HPLC Method Validation
Objectives:

I. To determine some common practical errors during validation studies in order to avoid them.

II. To define HPLC validation parameters.

III. To determine method robustness as one of the validation parameters using a design of experiment (DOE) software package (DryLab®).

References:

• *Pitfalls and errors in HPLC*, V. Meyer, Die Deutsche Bibliothek, 1997, Germany.

• *Modern HPLC for practicing scientists*, M. Dong, Wiley Interscience, 2006, USA.

• http://www.ich.org (ICH website)
I. Common Practical Errors (and hints) during HPLC method validation
1. Mixing of the mobile phase components

- Volume contraction = volume of a mixture < sum of volumes of components.
- Critical mixture: water-methanol.
why is C different from B?
Rasha Sayed Hanafi, 7/30/2010
2. Mobile phase pH

- Accurate control of mobile phase pH when ionic or ionizable compounds are to be separated.
- Acidic groups are ionized in basic pH.
- Basic groups are ionized in acidic pH.
- Ionization changes the elution pattern of the sample components.
- Robust conditions can be obtained if the mobile phase pH is 1 unit away from the pKₘ of the compound of interest.
3. Adjustment of mobile phase pH

- In case of addition of extra components such as neutral salts, solubility enhancers, ion pair reagents, or organic solvents, at which stage should the pH be adjusted?
- In all cases, the preparation of the mobile phase should be described in detail specifying the steps’ order.
4. Inadequate purity of reagents and solvents

- It is easy to find classic solvents in good HPLC quality, yet it is more difficult to find buffer salts, ion pair reagents and other additives in satisfactory quality.
- Isocratic separations are less prone to purity problems than gradient separations.
How does dissociation of the ion pair happen after it has eluted?
Rasha Sayed Hanafi, 8/2/2010
5. System peaks

- Detector-active mobile phases = the mobile phase itself gives rise to a detector signal.
- System Peaks vs. Ghost peaks?
- Problem: an additional peak causes problem in the separation
- Problem: Peak areas of regular peaks depend on their position relative to system peaks

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Why would a mobile phase give a peak? If it has absorbance at the detector wavelength, it should not be cause a baseline shifted up from zero.

Rasha Sayed Hanafi, 8/2/2010
6. Poor choice of sample solvent: Peak distortion

- Best generally: Mobile phase.
- Advantages:
  - Avoid precipitation within the column
  - Excessive band broadening
  - Unusual peak shape
- In gradients: mobile phase composition at time of injection. Exception: the solvent should be weaker eluent than the mobile phase (water in RP and methanol in NP) to concentrate the sample at the column entrance making peaks narrower.
7. Poor choice of sample solvent: Tailing

Disadvantages of tailing:
• Reduces peak height
• Hinders detection of trace components
• Reduces the resolution of adjacent peaks
• Makes peak integration difficult
8. Impurities in the sample

- The signal of a component in the UV detector is 232 nm.
- It is never permissible to postulate a certain mass ratio from a given peak size ratio!!
- An equally large peak can still be an impurity.
9. Other Sample difficulties

• Double peaks for stable conformers

• Chemical Reaction within the column
• Formation of a By-product in the sample
• Formation of an associate in the sample solution

solvent: methanol containing 3 ppm formaldehyde

solvent: formaldehyde-free methanol
10. Dwell volume

• It is the volume between the mixing point of the various solvents and the column entrance (mainly the mixing chamber).
• It delays the time until the gradient profile becomes effective at the column entrance.
• It becomes problematic if gradient method transfer is done between instruments.
• How to calculate the dwell volume?
10. a. How to determine the dwell volume?

1. Remove the column from the system and use a suitable length of capillary tubing (a blind connection) to connect the injector directly to the detector.

2. Fill the "A" reservoir with a suitable UV-transparent solvent (e.g., water, methanol, acetonitrile, etc.).

3. Fill the "B" reservoir with the same solvent doped with a UV-absorbing component to give approximately 1AU of detector signal (e.g., 0.1% acetone). Select an appropriate wavelength (e.g., for acetone, use 265 nm).

4. Run a typical gradient from 0 to 100% B (e.g., 0-100% in 20 minutes at 3 mL/min flow). Record the detector signal during this gradient.

5. Print out or display the "chromatogram" from the gradient run. Note that the signal stays flat at the beginning of the chromatogram, before the change in composition has washed through the dwell volume.

