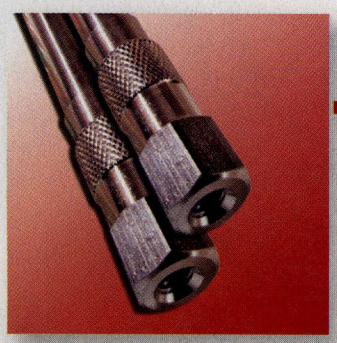

LC TROUBLESHOOTING

How Do I . . . ?

Are you stuck on which step to take next?

John W. Dolan
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One of my roles as "LC Troubleshooting" editor is to field questions from readers about various problems with liquid chromatography (LC) separations. I am happy to provide answers, usually by e-mail (John.Dolan@LCResources.com). I especially enjoy questions that are process-, technique-, or equipment-oriented. I also get frequent questions to the effect of "How do I separate A from B?" I will share a secret — I rarely answer questions of the latter kind, at least in the manner that the inquirer requests. There is a twofold reason for this. First, I do not have much experience in some of the specific separation areas and I am not willing to do someone else's literature search. Second, I am a firm believer in the old adage to the effect of "Give a man a fish and you feed him for today, teach a man to fish and you feed him forever." In a similar manner, if you know where to look for help, you will not have to wait for an answer from me and you will develop the skills to solve the problems on your own.

For this month's discussion, I will share some of the key resources that I recommend using when you have one of those "How do I?" questions. I also will answer a reader's question that fits nicely into this context. Finally, I need to correct an error that appeared in a previous "LC Troubleshooting" column.

Where Is It Written?

The church that I attend has a catchphrase, "Where is it written?" that encourages members to examine statements and decisions based upon written authority, in this case the Bible. I have found that this is a good guideline for me as a scientist, as well — it generally is not good to make up procedures and practices outside of the context of the scientific background laid down by others. So what are those resources for LC? I have listed a few of my favorites in the following.

Troubleshooting: I'll have to admit that I'm a bit prejudiced, but I don't think there is a more comprehensive troubleshooting book than *Troubleshooting LC Systems* (1). Although the publication date is rather old, this book gives a detailed treatment of equipment operation, troubleshooting, and preventive maintenance. Equipment models have changed over the years, but the general principles still hold true. The same goes for treatment of separations problems. A more recent book that I like is *Pitfalls and Errors of HPLC in Pictures* (2). In this clever treatment of LC troubleshooting, Veronika Meyer uses approximately 100 chromatograms and diagrams to illustrate various LC problems and accompanies each of these with a one-page discussion of the problem and solution. I read this book cover to cover in a couple evenings and found it quite good for chromatogram-oriented problems. This column in *LCGC*, of course, is a great source of troubleshooting information, both specific and general. I know that many readers tear out each column and collect them in a notebook. You can access the last several years of columns on the *LCGC* website (3). I have collected all 273 columns from the first one in 1983 through the end of 2007 in a CD collection that I call *The 2008 Troubleshooting Bible* (4). This comes with a PDF file of each column and a Windows help file

that allows you to search the collection by keywords. For a limited time (ending August 1, 2008), I'm willing to send U.S. readers a free copy of this if they want to request it from our website (http://www.lcresources.com/resources).

General principles: For as long as I can remember, the Snyder–Kirkland classic, *Introduction to Modern Liquid Chromatography* (5), has been the standard reference book in the field. The second edition is now almost 30 years old, and I've worn the cover off my original copy, but the core material about LC still has plenty of value. A third edition of this classic is in process for publication late this year. An alternative, easy-to-read resource is Michael Dong's *Basic HPLC* (6). This paperback book contains good coverage on the basics of LC. Written in the context of Michael's intimate experience in the pharmaceutical field, it makes a good selection for your library. Another excellent (and free!) source of general LC information is R.P.W. Scott's on-line Chrom-Ed Series (7). (This series also contains tutorials on gas chromatography, preparative chromatography, and other chromatography-related topics). Finally, don't forget Chromatography Forum (8), a free on-line discussion group of nearly every imaginable chromatography-related topic.

