

Performance Verification of HPLC

By Dr. Herman Lam

High-Performance Liquid Chromatography (HPLC) is one of the premier analytical techniques widely used in analytical laboratories. Numerous analytical HPLC analyses have been developed for pharmaceutical, chemical, food, cosmetic and environmental applications. The popularity of HPLC analysis can be attributed to its powerful combination of separation and quantitation capabilities. HPLC instrumentation has reached a state of maturity such that the majority of vendors are capable of supplying highly automated and sophisticated systems to meet user needs. In order to provide a high level of assurance that the data generated from the HPLC analysis are reliable, the performance of the HPLC system should be monitored at regular intervals. In this article, some of the key performance attributes for a typical HPLC system (consisting of a quaternary pump, an auto-injector, a UV-Visible detector, and a temperature-controlled column compartment) will be discussed.

The overall performance of the HPLC system can be evaluated by examining the key functions of the different modules that comprise the system, followed by a holistic testing, which tests the performance of the LC components as an integrated unit for its intended use. Modular testing can provide specific information related to the performance of the individual components of the LC. Information, such as the wavelength accuracy of the UV detector and the gradient accuracy of the pump, cannot be obtained by holistic testing alone. The holistic test can be as simple as running a frequently used HPLC method in the operating laboratory. This frequently used method can also be used as a means to compare the overall performance of different HPLC systems in the laboratory. The common performance attributes for each HPLC module, and the general expectations for each, are listed in Table 1.

Table 1: Performance Attributes for HPLC Modules

HPLC Modules	Performance Attributes	General Expectations
Pump	Flow rate accuracy	$\pm 1\%$ of the set flow rate
	Gradient accuracy	$\pm 1\%$ of the step gradient composition
	Pressure test	Proper functioning check valve No leak from the pump
Injector	Precision	1% RSD
	Linearity	$r \geq 0.999$
	Carryover	$< 1\%$
Detector	Wavelength accuracy	± 2 nm
	Linearity of response	$r \geq 0.999$
	Noise and drift	Noise: 10^{-5} AU Drift: 10^{-4} AU/hr

Pump Module:

Flow Rate Accuracy

One of the key performance requirements for the pump module is the ability to maintain accurate and consistent flow of the mobile phase, which will be necessary to provide stable and repeatable interactions between the analytes and the stationary phase. Poor flow-rate accuracy will affect the retention time of the separation.

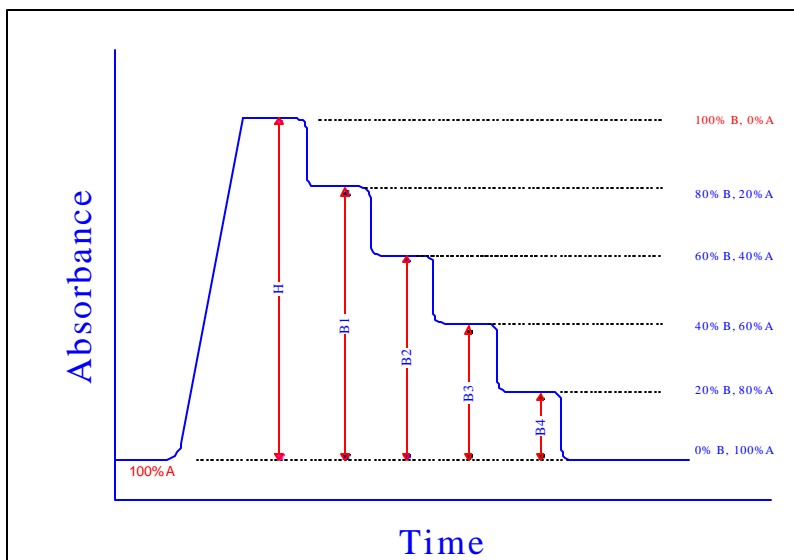
The flow-rate accuracy of the pump can be evaluated simply by calculating the time required to collect a predetermined volume of mobile phase at different flow-rate settings. For example, the flow-rate accuracy at 2 mL/min. can be verified by using a calibrated stopwatch to measure the time it takes to collect 25 mL of effluent from the pump into a 25 mL volumetric flask. The typical acceptance of the flow-rate accuracy is listed in Table 1.

Gradient Accuracy and Linearity

When it comes to gradient analysis, the ability of the pump to deliver the mobile phase at various solvent strengths over time by varying the composition of the mobile phase accurately in linear steps is crucial to achieve the proper chromatographic resolution and reproducibility.

