

Gradient Performance Checks

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Just how good are your gradients?

Gradient elution is used widely both in research and production liquid chromatography (LC) separations. For routine analyses of drugs in biological matrices, our laboratory prefers to use gradient elution rather than isocratic separations. A built-in cleaning step is part of every gradient method that finishes at a high percentage of strong solvent and gives longer column life with "dirty" samples. Modern equipment provides a precision and accuracy of gradient control that was not possible with previous generations of equipment. However, even the best equipment can malfunction occasionally and parts can fail, so it is wise to test the performance of the gradient periodically to be sure it meets your expectations. Of particular interest are gradient linearity and accuracy. These parameters can affect gradient reproducibility and are critical for smooth transfer of gradient methods from one system to another.

In our laboratory, we perform a semi-annual instrument performance check on each LC system to ensure that it is working properly and to identify any early warning signs of pending trouble. Although a system-suitability check is made before running any set of samples, it is possible for the system suitability to pass if the instrument is reproducible even though it might not be accurate. Consider the simple case of on-line blending of mobile phase for an isocratic run. The percent composition of the mixture could be in error by a few percent and still generate very reproducible retention times so that the system-suitability check passed. However, the accuracy would still be in error. The same can hold for a gradient

that is reproducible, but not accurate. This can be of minor concern for a method that is run on a single instrument, but if the same results are expected on another instrument, accuracy is also essential.

This month's "LC Troubleshooting" will take a case study format. We'll look at a couple of tests that we perform during our semi-annual instrument check and examine errors that were discovered for one instrument that uses low-pressure mixing. These will illustrate the need for periodic performance checks of LC instrumentation.

The Test

Our semi-annual check includes measurements of flow rate, pressure decay retention reproducibility and autosampler precision. The core of the performance test, however, is a check of the system's ability to generate accurate linear gradients. The test is quite simple. HPLC-grade water is placed in the A-solvent reservoir and water containing 0.1% acetone is placed in the B reservoir. The column is removed and replaced with approximately 1 m of 0.005 in. i.d. tubing. The detector wavelength is set to 265 nm, and the flow rate to 1–2 mL/min to create sufficient back pressure for reliable check valve operation.

For high-pressure mixing systems, there are just two tests. First, a series of 4 min steps is run in 10% increments (0% B, 10% B, 20% B... 100% B) plus an extra step at 45% B and 55% B. Second, a 15 min linear gradient of 0–100% B is run. For low-pressure mixing systems, an additional gradient proportioning-valve (GPV) test is added. This tests all combinations of the proportioning valves for accurate delivery.

For example, with a four-solvent system, the test would place inlet lines for A and B in the water reservoir and C and D in the water-acetone reservoir. A series of 2 min steps is run from 50:50 A–B to 90:10 A–C to 50:50 A–B to 90:10 A–D to 50:50 A–B to 90:10 B–C and so forth, until all valve combinations have been run. The 50:50 A–B (water only) serves as a baseline, with the 90:10 mixtures generating steps containing UV-absorbing solvent.

GPV Results

When low-pressure mixing systems are tested, it is usually easiest to run the proportioning-valve test before the other gradient performance tests. The results of the GPV test are shown graphically in Figure 1 and Table 1, Test 2. Visual examination of Figure 1 shows that the first step (A–C) is lower than the remaining steps and the second step (A–D) is higher. To pass the test, the difference between the maximum and minimum plateau heights must be less than 5% of the average height. Table 1, Test 2 shows that the limit is exceeded. In our experience, the 5% limit is liberal; passing results are generally $\leq 1\%$.

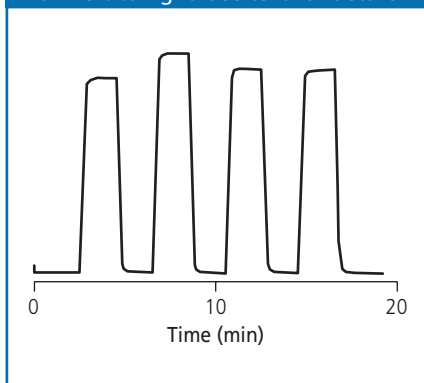
When a failed test is observed, troubleshooting should be undertaken to identify and correct the root cause. Three areas are most likely to cause problems: general pump performance, blockage of inlet line frits and proportioning-valve failure. General pump reliability problems can be the result of improper degassing of mobile phase components, check-valve problems or pump seals. We degassed the mobile phase again using helium sparging. The check valves were removed from the system and sonicated in methanol, a

technique that will usually remove particulate matter that might cause check-valve leakage. The pump seals had been replaced recently, so this was not a likely failure point. The test was rerun, and the results showed no significant improvement.