The dwell volume can be determined in one of two ways:

- **Graphically.** Draw the best straight line fit to the flat portion at the beginning of the plot. Draw the best straight line fit to the linear ramp of the gradient. The time at which these two lines intersect is the dwell time (tD). The dwell volume is the product of the dwell time and the flow rate:

  \[ V_D = t_D \times F \]

- **Computationally.** Find the time at which the baseline has increased by 50% (the midpoint of the linear gradient ramp, t50). Find the dwell time by:

  \[ t_D = t_{50} - (0.5 \times t_G) \]

The dwell volume is the product of the dwell time and the flow rate.
10. b. Checking proportioning accuracy - ”The step test”

Gradient linearity problems often result from inaccurate proportioning in the solvent delivery system.

Instead of running a linear gradient, "stair-step" the composition (e.g., 0% for 5 minutes, 10% for 5 minutes, 20% for 5 minutes, etc.).

· If the absorbance shift at each step is "identical" (i.e., the variations are within the manufacturer's specification), so the proportioning system is functioning correctly.

· If the variation is absorbance is greater than that specified for the proportioning system, the system should be serviced. Proportioning problems can result from sticking or defective switching valves, sticking, dirty, or defective check valves, air bubbles in check valves or pump heads, or software errors.

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11. Influence of temperature on separation

Partition equilibrium on which chromatography is based are temperature dependent. Without knowledge of compounds thermodynamic data, it is impossible to predict whether an increase or decrease of the temperature would be advantageous to a given problem. If the analysis is performed without thermostating, it is not surprising to see changes in the chromatogram which result from fluctuations in ambient temperature during a day or a year.
II. HPLC System and Method Validation
ICH

» The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is a unique project that brings together the regulatory authorities of Europe, Japan and the United States and experts from the pharmaceutical industry in the three regions to discuss scientific and technical aspects of product registration.

» Method validation is the process of ensuring that a test procedure is accurate, reproducible, and robust within the specified analyte range for the intended application.

» The following will highlight validation parameters and procedures for HPLC methods under ICH guidelines Q2B.

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1. HPLC System Qualification

» Design Qualification (DQ)
» Installation Qualification (IQ)
» Operational Qualification (OQ)
» Performance Qualification (PQ)
» Documentation
» System Calibration
A schematic diagram illustrating the timeline and documents in the various stages in HPLC system qualification.
System calibration

- “System calibration” refers to the periodic operational qualification of the HPLC, typically every 6 to 12 months in most regulated laboratories.

- This calibration procedure is usually coordinated with an annual preventative maintenance (PM) program and is performed immediately after PM.

- A calibration sticker is placed on the instrument to indicate its calibration status and readiness for GMP work.

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# Summary of Typical HPLC Calibration Test Procedures and Acceptance Criteria

<table>
<thead>
<tr>
<th>HPLC module</th>
<th>Typical test</th>
<th>Procedure (suggested)</th>
<th>Acceptance criteria (suggested)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detector</strong></td>
<td>Wavelength accuracy</td>
<td>Measure $\lambda_{\text{max}}$ or maximum absorbance of an anthracene solution (1 µg/mL)</td>
<td>251 ± 3nm</td>
</tr>
<tr>
<td>UV/Vis or PDA Pump</td>
<td>Flow accuracy</td>
<td>Run pump at 0.3 and 1.5 mL/min (65% methanol/water) and collect 5 mL from detector into a volumetric flask. Measure time</td>
<td>340 ± 3nm</td>
</tr>
<tr>
<td></td>
<td>Flow precision</td>
<td>Determine retention time RSD of six 10-µL injections of ethylparaben (same as in autosampler test)</td>
<td>$&lt;\pm 5%$</td>
</tr>
<tr>
<td></td>
<td>Compositional accuracy</td>
<td>Test all solvent lines at 2 mL/min with 0.1% acetone/water, step gradients at 0%, 10%, 20%, 50%, 90%, and 100%. Measure peak heights of respective step relative to 100% step</td>
<td>$\pm 1%$ absolute</td>
</tr>
<tr>
<td><strong>Autosampler</strong></td>
<td>Precision</td>
<td>Determine the peak area RSD of six 10-µL injections of ethylparaben (20 µg/mL)</td>
<td>RSD</td>
</tr>
<tr>
<td></td>
<td>Linearity*</td>
<td>Determine coefficient of linear correlation of injection of 5, 10, 40, and 80 µL of ethylparaben solution</td>
<td>$&lt;\pm 0.5%$</td>
</tr>
<tr>
<td></td>
<td>Carryover</td>
<td>Determine carryover of peak area from injecting 80 µL of mobile phase following 80 µL injection of ethylparaben</td>
<td>$&lt;0.1%$</td>
</tr>
<tr>
<td></td>
<td>Sampling accuracy</td>
<td>Determine gravimetrically the average volume of water withdrawn from a tared vial filled with water after six 50-µL injections</td>
<td>50 ± 2 µL</td>
</tr>
<tr>
<td><strong>Column oven</strong></td>
<td>Temperature accuracy</td>
<td>Check actual column oven temperature with validated thermal probe</td>
<td>30 ± 2°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 ± 2°C</td>
</tr>
</tbody>
</table>

