Introduction

Once one has developed a suitable chromatographic method for the analysis of a sample, it is often necessary to validate the method to demonstrate that the method is suitable for its intended purpose or to present the validated analytical method in the registration application process. Validating a method can be especially important for procedures created for routine analysis, quality assurance testing and high end applications such as medical devices and pharmaceuticals.

The purpose of this paper is to describe Jordi Labs' general approach to validating a chromatographic method and to help provide you with the tools you need in creating a high quality validation system in your laboratory. We have drawn on guidance documents provided by the *US Food and Drug Administration (FDA)* and the *European Medicines Agency (EMEA)* and on our expertise in chromatography to evaluate the suitability of an analytical method.

This paper is not intended to cover all aspects that may be required when submitting an analytical procedure for registration to regulatory bodies in the United States, Europe or other regions of the globe, but to introduce the reader to Jordi's adaptation of a rigorous method validation design by way of defining terms and parameters referenced by regulatory guidance documents and providing examples of typical validation experiments.

Method Validation Parameters

Accuracy

Accuracy is defined as the measure of how close the experimental value is to the actual value. The analyst could examine this parameter by analyzing reference standards of known value. The resultant *calculated values* for the standards should be compared to the *actual values* for the standards. Let us engage an example.

A lab is formulating a new topical acne treatment whose active ingredient (API) is ***salicylic acid*** *at 0.1%. The method developed prior to validation should demonstrate that the technique and the detection method are conducive to the chemistry of the API. For the purposes of this example, High Performance Liquid Chromatography with Ultraviolet Detection (HPLC-UV) will be the method of choice.*

Reference standards containing pure salicylic acid should be prepared at a minimum of three known concentrations bracketing 0.1% (the desired concentration), and run using the HPLC-UV method. The analyst should create a calibration curve based upon resultant peak areas for the standards. Three independent standards solutions containing pure salicylic acid at 0.1% should be analyzed and the experimental concentration calculated against the calibration curve. How closely the calculated concentration of the standards are to 0.1% provides a measure of accuracy.

Precision

Precision is defined as the measure of how close experimental data values are to each other for a continuous series of measurements under the same analytical conditions. The focus of this experiment is to ensure that the method produces a high level of repeatability within a single run and on different occasions. According to EMEA's guidance document entitled Part I: Validation of Analytical

Procedures: Definitions and Methodology, two main parameters should be explored to evaluate precision: (1) Repeatability and (2) Intermediate Precision.

Injection repeatability, or the precision within a single run, can be measured by analyzing at least six (6) injections of a homogeneous preparation of the experimental sample in a single run[1]. All runs should be executed on a single day, by one analyst and under identical conditions.

Six (6) consecutive injections of a single sample preparation should be run and the level of salicylic acid calculated against the calibration curve. The calculated concentration for salicylic acid among the six (6) injections should be consistent to indicate repeatability.

Intermediate Precision involves evaluating the reliability of the method in an environment other than that used during method development. The purpose of this work is to identify the effects caused by select variables, such as running on two separate occasions, using different instruments or different analysts to perform the work, etc. In our experience, the reproducibility of the analytical procedure should be evaluated on two separate occasions to give a more accurate indication of intermediate precision.

Five consecutive injections of a single sample preparation should be run and the level of salicylic acid calculated on two separate dates. We would recommend allowing at least one week between analyses to demonstrate the repeatability of the methodology and instrumentation as a function of time. The resultant concentrations for salicylic acid on "day 1" and "day 2" should be within 5% of each other.

The FDA provides an additional parameter which could be evaluated as part of the precision experiment. **Analysis repeatability** can be measured in conjunction with accuracy by reporting the RSD value for the analysis of three independent preparations of a known standard analyzed within a single run. The significance of this measurement lies in generating reproducible results for the calibration standards against which the experimental would be calculated. Inferior precision among reference standards could translate into variations in the experimental calculations for samples within a run.

Range

Every method has its limitations, and it is important to determine what these are to ensure that your calculated results are accurate. One should evaluate the limits of the method by examining the **range** of values that can be accurately tested using the desired method. The EMEA suggests that standards at ±20% from the expected value should be analyzed during this experiment. The goal of this study is to demonstrate that accuracy and precision at these extremes are sound.