If an inlet line frit were partially blocked, it could restrict the flow of solvent from the mobile phase reservoir to the proportioning valves and result in poor proportioning performance. The easiest way to check the inlet line valves is to remove the connection at the proportioning valve and observe if solvent syphons freely through the tube from the reservoir. We like to see at least 10 mL/min flowing by syphon action to ensure that adequate solvent is available at the proportioning valves. There was no indication of restriction in the test instrument, so this was dismissed as a cause.

The remaining potential problem area was the proportioning-valve assembly. Failure of proportioning valves can occur in two major ways. The failure of a seal or other mechanical failure will require valve replacement. If, on the other hand, the failure is related to improper control of the valve cycles, it might be possible to correct the problem. The particular instrument used in the current discussion has a calibration routine that can be run to aid in correction of errors in valve timing. We ran the calibration routine and obtained the results shown in Table 1, Test 3. The performance definitely improved, but it did not pass the $\leq 5\%$ acceptance criteria. An additional four calibrations were performed and the resulting changes were inconsistent. In no case did the GPV test pass after recalibrating the valves.

Figure 1: GPV test results for the failed test of Test 2. Baseline is generated by 50:50 A–B and the remaining plateau values are 90:10 A–C, A–D, B–C, and B–D from left to right. See text for details.



One might question the value of the step test, which in the present case passed specifications, even though the system was not operating properly.

The data showed us that part of the initial proportioning-valve performance problem was due to calibration because a dramatic improvement was shown after the first recalibration. However, the inability of the calibration routine to further improve performance indicated that the remaining problem was not related to control or calibration. At this point, the entire proportioning-valve assembly was replaced and the calibration routine was repeated one more time. The results of the GPV test are shown as Test 4 in Table 1. It can be seen that the variability between the different valve combinations improved dramatically when compared with the earlier tests (Tests 2 and 3). The results also compare favourably with the proportioning-valve performance of the prior semi-annual test shown as Test 1 in Table 1.

Step-Test Results

A graphical representation of the gradient step-test results is shown in Figure 2 (corresponding to the GPV results of Test 2). Visually, the steps look good. They should appear as flat steps with a slight rounding at the edge of each step. The step size was calculated for each step and compared to the theoretical (programmed) value. The largest error was 0.4%, which is less than the 1% allowable maximum, so the step test passed under the initial (Test 2) conditions.

Gradient Linearity Results

The results for the linear gradient plot are shown in Figure 3. The plot should appear as a straight ramp between the initial and final plateaus. Note the offsets in the plot (arrows in Figure 3) at approximately 25%,

50% and 75% B. Some low-pressure mixing systems change the mobile phase proportioning algorithm in different parts of the gradient to optimize mixing of different proportions of mobile phase components. For the test system used here, it appears that the changes occur at the noted points on the curve. It is interesting to note that although the step test passed the acceptance criteria, the resulting linear gradient is not satisfactory. This required further investigation.

Step Test Revisited

The suspect region of the gradient plot of Figure 3 was examined more closely. The system was programmed to produce 1% steps between 45% and 55% B. The plot of the resulting run is shown in Figure 4. It is obvious that the step from 50% to 51% is in error by about half. Although this would pass the 61% acceptance criteria for step accuracy, there is definitely something wrong. The evenness of the step sizes is as important as the absolute accuracy. Following replacement of the proportioning-valve assembly, the step sizes evened out and the extra bumps in the linear gradient plot disappeared.

Dwell Volume Determination

The dwell volume of the LC system is the volume from the points at which the solvents are mixed until they reach the head of the column. For a low-pressure mixing system, this includes the proportioning-valve assembly, the mixer (if separate) and all connecting tubing between the proportioning valves and the head of the column, the pump head volume and the injector loop volume. The presence of system dwell volume results in the appearance of an isocratic hold step at

Figure 2: Plot of step test using faulty proportioning valves; conditions of Test 2.

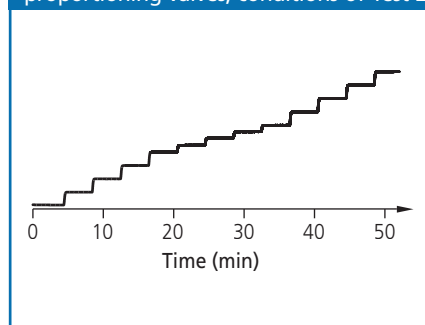


Figure 3: Plot of linear gradient with faulty proportioning valves; conditions of Test 2. Arrows indicate deviations from linear behaviour.