Even though lower-pressure gradient LC pumps are usually equipped with quaternary proportioning valves, which can handle up to four solvents, typical low- and high-pressure gradient runs involve two solvent systems. The accuracy and linearity of the gradient solvent delivery can be verified indirectly by monitoring the absorbance change as the binary composition of the two solvents changes from two different channels. For example, an LC gradient has four channels: A, B, C and D. The test will be performed for two channels at a time. Channel A is filled with a pure solvent such as methanol, while channel B is filled with a solvent containing a UV-active tracer such as caffeine. The gradient profile is programmed to vary the composition of the mixture from 100% A to 100% B in a short period of time, and changed back to 100% A in a stepwise manner (See Fig. 1). The absorbance change from 100% A (baseline) to 100% B is measured and expressed as height H in the plot of absorbance versus solvent composition. As the percentage of solvent B decreases in the solvent mixture, the UV absorbance of the mixture should decrease accordingly. If the composition of the 20% A and 80% B is accurate, the height B1, which corresponds to the absorbance at 80% B, should be close to 80% of H. Similarly, accuracy verifications can be determined at 60%, 40%, 20% and 0% B. The linearity of the gradient delivery can be verified by plotting the absorbance at various mobile-phase compositions versus the theoretical composition. The entire process can be repeated for channels C and D.

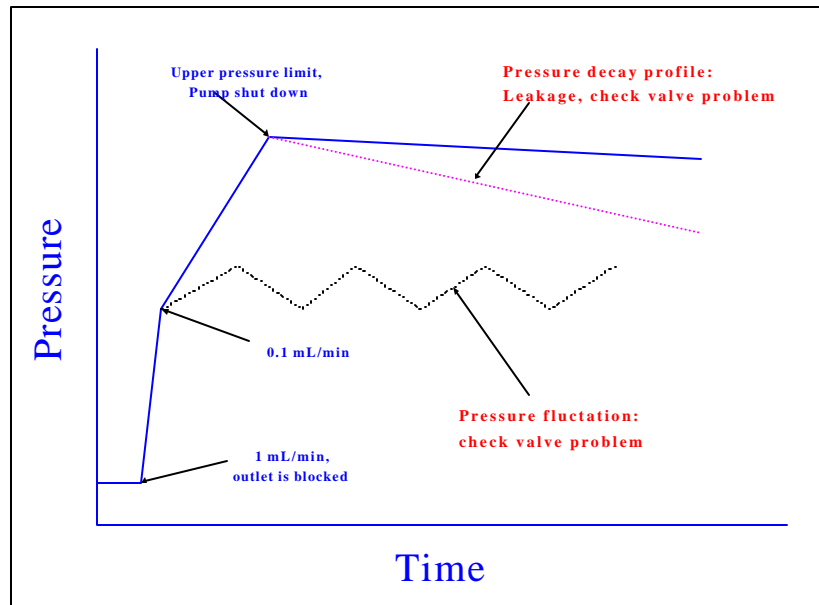
Figure 1: Gradient accuracy and linearity measurement



Pressure Test

The performance of the LC pump depends on the proper functioning of the check valves and the proper connection of the tubing. Properly functioning check valves and tubing connections (seals) are important in maintaining stable mobile-phase flow and system pressure. For pump systems that output the pressure reading in the pump head over time, a simple pressure test can be a useful qualitative test to check the condition of the check valves and to determine whether or not there are any leaks in the system. The first step of the pressure test is to plug the outlet of the pump using a dead-nut and by setting the automatic pump shutdown pressure to 6,000 psi. The pump-head pressure signal output is connected to a recorder. Pressurize the pump by pumping methanol at 1 mL/min. The pressure inside the pump head increases quickly as the outlet of the pump is blocked. As the pressure increases to about 3,000 psi, the flow rate is reduced to 0.1 mL/min. The pressure will gradually rise to the shutdown pressure if the check valves are able to hold the mobile phase in the pump chamber as would be normally expected (Fig. 2). If the check valve is not functioning properly, the pressure will fluctuate at about 3,000 psi instead of reaching the shutdown pressure. The pressure in the pump head decreases slowly over time after the automatic shutdown. A steep decrease in pressure over time implies poor check-valve performance or leaks within the pumping system.

Figure 2: Pressure test of the pump module



Injector Module:

Precision

The ability of the injector to draw the same amount of sample in replicate injections is crucial to the precision and accuracy for peak-area or peak-height comparison for external standard quantitation. If the variability of the sample and standard being injected into the column is not controlled tightly, the basic principle of external standard quantitation is seriously compromised. No meaningful comparison between the responses of the sample and the standard can be made. The absolute accuracy of the injection volume is not critical as long as the same amount of standard and sample is injected.

The precision of the injector can be demonstrated by making at least six replicate injections from a sample. The relative standard deviation (RSD) of the response of the injections is then calculated to evaluate the precision.

Linearity

Most of the automated LC injectors are capable of varying the injection volume without changing the injection loop. Variable volume of sample will be drawn into a sample injection loop by a syringe or other metering device. The uniformity of the sample loop and the ability of the metering device to draw different amounts of sample in proper proportion will affect the linearity of the injection volume. The linearity is important for methods that require the use of variable injection volumes, such as the high-low method in the quantitation of impurities.