Advanced topics: The list of specialty books in LC might seem to be endless; I will point out just two. First is a reference that should be in every chromatographer's personal library if method development is ever undertaken. That's the classic Snyder, Glajch, and Kirkland *Practical HPLC Method Development* (9). In my opinion, if you are going to own just one book on chromatography, this probably would be the choice. It is exactly as the title says — practical. It covers the basics and builds on them to present a very logical and effective strategy for the development of LC methods — whether you do this manually or with the aid of method development software. If you work with gradient elution, you'll want a copy of *High Performance Gradient Elution* (10). Here you'll find everything you ever wanted to know about gradients — and maybe more! You will not find another text with such a comprehensive treatment of the subject. For current, application-specific information, consult the websites of the various column manufacturers — their extensive applications note libraries can contain just the information you need. And don't forget the mainstay chromatography journals, *Journal of Chromatography A* and *Journal of Chromatography B*. A free on-line search engine, such as Pub-Med (11) will access these, as well as chromatography-related articles in many other journals.

Reader's Question: Column Length

Here is a typical e-mail question that I receive, accompanied by my answer: R.G. writes, "I put a fair amount of effort into optimizing the separation of a complex mixture of amino acids. I was mostly successful, but ultimately I couldn't quite get all the peak pairs resolved to my satisfaction. I had the idea that I could achieve a better separation by simply replacing my column with another column that is the same, except for being twice as long. Certainly this should give me more plates, but would my method transfer over to the new column easily? Would I have to start the method development process over again?"

And here is my reply: You are right that an increase of column length and, thus,

plate number will increase resolution — if you do things correctly. If you are making an isocratic run, it is quite simple — just increase the column length. For example, connect two 150 mm × 4.6 mm columns in series or use a 250 mm × 4.6 mm column packed with the same description of packing material from the same manufacturer. The retention time will increase in proportion to the column length, as will the column plate number and system back pressure. However, resolution will increase only by the square root of the length increase. This might be enough to get the additional separation you need. Another alternative to increase the plate number is to reduce the particle size, for example, from 5 µm to 3 µm diameter. The plate number will change in proportion to the reduction in particle size, and the retention should be unchanged. However, the pressure will increase in proportion to the square of the change. So, for example, a twofold reduction in particle size would double the plate number, increase the resolution by the square root of two, and increase the pressure by a factor of four. The increased pressure might require you to lower the flow rate to keep the pressure within reasonable limits. Retention will increase in direct proportion to the flow rate reduction. You also might observe a small increase in column plate number with a reduction in flow rate, but this often is insignificant with 3- and 5-µm diameter particles. So you can see that changes in the column with isocratic separation are straightforward and logical.

On the other hand, if you are running a gradient, the story is quite different. This is because changes in the column length and flow rate can generate selectivity changes in gradients that are not observed in isocratic separation. The key equation is:

$$k^* = (t_G\ F) / (\Delta\Phi\ V_m\ S) \qquad [1]$$

where k^* is the gradient retention factor, t_G is the gradient time (for example, a 20-min gradient), F is the flow rate, $\Delta\Phi$ is the gradient range (for example, 5–95% B-solvent = 0.9), V_m is the column internal volume, and S is the slope of the retention factor versus Φ plot (a value of $S = 5$ is suitable for estimates of k^* with analyte molecular weights less than ≈1000 Da). To have selectivity remain constant, k^* must remain constant. Thus, a change in one parameter often requires a compensatory change in another one. So if the column length is doubled, V_m will double, and k^* will then double. To keep k^* constant, either t_G or F could be doubled. Doubling the gradient time will mean that all the retention times will be longer, but k^* and, thus, selectivity, should stay the same. A better separation should be obtained because the column plate number also will double. A change in flow rate by a factor of two probably will not be practical — twice the column length will double the pressure, as will the flow rate increase, for a fourfold increase in pressure. This is unlikely to be acceptable unless the current method has a very low pressure. If the pressure is too high with two columns in series, you will have to lower the flow rate and then make a further increase in the gradient time to compensate.