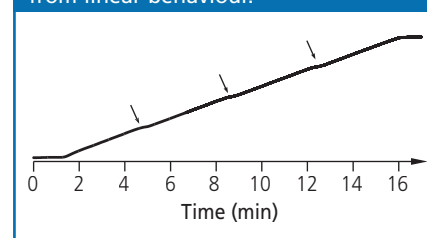
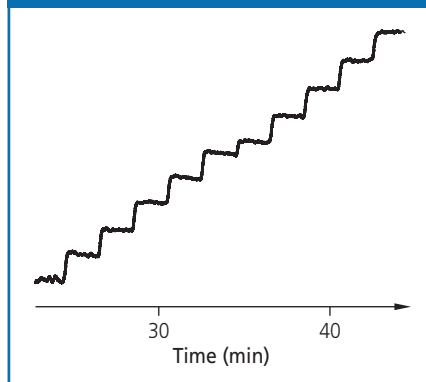


Figure 4: Step test similar to Figure 2, but for 1% steps between 45% and 55% B. Note small step at the 50–51% interval.



the beginning of every gradient. Because the dwell volume can vary from one LC system to the next and can influence retention times, particularly of early-eluted compounds, it is important to know the dwell volume of every gradient LC system. Differences in dwell volume are a primary source of difficulty in transferring gradient methods from one system to another.

System dwell volume is determined easily from the linear gradient plot obtained during the gradient tests; so we measure it with each semi-annual calibration check. Measure the dwell volume as follows. First, find the point on the gradient curve (the linear portion of Figure 3) at which the gradient has reached 50% of the maximum deflection. From the “retention” time at 50%, subtract half the gradient time. The remaining time is the dwell time, which can be converted to the dwell volume by multiplying by the flow-rate. For the test system, the 50% point was reached at 8.43 min. Half the gradient time was $15 \text{ min}/2 = 7.5 \text{ min}$, so the dwell time was $8.34 - 7.50 = 0.93 \text{ min}$. This converts to 0.93 mL at a flow-rate of 1.0 mL/min . This value is fairly typical for a well-plumbed, modern, low-pressure mixing LC system.

Conclusions

Let’s examine the results obtained from the various tests and see what we can learn from this process. In the present case, the proportioning-valve test plus visual inspection of the linear gradient pointed to a problem with gradient formation. The obvious easy fixes were examined (dirty check valves, blocked frits or calibration errors) and eliminated. A closer look at the problem region of the step test revealed a step error, giving further evidence of a

Table 1: Gradient proportioning valve test results.

Valves	Plateau Height			
	Test 1	Test 2	Test 3	Test 4
A and C	16 681	264 065	15 964	11 927
A and D	16 755	298 911	15 774	12 040
B and C	16 712	278 405	15 221	12 040
B and D	16 667	279 949	15 147	12 019
Maximum difference	0.5%	12.4%	5.3%	0.9%

proportioning problem. Once the easy fixes were tried unsuccessfully, the proportioning-valve assembly was replaced and recalibrated. All the tests were repeated and passed successfully.

One might question the value of the step test, which in the present case passed specifications, even though the system was not operating properly. We have noted with other systems, particularly high-pressure mixing systems, that a failing pump seal or check-valve can be picked up by failures in the step test. The additional tool of the proportioning-valve test is not available for high-pressure mixing systems, so the step test takes on more importance. However, it is the combination of test results from all the tests that determines whether or not the system passes its calibration check. In the present case, although the step test passed, the proportioning and gradient linearity tests failed.

Finally, what does a proportioning-valve failure imply in terms of reliability of data collected just before the tests? A system-suitability test run before each set of samples should determine if the system is suitable for use. In addition, quality control samples that contain known quantities of the analyte should be run intermixed with samples to ensure that the expected results are obtained. If these tests pass during analysis, they are considered evidence that the system is working adequately to produce results at the necessary level of precision and accuracy. Truly erratic proportioning-valve performance would be expected to result in retention time variations larger than expected for a series of runs. This was not the case for the results obtained for the current system. Problems would be more likely to be observed if a method developed and validated on a faulty system were then transferred to a properly working one.

Spending the time and money to test each LC system twice a year might seem a

bit extreme for some labs. However, the present example shows how such tests can identify potential problems before they reach the point that they compromise analytical results.

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“LC troubleshooting” editor **John W. Dolan** is vice-president of BASi Northwest Laboratory of McMinnville, Oregon, USA; a training consultant for Rheodyne, LLC, the LC Resources Training Group, of Walnut Creek, California, USA; and a member of the Editorial Advisory Board of *LC•GC Europe*. Direct correspondence about this column to “LC Troubleshooting,” *LC•GC Europe*, Advanstar House, Park West, Sealand Road, Chester CH1 4RN, UK. Readers can also direct questions to the on-line Chromatography Forum at <http://www.chromforum.com>.

NEXT MONTH in LC Troubleshooting “My Favourite Shortcuts” by John W. Dolan. This column discusses simple shortcuts and rules of thumb that allow quick estimates to guide method development.