The linearity of the injector can be demonstrated by making injections to cover a range of 0 to 100 μL . The response of the injections at each injection volume is plotted against the injection volume. The correlation coefficient of the plot will be used in the evaluation of the injection linearity.

Carryover (Not all vendors do this test because it is very dependent on the analyte.)

Small amounts of analyte may get carried over from the previous injection and contaminate the next sample to be injected. The carryover will affect the accurate quantitation of the subsequent sample. The problem is more serious when a dilute sample is injected after a concentrated sample. In order to avoid cross contamination from the previous sample injection, all the parts in the injector that come into contact with the sample (the injection loop, the injection needle and the needle seat) have to be cleaned effectively after the injection. The effectiveness of the cleaning can be evaluated by injecting a blank after a sample that contains a high concentration of analyte. The response of the analyte found in the blank sample expressed as a percentage of the response of the concentrated sample can be used to determine the level of carryover.

UV-Visible Detector Module:

Wavelength Accuracy

Wavelength accuracy is defined as the deviation of the wavelength reading at an absorption or emission band from the known wavelength of the band. The detrimental effects of wavelength deviation on the qualitative and quantitative UV-Vis measurements have been discussed in detail previously in an article on the performance of UV-Vis spectrophotometer (*Laboratory Focus-Gazette Edition*, April 2000, pg. 8). In short, the accuracy and sensitivity of the measurement will be compromised if there is a wavelength accuracy problem.

There are many ways to check the wavelength accuracy of a UV-Vis detector. For the built-in wavelength verification, the deuterium line at 656 nm and the absorption bands at 360, 418, 453 and 536 nm in a holmium oxide filter are often used. The deuterium line and the holmium oxide bands are easy to use, but are restricted to the visible range. The wavelength verification of the UV range, where most quantitative analysis is done, is performed by filling a flow cell with a solution of a compound with a well-known UV absorption profile, and scanning the solution for absorption maxima and minima. The λ_{\max} or λ_{\min} from the scan profile is then compared to the known λ_{\max} or λ_{\min} of the compound to determine the wavelength accuracy. Solutions of potassium dichromate in perchloric acid and holmium oxide in perchloric acid can be used. However, these acidic solutions are difficult to work with as the flow cell has to be thoroughly cleaned after the measurement to remove any traces of fluorescence from the potassium dichromate solution. Aqueous caffeine solution, which is easy to prepare and handle, with λ_{\max} at 272 nm and 205 nm, and λ_{\min} at 244 nm, can also be used.

Linearity of Response

Since the analytes of interest may vary in concentration, the ability of a detector to produce a linear response to concentration variation within a reasonable range is crucial to the accuracy for peak-area and peak-height comparison between standards and samples. The linearity of the detector response can be checked by injecting or by filling the flow cell with a series of standard solutions of various concentrations. Aqueous caffeine solutions are convenient for the linearity measurement. The concentration range typically should generate responses from zero to at least 1.0 AU. Absorbencies beyond 1.5 AU are more prone to deviation due to stray light. From the plot of response versus the concentration of the solutions, the correlation coefficient between sample concentration and response can be calculated to determine the linearity.

Noise and Drift (Not all vendors perform this test. Older systems may not be able to meet the same signal-to-noise ratio specified for the new equipment.)

Electronic, pump and photometric noise, poor lamp intensity, dirty flow cell, and thermal instability contribute to the overall noise and drift in the detector. Excessive noise can reduce the sensitivity of the detector and hence affect the quantitation of low-level analytes. Detector drift may affect the baseline determination and peak integration. Many procedures for detector noise and drift estimation are based on the ASTM (American Society for Testing and Material) Method E 685. Nowadays, most chromatographic software is capable of calculating the detector noise and drift. Typically, the detector should be warmed up prior to the test, and any temperature fluctuations should be avoided during the test. For a dynamic testing condition, methanol is passed through the flow cell at 1 mL/min. A backpressure of about 500 psi is maintained to prevent bubble formation.

Discussion

In reality, the performance of the LC system will deteriorate over time. If the performance verification tests do not pass the predetermined acceptance criteria, an impact assessment should be done to evaluate the effect of the failure on the quality of the data generated by the system. The impact assessment should cover all the analyses done on the system since the last performance verification, as there is no effective way of determining when the failure occurred. The system suitability data generated together with the analyses will be very useful in

demonstrating that the system performance was adequate for the application at the time of analysis, so that any data generated was reliable.

However, running system suitability cannot replace the need to do instrument performance verification tests at regular intervals. System suitability only demonstrates that the instrument is suitable for a particular analysis at the time of analysis. It cannot reveal marginal performance of the system. For example, the system suitability test for an HPLC assay using UV detection is unlikely to pick up any wavelength accuracy problems since both the standards and the samples are quantitated at the same wavelength. Marginal performance is an early warning of system failure. A follow-up maintenance is a good way of preventing unwelcome critical system failure during an important analysis.

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