or undesirable. A mismatch between the injection solvent and the mobile phase can have two important effects: it can upset the mobile phase–stationary phase equilibrium and change the solvent strength.

Some modes of chromatography are more prone to equilibrium problems than others. For example, with ion pairing, uptake of ion-pairing reagent from the mobile phase dramatically modifies the stationary phase. Thus, any change in the mobile-phase composition will cause a corresponding change in the stationary-phase chemistry and subsequent changes in retention and selectivity. For this reason, it is very important to use the mobile phase as the injection solvent in ion-pair chromatography. On the other hand, with size-exclusion chromatography, the mobile phase is chosen primarily for convenience. Good sample solubility, no detection problems, and minimal unwanted stationary-phase interactions are characteristics of a good size-exclusion mobile phase. Because this mode of chromatography does not rely on a delicate chemical balance between the stationary phase and mobile phase, the injection solvent is much less important than with ion pairing. Reversed-phase separations fall somewhere between these two examples. It is best to keep the injection solvent similar to the mobile phase, but a perfect match often is unnecessary.

The injection-solvent strength can play an important role in reversed-phase separations. From a practical standpoint, the combination of injection-solvent strength and injection volume is the key. Recall the *Rule of Three,* which states that retention changes approximately threefold for a 10% change in mobile-phase organic solvent concentration. This rule means that a peak eluted at 15 min with a 50% organic solvent mobile phase will be eluted at approximately 5 min with 60% organic solvent in the mobile phase. A very large injection volume of a strong solvent is equivalent to switching to a stronger mobile phase. The stronger mobile phase would cause sample molecules to travel down the column more quickly until the injection solvent was fully diluted. The result would be shorter retention times. Conversely, a weaker injection solvent tends to momentarily stop elution and can result in longer retention times. In the extreme, an analyst could load a sample on the column in water, hold the sample at the top of the column during injection, and elute it in the normal manner after restoring the regular mobile phase. This process is called *on-column concentration* and can be used to your advantage if large quantities of dilute sample must be injected.

So how much sample can be injected in solvents that don't match the mobile phase? If the injection solvent is stronger than the mobile phase by no more than approximately 20%, analysts usually can inject 25 µL of sample without retention or peak-shape problems. If the injection solvent is 100% organic solvent, it is best to keep the injection volume no larger than approximately 10 µL. If the injection volume is small enough, nearly any injection solvent can be used. For example, my laboratory developed a method for in-process analysis in which the sample arrived dissolved in methylene chloride. We were able to inject 5 µL of this sample directly into a reversed-phase acetonitrile–water mobile phase without problems. A larger volume certainly would have caused miscibility and solvent strength problems, but the small volume was soluble in the bulk mobile phase and greatly simplified sample preparation.

When injection solvents weaker than the mobile phase are used, much larger sample volumes can be tolerated. If the injection solvent is weaker than the mobile phase by 10% or more, injection volumes of more than 100 µL may be possible. In the extreme and when using gradient elution, analysts can inject 1 L or more of aqueous sample to take advantage of on-column concentration and elute the sample as if it were injected in a much smaller volume.

As with any chromatographic condition that may have questionable robustness, I recommend checking the injection volume before finalizing a method to ensure that the injection volume does not negatively impact the final results. The easiest way to check the injection volume is to inject the desired volume as well as injections half and twice as large. Watch closely for changes in retention, peak shape, and resolution. If double the normal injection volume gives no change or an acceptable change in these parameters, the target volume can be used. I like to operate with a factor of two safety — this guideline helps produce method tolerance for unforeseen changes.

SUMMARY

Several guidelines help address the question of how much sample can be injected onto an LC column. Too large a sample mass will overload the column, causing peak distortion and shorter retention times. Some of these same effects can be observed if too much of too strong a solvent is injected. It is best to stay on the safe side and be conservative with the mass, volume, and injection solvent strength used for routine methods. Sometimes overload is unavoidable, especially if one analyte is at high levels and another is at trace levels. One way around this problem is to make two injections, a small one for the parent drug and a large one for the trace components. Whatever choice is made regarding the injection solvent and sample size, it is wise to determine that the selected conditions are stable by finding out what happens if the injection size is increased.

REFERENCE

(1) N.S. Wilson, J.R. Kern, and J.W. Dolan, *LC•GC* **16**(5), 442–446 (1998).

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