**Additional tests recommended during operation qualification**

| Detector | Baseline noise and drift | ASTM method E19.09 | $<2 \times 10^{-5}$ AU |
| PDA or UV/Vis | Dwell volume | Perform linear gradient with 0.1% acetone/water in 10 min at 1 mL/min without column and measure the intersection of baseline with extrapolate gradient profile (see Fig. 4.5) | $<2.5 \times 10^{-5}$ AU |
| Required for systems using Fast LC or <2-mm i.d. columns | Bandwidth (dispersion) | Measure the 4σ bandwidth of a 1-µL injection of a 0.1% caffeine solution without the column (see Fig. 4.19) | $<40$ µL |

Table adapted and updated from procedures and data found in reference 8 with additional recommendations for OQ testing.

*Linearity of the UV detector and the data system is also verified.

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2. HPLC method validation

- Specificity
- Linearity
- Accuracy
- Limit of Detection
- Limit of Quantitation
- Precision
- Range
- Robustness
- System Suitability

A diagram listing of common method validation parameters.
## Validation Requirements for Each Type of Pharmaceutical Analysis Method

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Assay Category I</th>
<th>Assay Category II Quantitative</th>
<th>Assay Category II Limit Test</th>
<th>Assay Category III</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Linearity</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Precision</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Range</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>LOD</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>No</td>
</tr>
<tr>
<td>LOQ</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>No</td>
</tr>
<tr>
<td>Robustness</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>*</td>
<td>No</td>
</tr>
</tbody>
</table>

Table adapted from the USP and ICH guidelines Q2B. *May be required depending on the specific method.
Specificity, Accuracy

» **Specificity** is the ability of a method to discriminate between the intended analyte(s) and other components in the sample. Specificity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients.

» **Accuracy** is the closeness in agreement of the accepted true value or a reference value to the actual result obtained. Accuracy studies are usually evaluated by determining the recovery of a spiked sample of the analyte into the matrix of the sample (a placebo) or by comparison of the result to a reference standard of known purity. If a placebo is not available, the technique of standard addition is used.
An example demonstrating the noninterference of placebo in an impurity testing of a drug product. The upper chromatogram shows the separation of key analytes (API and various impurities and degradants) in an extract of the drug product. The lower chromatogram shows a similar extract of the placebo showing the absence of these key analytes in the placebo extract.
LOD, LOQ

» **Limit of detection (LOD)** is the smallest amount or concentration of analyte that can be detected. There are a number of ways for the calculation of LOD, as discussed in the ICH guidelines on method validation. The simplest method to calculate LOD is to determine the amount (or concentration) of an analyte that yields a peak height with a signal-to-noise ratio (S/N) of 3.

» **Limit of quantitation (LOQ)** is the lowest level that an analyte can be quantitated with some degree of certainty (e.g., with a precision of ±5%). The simplest method for calculating LOQ is to determine the amount (or concentration) of an analyte that yields a peak with a signal-to-noise ratio of 10. Thus, LOQ is roughly equal to 3 times of LOD.
Chromatograms showing analyte peaks at limit of detection (LOD, S/N = 3) and limit of quantitation (LOQ, S/N = 10).
Linearity, Range

» **Linearity** of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range, using the relationship between detector response (peak area or height) and sample concentration (or amount).

» The **range** of an analytical method is the interval between the upper and lower analytical concentration of a sample that has been demonstrated to show acceptable levels of accuracy, precision, and linearity.
## Linearity Ranges and Acceptance Criteria for Various Pharmaceutical Methods

<table>
<thead>
<tr>
<th>Test</th>
<th>Levels and ranges</th>
<th>Linearity</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay and content uniformity</td>
<td>five levels</td>
<td>correlation coefficient, $R$</td>
<td>$R \geq 0.999$</td>
</tr>
<tr>
<td></td>
<td>50–150% of label claim</td>
<td></td>
<td>% y-intercept NMT 2.0%</td>
</tr>
<tr>
<td>Dissolution</td>
<td>five to eight levels</td>
<td>$R \geq 0.99^*$</td>
<td></td>
</tr>
<tr>
<td>Related substances</td>
<td>five levels</td>
<td></td>
<td>$R \geq 0.99^*$</td>
</tr>
<tr>
<td></td>
<td>LOQ to acceptance criteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleaning surface validation</td>
<td>five levels</td>
<td>$R \geq 0.99^*$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LOQ to 20 times LOQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioanalytical</td>
<td>six to eight levels covering the dynamic range</td>
<td>$R \geq 0.99^*$</td>
<td></td>
</tr>
</tbody>
</table>

