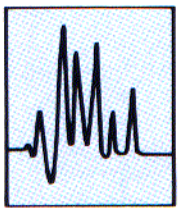
TROUBLESHOOTING

Extracolumn Effects: Two Case Studies

JOHN W. DOLAN



Chromatographers are becoming increasingly aware of "extracolumn effects" in HPLC as the trend toward the use of small-volume columns continues. These extracolumn effects are the added volume contribution from the injector sample loop, the detector cell and time constant, and the tubing connecting the column to the injector and detector.

Extracolumn contributions are often expressed as

$$V^2 = V_p^2 + V_l^2 + V_t^2 + V_d^2 + V_\tau^2 \quad [1]$$

where V is the total peak volume, V_p is the peak volume in absence of extracolumn effects, V_l is the volume contribution from the sample loop, V_t is from the connecting tubing, and V_d is from the detector cell; V_τ is the broadening contribution from the detector time constant. Clearly, if V_p is much larger than the other factors, these factors are not very important to the overall peak volume. When the other factors become significant, we say that extracolumn band-broadening has occurred or that extracolumn effects are significant.

Most commercial LC systems are designed so that extracolumn effects are insignificant if tubing runs are kept short and if standard columns (for example, 15 cm × 4.6 mm, 5-μm particles) are used. If smaller-dimension or smaller-particle columns are used, however, extracolumn effects can be important, as discussed below in two case studies.

Calculation of extracolumn effects has been thoroughly discussed in the literature (for examples, see references 1–3), but manual calculations can be tedious. For this reason, commercial computer software (4) has been used for all calculations presented here.

CASE STUDIES

Case 1 — A "good" system with "better" columns: The first case involves workers who were evaluating a change from 15-cm, 5-μm columns to shorter, 3-μm columns so that they could increase throughput in their quality assurance lab. The use of very short columns for rapid analysis has been well documented (for example, see reference 5), so this was a logical choice. Four columns of each size were obtained, as shown in Table I.

TABLE I: DATA FOR CASE STUDY 1

	Column plate number *N*			
Column	**mfg***	**expt****	**0/0†**	**25/0.1‡**
15 cm, 5 μm	12657	9280	12950	8520
8.3 cm, 3 μm	11940	6595	12073	7905
3.3 cm, 3 μm	4201	1700	3880	1805

* mfg = manufacturer's test data
** expt = experimental data, average for four columns of each size
† 0/0 = extracolumn band-broadening = 0 μL, τ = 0 s; calculated using software (reference 4)
‡ 25/0.1 = extracolumn band broadening = 25 μL (1 σ), τ = 0.1 s; calculated using software (reference 4)

The columns were used for the assay of several parabens in a hand-cream formulation; average N values for one paraben are shown in Table I (N_{expt}). Immediately, the analyst noticed that the plate numbers were about half of what he expected for the 3-μm columns. That puzzled him because the test chromatograms from the manufacturer indicated that the small-particle columns were good. Furthermore, the 5-μm columns functioned just as they always had, so he felt confident that the system was working well. He wasn't sure what to do next.

This is a case in which extracolumn effects should have been suspected immediately. If columns that produce smaller peak volumes (for example, shorter columns and/or smaller-particle columns) perform poorly compared with larger-peak-volume columns, extracolumn effects are likely to be the cause. In this case, the computer was used to calculate the plate number under the assumption that no extracolumn effects were present. The results of these calculations are shown in Table I ($N_{0/0}$). The N values agree quite well with the manufacturer's test chromatograms. We concluded that the columns were good because of this agreement and that the manufacturer used an LC system that had been optimized to eliminate extracolumn effects. Neither conclusion is surprising; it is rare to receive a bad column if it has been tested by the manufacturer, and it is expected that the manufacturer will test the columns under conditions that allow them to perform at their best.

Once we knew the columns were good, we tried to find the source of the problem in the analytical LC system. We made a series of calculations to determine the result if extracolumn effects were added to the system. One set of the results is shown in Table I ($N_{25/0.1}$) for an LC system with 25-μL (1 σ) of extracolumn band broadening and a detector time constant of 0.1 s. The values are typical for an LC system in routine use and agree fairly well with the experimentally determined N values, which means that the LC system is fine for assays using 15-cm, 5-μm columns. If the system is to be used for smaller-peak-volume columns, however, extracolumn effects must be addressed. Generally, that calls for use of a smaller-volume detector cell and for very short lengths of 0.007-in. or 0.005-in. i.d. tubing if short, 3-μm columns are used.

Case 2 — A "bad" system for "good" columns: This example involves a lab in which gradient elution is used for the assay of biological samples. Workers in the lab were investigating the feasibility of switching to 5-cm, 3-μm cartridge columns from 15-cm, 5-μm columns. The column specifications indicated that this would be possible, and the cost savings would be significant if the approach were viable. Columns would be used for the assay under gradient conditions, but would be periodically returned to isocratic column-test conditions to see how they were

holding up. When the initial isocratic runs were made on the short columns, however, the results were disappointing (Table II).

