Validation of HPLC Methods

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Introduction

HPLC method validation is the process used to confirm that the HPLC procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of HPLC results, and it is an integral part of any good analytical practice.

Method validation has received considerable attention in literature and from industrial committees and regulatory agencies. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use [1] has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics. ICH has also developed appendices with more detailed methodology [2].

The United States Food and Drug Administration (US FDA) has proposed guidelines on submitting samples and analytical data for methods validation [3,4]. The United States Pharmacopoeia (USP) has published specific guidelines for method validation for compound evaluation [5].

The US FDA has added section 211.222 on ‘methods validation’ to cGMP regulations [6]. This requires the manufacturer to establish and document the accuracy, sensitivity, specificity, reproducibility, and any other attribute necessary to validate test methods. The validation is also required to meet the existing requirements for laboratory records provided at Sec. 211.194 (a). These requirements include a statement of each method used in testing the sample to meet proper standards of accuracy and reliability, as applied to the tested product.

Representatives of the pharmaceutical and chemical industry have published papers on the validation of analytical methods. Hokanson [7,8] applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods. Green [9] gave a practical guide for analytical method validation, with a description of a set of minimum requirements for a method. Renger and his colleagues [10] described the validation of a specific analytical procedure, for the analysis of theophylline in a tablet using high performance thin layer chromatography (HPTLC). The validation procedure in this particular article is based on requirements for European Union multi-state registration. Winslow and Meyer [11] recommend the definition of a master plan for validating analytical methods.

This paper gives a strategy for the validation of HPLC methods developed in house and a recommendation on the implementation and documentation that should be produced during, and on completion of, validation. It does not discuss in detail parameters and limits. It also does not discuss validation of standard methods, ad hoc methods and revalidation.
Strategy for Validation of HPLC Methods

The preparation and execution of HPLC method validation should follow a validation protocol, preferably written in a step by step instruction format. If generic validation protocol templates for HPLC method validation are not available, they should be developed as well as a standard operating procedure and templates for implementation. This standardization will ensure consistent use and improve the overall long term efficiency in the laboratory and throughout the organization.

Possible steps for a complete HPLC method validation are listed in table 1. This proposed procedure assumes that the instrument has been selected, the method has been developed and meets criteria such as ease of use, ability to be automated and to be controlled by computer systems, costs per analysis, sample throughput, turnaround time and environmental, health and safety requirements.

1. Define the application purpose and scope of the HPLC method
2. Define the performance parameters and acceptance criteria
3. Define validation experiments
4. Verify relevant performance characteristics of the Liquid Chromatograph, e.g. baseline noise of the UV detector, the precision of peak areas and retention times using generic standards (for details see reference 13).
5. Check the quality materials, e.g. standards and reagents for purity, accurate amounts and sufficient stability.
6. Perform pre-validation experiments
7. Adjust method parameters or/and acceptance criteria if necessary
8. Perform full internal (and external) validation experiments
9. Develop SOPs for executing the method in the routine
10. Define criteria for revalidation
11. Define type and frequency of system suitability tests and/or analytical quality control (AQC) checks for the routine, together with acceptance criteria and recommendations to the operators in case the acceptance criteria are not met.
12. Document validation experiments and results in the validation report

Table 1. Steps in Method Validation


Scope of the method and validation parameters

The scope of the method and its validation parameters and acceptance criteria should be defined early in the process. These include:

- what analytes should be detected?
- what are the expected concentration levels?
- what are the sample matrices?
- are there interfering substances expected and, if so, should they be detected and quantified?
- are there any specific legislative or regulatory requirements?
- should information be qualitative or quantitative?
- what are the required detection and quantitation limits?
- what is the expected concentration range?
- what precision and accuracy is expected?
- how robust should the method be? For example, should the method work at a specific room temperature or should it run independent from room temperatures?
- which type of HPLC should be used, is the method for one specific model from a specific vendor or should it be used by all models from all vendors. This is especially important for HPLC gradient methods, because different instrument may have different delay volumes ranging from 0.5 up to 8 mL. This can have a tremendous impact the separation and elution order of the compounds.
- will the method be used in one specific laboratory or should it be applicable in all laboratories in your organization?
- what skills should the anticipated users of the HPLC method have?

The method’s performance characteristics and acceptance criteria should be based on the intended use of the method. It is not always necessary to validate all parameters that are available for HPLC. For example, if the method is to be used for qualitative trace level analysis, there is no need to test and validate the method’s limit of quantitation, or the linearity, over the full dynamic range of the equipment. Initial parameters should be chosen according to the chromatographer’s experience and best judgment. Final parameters should be agreed between the lab or analytical chemist performing the validation and the lab or individual applying the method. Table 2 gives examples of which parameters might be tested for a particular analysis task.

<table>
<thead>
<tr>
<th></th>
<th>major compounds</th>
<th>major compounds and traces</th>
<th>traces quantitative</th>
<th>traces quantitative</th>
</tr>
</thead>
<tbody>
<tr>
<td>limit of detection</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>limit of quantitation</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>linearity</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>
Table 2. Validation parameters for different analysis tasks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>range</th>
<th>precision</th>
<th>accuracy</th>
<th>specificity</th>
<th>ruggedness</th>
</tr>
</thead>
<tbody>
<tr>
<td>USP</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>ICH</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

For submitting data to regulatory agencies, both the United States Pharmacopeia [5] and the ICH [1,2] give guidelines on which parameters should be validated for a specific analysis task. A comparison of USP and ICH requirements is described in reference 12.