NMT = Not more than. *$R$ can be higher as specified by the company’s SOP.*
Linearity data of an assay method showing the peak area responses at 50, 75, 100, 125, and 150% of the drug substance concentrations. Regression analysis of the data shows a good coefficient of linear correlation ($r > 0.999$) but an obvious bias since the %y intercept is about 7.2%. This bias is typically caused by the nonlinearity of the UV detection in the method.
Precision

» **Method precision** is a measure of the ability of the method to generate reproducible results. The precision of a method is evaluated for repeatability, intermediate precision, and reproducibility.

» **Repeatability** is a measure of the ability of the method to generate similar results for multiple preparations of the same homogeneous sample by one analyst using the same instrument in a short time duration (e.g., on the same day). For instance, method repeatability for pharmaceutical assays may be measured by making six sample determinations at 100% concentration, or by preparing three samples at 80, 100, and 120% concentration levels each.

» **Intermediate precision, synonymous with the term “ruggedness,”** is a measure of the variability of method results where samples are tested and compared using different analysts, different equipment, and on different days, etc. It is a measure of the intra-laboratory variability and is a measure of the precision that can be expected within a laboratory.

» **Reproducibility** is the precision obtained when samples are prepared and compared between different testing sites. Method reproducibility is often assessed during collaborative studies at the time of method transfer (e.g., from a research facility to quality control of a manufacturing plant).
Robustness

Robustness is a measure of the performance of a method when small, deliberate changes are made to the specified method parameters. The intent of robustness validation is to identify critical parameters for the successful implementation of the method. Robustness validation is a formalized evaluation of the written method by varying some of the operating parameters within a reasonable range. These factors can be evaluated one at a time, or preferably by the use of experimental design such as that of “Plackett and Burman” or other design of experiment (DOE) software packages such as DryLab®. Results of the robustness study are used to define system suitability acceptance criteria.
System Suitability Testing

- **System suitability testing (SST)** is used to verify resolution, column efficiency, and repeatability of the analysis system to ensure its adequacy for performing the intended application on a daily basis.

- SST acceptance limits should represent the minimum acceptable system performance levels rather than typical or optimal levels.

- Recent ICH guidelines indicated that SST must be performed before (initial) and throughout all regulated assays. It is no longer sufficient to assume that the system will function properly during the experiment after passing initial SST. The use of a single-component calibration solution to check system suitability is not adequate because the system’s separation capability is not demonstrated. Rather, the use of system suitability samples (SSS) or resolution test mixtures containing both main components and expected impurities is required. For impurity testing, it is customary to include one of several key impurities in SSS to demonstrate resolution and system sensitivity.

- If initial SST fails, the analyst should stop the sequence immediately, diagnose the problem, make necessary adjustments or repairs, and re-perform SST.

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## Comparison of SST Criteria According to FDA’s and Hsu and Chien

<table>
<thead>
<tr>
<th>SST limits</th>
<th>CDER guidelines</th>
<th>Hsu and Chien’s recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability (RSD) of peak response</td>
<td>≤1.0% for five replicates</td>
<td>≤1.5% general</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5–15% for trace</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;5% for biologics</td>
</tr>
<tr>
<td>Resolution (R)</td>
<td>&gt;2.0 in general</td>
<td>&gt;2.0 general</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;1.5 quantitation</td>
</tr>
<tr>
<td>Tailing factor (T₁)</td>
<td>≤2.0</td>
<td>&lt;1.5–2.0</td>
</tr>
<tr>
<td>Plate count (N)</td>
<td>&gt;2,000</td>
<td>NA</td>
</tr>
<tr>
<td>Capacity factor (k)</td>
<td>&gt;2</td>
<td>2 to 8</td>
</tr>
</tbody>
</table>

NA = not available.
HPLC chromatogram of a system suitability solution (SSS) for a challenging impurity testing method for a drug product containing two APIs. This SSS contains both APIs and several key degradants and impurities at their expected concentrations (as retention time markers). One of the component DGA1 present at 0.10% level also serves as a sensitivity check and must meet the acceptance criterion of having S/N > 10.
III. DryLab®

- Introduction to computer-assisted estimation of HPLC method robustness.
- Example using the software.
Prof. Hassan Aboul-Enein, Dr. Rasha Hanafi and trainees from the pharmaceutical industry and academia.

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