Reference standards containing pure salicylic acid should be prepared at a minimum of three known concentrations bracketing 0.1%, and run using the method developed to create a calibration curve. Two independent standards solutions containing pure salicylic acid at 0.12% (upper limit) and 0.08% (lower limit) should be analyzed. The calculated concentrations should be consistent with the known values. Experimental samples falling beyond these limits should be considered as being outside of the validated test range for this method.

Robustness
Robustness relates to the measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Many parameters can be tested as a part of evaluating the robustness of a method, including sample concentration, chromatography column, temperature, flow rate, mobile phase composition, sample dissolution time, among others.[1]

Concentration Study
In our experience, variation of sample concentration should be introduced by 10% in either direction, and the effect on the calculated value measured. This parameter is useful in that it allows the analyst to identify the optimum concentration(s) at which the samples can be analyzed reliably using the established methodology.

Column Study
The analysis should be performed on two different column sets, manufactured from two different batches of chromatographic resin. The calculated values should reproduce regardless of the column used.

Sample Solution Stability Study
Some molecules can potentially degrade in solution as a function of time, thus it is important to evaluate the stability of the polymer in solution at time intervals relevant to the testing time requirements. We typically analyze the experimental sample at 6, 12, and 24 and 48 hours after dissolution. Additional time points can be included depending upon the application. The purpose of this experiment is to determine if, and at what time point the molecule of interest appears to degrade in solution, introducing the risk of false negatives.

System Suitability Tests
System Suitability Tests are intended to provide a measure of system performance during routine sample analysis. Experimental samples should be analyzed in at least duplicate or triplicate to evaluate this characteristic.

Analysis of Known Standards Determination of the molecular weight of reference standards should be included as a part of the final analytical methodology. One narrow standard and one broad standard should be analyzed at the same time as the samples. The broad standard should be analyzed in a minimum of triplicate injections and the percent RSD for the weight average molecular weight should not exceed 5%.

Acceptance Criteria
Rule of thumb for accepting (or reevaluating) results: Calculated values should be within 5% of each other in each experiment of the validation to demonstrate suitable repeatability and reliability.

Other Factors to Consider…

Stress Testing

In some cases, the sensitivity of the sample material to its immediate environment is unknown, and can affect the results of the analysis. Factors such as air and light sensitivity could affect the stability of the material, leading to skewed results. By analyzing the material after it has been subjected to forced degradation attempts, it is possible to determine if degradation has occurred, indicated by evidence of a decrease in molecular weight in the case of GPC or by the appearance of peaks contributed by degradation products in the case of HPLC, for example. This type of evaluation would be considered a stability indicating assay, and is intended to rule out (or in) the possibility that the sample is affected by its environment.

Light Sensitivity

Let us continue with the example of a topical acne treatment containing salicylic acid. It is possible that the material in solution is sensitive to light. One could expose the sample solution to interior lighting or UV light (or both) for 48 hours then analyze the solution. In the case of HPLC, one should look for the appearance of new peaks that were not observed in previous experiments. The purpose of the stability indicating study is not only to determine if degradation products are created, but also to determine if the degradation products and the API can be adequately separated to allow for accurate measurements.

Heat Sensitivity

Lack of adequate heat stabilization can be detrimental to the integrity of molecules exposed to high temperatures. The molecule(s) of interest could have the potential to become oxidized, which can change the properties of the material. This could result in a variation in the methods suitability to the sample. To evaluate the effects of elevated temperature and time exposure on the sample, the material can be placed in a vacuum oven well above ambient (90°C or higher). The sample should be analyzed after at least 24 hours and potential changes in calculated value noted. One should also look for new observable peaks in the resultant chromatogram, which could suggest the creation of degradation products during the heat exposure.

pH Sensitivity

Many molecules have the potential to hydrolyze under extremely basic or acidic conditions. Exposing the material to pH 2 and pH 11 for a given time period (we recommend 24 hours) then analyze the material to look for differences in calculated values from the known value.

Guidance for validating a chromatographic method is provided by the Center for Drug Evaluation and Research at the FDA in the document entitled “Reviewer Guidance: Validation for Chromatographic Methods.” This document details the parameters which should be considered when designing a validated chromatographic method.