The lab results for each of two cartridge columns (A and B, Table II) showed that the columns had about one-third the plate number that they had at the factory and that the bands tailed severely (As = 1.4). Tailing bands and poor performance suggested that extracolumn effects might be involved, so the columns were connected in series and the experiment rerun. (A longer column has a larger V_p, but other factors in Equation 1 should remain constant so extracolumn effects should be less significant.) Now, with two columns in series (70% methanol, A + B, Table II), the plate number increased to about half the factory value, and the asymmetry dropped. This prompted the analyst to run the column test with a weaker mobile phase. A larger retention volume would be expected to reduce extracolumn effects because V_p in Equation 1 increases while other factors remain constant. Under those conditions (60% methanol, Table II), the band shape is satisfactory (As = 1.0), and the agreement between experimental and factory plate numbers is better.

At this point, the computer was used to quantify the magnitude of the extracolumn effects (although time would have been saved had this been done before additional experiments were run). When an extracolumn volume of 110 μL was used, the calculations agreed quite well with the experiment results

TABLE II: DATA FOR CASE STUDY 2

Column	Column plate number *N* (*As*)				
	70% methanol			60% methanol	
	mfg*	expt**	calc†	expt**	calc†
A	4500	1700 (1.4)	1700	2700 (1.0)	2700
B	4400	1700 (1.4)	1700	2900 (1.0)	2700
A + B	8700	4700 (1.2)	1700	5700 (1.0)	6600

conditions: columns A and B = 5 cm, 3 μm; 60% or 70% MeOH–water mobile phase; flow rate = 1 mL/min
* mfg = from manufacturer's test chromatogram
** expt = experimental *N* values (*As* values)
† calc = calculated *N* values using software (reference 4); extracolumn band-broadening (1 σ) = 110 μL; τ = 0.5 s

in Table II. This volume is five to ten times as large as typical (for example, 10–25 μL) values for well-plumbed LC systems.

An inspection of the plumbing of the LC system revealed two major problems. First, a column-switching valve was being used, a valve not designed for use with small-volume peaks. Second, the autosampler was connected to the column with an excessive length of 0.020-in. i.d. tubing.

CONCLUSIONS

These case studies illustrate several important points that should be considered whenever a new method is developed or an old method is significantly altered. These points are summarized in Table III and below.

First, in both cases the LC systems worked well under normal operating conditions — that is, neither system was inherently "bad." The first system worked well with a 15-cm, 5-μm column. The second system, although it had excessive extracolumn volume for isocratic assays, worked well under gradient conditions. With gradient runs, large volumes of dilute sample can be injected without adverse effects because the sample is concentrated at the head of the column before the gradient begins. That is the reason the second (gradient) system worked well — the sample

TABLE III: INDICATIONS OF POTENTIAL EXTRACOLUMN EFFECTS

- **System yields smaller plate numbers than expected; worse with early peaks**
- **Coupled columns give higher plate numbers than the sum of the individual columns**
- **Short, small-particle columns give more band-broadening problems than larger columns**
- **Tailing bands are present under column test conditions**
- **LC system gives poor plate numbers and/or band shape if converted from gradient to isocratic operation**

was diluted in the large transfer tube, but on-column concentration masked this effect.

Second, in both cases the column plate number became worse with smaller-peak-volume columns. The chromatograms also show that later peaks were less affected than early peaks. Lower plate numbers with smaller-volume peaks indicate that extra-column effects may be important. Be aware, however, that low-k' peaks often have lower N values than later-eluting peaks when everything is normal.

Third, when bands tail badly under "ideal" column-test conditions, something is wrong. Once again, extracolumn effects are a prime suspect in this case.

Finally, extracolumn effects can be verified in one of two ways. If you increase the peak volume by coupling columns or decreasing the mobile phase strength (as in Case 2), you can empirically check for extracolumn effects. If plate numbers are larger and As values closer to 1.0 under these conditions, extracolumn effects are likely. Alternatively, the influence of extracolumn effects can be calculated using well-established techniques (1–4).

These two examples demonstrate that extracolumn effects create real problems in typical applications. Although short, small-particle columns can improve assays in many ways, special precautions must be taken to keep extracolumn effects to a minimum with such columns.

REFERENCES

(1) R.W. Stout, J.J. DeStefano, and L.R. Snyder, *J. Chromatogr.* **282,** 263 (1983).

(2) K.W. Freebairn and J.H. Knox, *Chromatographia* **19,** 37 (1984).

(3) L.R. Snyder and P.E. Antle, *LC, Liq. Chromatogr. HPLC Mag.* **3,** 98 (1985).

(4) DryLab software (LC Resources, San Jose, California, 1986).

(5) M.W. Dong and J.R. Gant, *LC, Liq. Chromatogr. HPLC Mag.* **2,** 294–303 (1984).

John W. Dolan is president of LC Resources Inc., of San Jose, California, and is a consulting editor for LC•GC.