Before an HPLC is used to validate a method, its performance specifications should be verified using generic chemical standards. Satisfactory results for a method can only be obtained with HPLC equipment that is performing well. Special attention should be paid to those equipment characteristics that are critical for the method. For example, if detection limit is critical for a specific method, the instrument’s specification for baseline noise and, for certain detectors, also the response to specified compounds, should be verified.

Any chemicals used to determine critical validation parameters, such as reagents and reference standards, should be

- available in sufficient quantities
- accurately identified
- sufficiently stable and
- checked for exact composition and purity.

Any other materials and consumables, for example HPLC columns, should be new. This ensures that one set of consumables can be used for most experiments and avoids unpleasant surprises during method validation.

If there is little or no information on the method’s performance characteristics, it is recommended to prove the suitability of the method for its intended use in initial experiments. These studies should include the approximate precision, working range and detection limits. If the preliminary validation data appear to be inappropriate, either the method itself, the HPLC equipment or the acceptance limits should be changed. HPLC method development and validation is therefore an iterative process. For example, selectivity is achieved through selection of mobile phase composition. For quantitative measurements, the resolution factor between two peaks should be 2.5 or higher. If this value is not achieved, the mobile phase composition needs further optimization.

Sequence of validation experiments

There are no official guidelines on the correct sequence of validation experiments and the optimal sequence may depend on the method itself. Based on our experience, for a liquid chromatographic method, the sequence in table 3 has proved to be useful.
# Validation parameters | Measurement methods
---|---
1. Specifity with standards | Sufficient separation of all compounds. resolution factor >2.5
2. Linearity | Inject 5 standards containing the full working concentrations. Inject each standard 3 times. Average the peak area. Plot the averaged peak area vs. concentration. Calculate the linear regression.
3. Precision of the amounts | Inject a standard at three different concentrations 5 times. Calculate relative standard deviation of peak areas.
4. Accuracy | Spike a blank sample with the analyte at three different concentrations. Calculate the deviation of the results obtained with the HPLC method to be validated with the true value.
5. Intermediate precision | Inject 3 standards at different concentrations over 15 working days. The analysis should be conducted by 3 different operators using columns from 3 different batches. Measure the precision of amounts.
6. Limit of detection (LOD) | Inject a standard with a concentration close to the detection limit 3 times. Average signal height and baseline noise.
   \[ \text{LOD} = 3 \times \text{signal height} \times \text{standard amount} / \text{baseline noise} \]
7. Limit of quantitation (LOQ) | Specify a precision limit for the amount at the limit of quantitation. Prepare six standard solutions with the amounts in the range from the expected limit of quantitation to 20 times this amount. Inject all samples 6 times and calculate the standard deviations of the amounts. Plot the standard deviations versus the amounts. Take the specified standard deviation at the corresponding LOQ amount from the plot.
8. Specifity with real samples | Use samples with analytes. Check peak purity with a diode-array detector and/or a mass selective detector. Run the sample under different chromatographic columns and/or with different columns.
9. Ruggedness | Check precision and accuracy in different laboratories
10. Robustness | Systematically change chromatographic conditions. Examples: column temperature, flow rate, gradient composition, pH of mobile phase, detector wavelength. Check influence of parameters on separation and/or peak areas.

**Table 3. Proposed sequence of validation experiments (for details see reference 12)**

During method validation the parameters, acceptance limits and frequency of ongoing system suitability tests or quality control checks should be defined. Criteria should be defined to indicate when the method and system are beyond statistical control. The aim is to optimize these experiments so that, with a minimum number of control analyses, the method and the complete analytical system will provide long-term results to meet the objectives defined in the scope of the method.
Validation report

Once the method has been developed and validated, a validation report should be prepared with items in table 4.

- objective and scope of the HPLC method (applicability, type)
- summary of methodology
- type of compounds and matrix
- all chemicals, reagents, mobile phases, reference standards, quality control samples with purity, grade, their source or detailed instructions on their preparation.
- procedures for quality checks of standards and chemicals used
- safety precautions
- a plan and procedure for method implementation from method development lab to routine
- method parameters
- critical parameters taken from robustness testing
- listing of equipment and its functional and performance requirements, e.g. cell dimensions, baseline noise, column temperature range. For complex equipment a picture or schematic diagrams may be useful.
- detailed conditions on how the experiments were conducted, including sample preparation. The report must be detailed enough to ensure that it can be reproduced by a competent technician with comparable equipment.
- statistical procedures and representative calculations
- procedures for quality control in the routine (e.g. system suitability tests), with acceptance criteria
- representative plots, e.g. chromatograms, spectra and calibration curves
- method acceptance limit performance data
- the expected uncertainty of measurement results
- criteria for revalidation
- the person who developed and initially validated the HPLC method
- references (if there are any)
- approval with names, titles, date and signature of those responsible for the review and approval of the analytical test procedure

Table 4. Contents of a validation report

References


4. US FDA, Guidelines for submitting samples and analytical data for method validation, Rockville, MD, Center for Drugs and Biologics Department of Health and Human Services, Feb. 1987


13. L. Huber, Qualification of High-Performance Liquid Chromatography Systems *BioPharm, Vol 11 Number 11, November 1998, pages 41 and 65/66*