You can see that manipulation of the gradient parameters can be a bit complicated. However, you will be OK if you remember that any change you make in one of the parameters on the right side of equation 1 must be accompanied by one or more other changes such that k^* remains constant. If you follow these guidelines, your current method should transfer to the longer column without changes in selectivity, so you would not need to start over the method development process.

Erratum

One of my favorite aviation writers, Rod Machado, wrote in one of his recent columns (12), "You really don't know what you don't know until you write about it. Then, everyone knows what you don't know." Every once in a while there is an error in one of my columns — typographic errors, unclear explanations, and just plain mistakes. Fortunately, it doesn't happen very often — only about a dozen times over the last 25 years that required some kind of written correction. Well, here's another (thanks to readers I.K. and R.B. for alerting me to this error).

Equation 1 in the February 2007 "LC Troubleshooting" column (13) would have been better expressed as

$$\mathrm{LLOD} = f\{[MW,\ V_m,\ (1 + k)] / [(S/N),\ CV,\ N^{0.5},\ L_{fc},\ \epsilon]\} \qquad [2]$$

where LLOD is the lower limit of detection, *MW* is the solute molecular weight, V_m is the column volume, *k* is the retention factor, S/N is the signal-to-noise ratio, CV is the coefficient of variation required by the method, $N^{0.5}$ is the square-root of the column plate number, L_{fc} is the path length of the detector flow cell, and ϵ is the extinction coefficient. The change over the original equation is that S/N is moved to the denominator. The purpose of equation 2 is to guide the user in changes that can be possible when attempting to lower the detection limit of a method. The main error in the original explanation was related to confusion about the role of molecular weight. Larger molecular weight compounds have smaller diffusion coefficients (not larger, as stated). This means that they diffuse more slowly, creating broader and, thus, shorter peaks; peak height is an inverse function of molecular weight. Shorter peaks for the same mass-on-column mean that the limit of detection will be larger (worse). So you will not obtain as low of detection limits with large molecular weight molecules as you do for small ones.

I will recap the role of the other variables so that you do not have to go back and find your old issue of *LCGC*. Smaller column volumes, V_m, and retention factors, *k*, result in smaller peak volumes. Smaller peak volumes generate narrower peaks, so they will be taller (area is conserved) and detection limits will be smaller. Large values of S/N mean that the peak will be large relative to the noise. Large values of the allowed CV mean that small S/N can be tolerated. These two factors work together to give lower detection limits. Larger values of the column plate number give narrower, taller peaks, and lower detection limits. Longer flow cells mean that more molecules are in the cell for detection, so a larger signal is seen. Similarly, a larger extinction coefficient means better response for a given mass. Thus, longer flow cells and larger extinction coefficients also work to lower detection limits.

In summary, when trying to improve detection limits, some factors are under your control (for example, V_m, *k*, *N*), some are limited by the sample (*MW*, ϵ), some by the instrument (L_{fc}), some by regulations (*CV*), and some by a combination of factors (S/N). Equation 2 can help to guide you when you try to improve detection limits. Consult the original article (13) for a more detailed discussion.

References

(1) J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems* (Humana Press, Clifton, New Jersey, 1989).

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(5) L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography,* 2nd ed. (Wiley, New York, 1979).

(6) M.W. Dong, Basic HPLC: *A Guide for Practicing Scientists* (Wiley, New York, 2006).

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(12) R. Machado, *AOPA Pilot* **51**(3), 60 (2008).

(13) J.W. Dolan, *LCGC* **25**(2), 142-152 (2007).

For more information on this topic, please visit www.chromatographyonline.com/dolan

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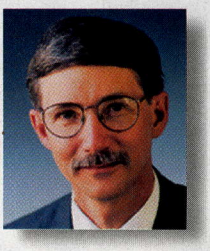

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For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at http://www.chromforum.com.