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"Method Validation Activities in GxP Regulated Environment"

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Abstract

The focus of this research is to evaluate the method validation processes in ELISA (enzyme-linked Immunosorbent assay), particularly in the development of drugs and biologics and subsequent method validations following strictly regulated rules in GxP controlled environment.

In an effort to bolster the existing formal system of controls at pharmaceutical companies through the CGMP regulations, the Food and Drug Administration (2011) has established general principles and practices for the validation process. These general principles and practices are suitable elements that pharmaceutical companies should use in process validation for the manufacture of animal and human biological and drug products, including the active pharmaceutical ingredients (APIs).

Keywords: *ELISA, validation, GxP, FDA, critical reagents, regulations.*

Introduction

The Food and Drug Administration (2011) defines process/method validation as "the collection and evaluation of data, from the process design stage through commercial production, which establishes scientific evidence that a process is capable of consistently delivering quality product". The process entails a series of activities that take place over a product's lifecycle and process. FDA guide on the general principles and procedures for process validation describes the validation activities in three key stages: process design, process qualification, and continued process verification.

Based on a summary of results outlined at the first and second Bioanalytical Method Validation (BMV) workshops that were held in collaboration with the American Association of Pharmaceutical Scientist (AAPS), the United States Food and Drug Administration (FDA), the International Pharmaceutical Federation (FIP), Health Protection Branch (HPB), and the Association of Analytical Chemist (AOAC), two main important outcomes were identified (Vinodh P, Shah, 2007). First set up the Acceptance Criteria (AC) for a Bioanalytical assay after a method has been developed and second - set up an in-study validation parameters such as accuracy, precision, selectivity, limit of quantification (LQ), and reproducibility, necessary for acceptability of the analytical method performance. Even though the first workshop had met very wide popularity among the pharmaceutical companies, it had never been published as an official document of the FDA. A draft guidance, based on the results from the first workshop was developed and published in 1999.

Another focus of this article is on the second workshop that was held in 2000, a year after the draft guidance was published. A second workshop, sponsored by AOAC and FDA was held in 2000 where all requirements for different types of Validation activities - a full Validation, partial Validation, and Cross Validation were briefly discussed and the result was a basis for FDA Guidance on Bioanalytical Methods Validation.

Thus, FDA has established a guideline that pharmaceutical companies should use to validate bioanalytical procedures such as high-pressure liquid chromatography (LC), gas chromatography (GC), combined LC and GC mass spectrometric (MS) procedures such as LC-MS-MS, GC-MS-MS, and LC-MS carried out for the quantitative determination of metabolites and/or drugs in biological matrices such as urine, serum, or blood (Food and Drug Administration, 2001). Food and Drug Administration (2001) adds that this bioanalytical method validation guidance for the pharmaceutical industry also applies to other bioanalytical procedures such as microbiological and immunological procedures, and to other biological matrices, for example, skin and tissue samples. Moreover, the guidance can be used in enzyme-linked immunosorbent assay (ELISA) tests as well. Since there is not unified pattern for performing validation of analytic methods such as an ELISA, and there is no clear instructions on how

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to proceed with the validation activities on different ELISA platforms, a unified approach needs to be found and applied.

ELISA

Enzyme-linked immunosorbent assay (ELISA) is a serologic technique which is currently used as a diagnostic tool to detect variety of target molecules such as antigens, allergens and food contaminants. The main step in ELISA is the direct or indirect detection of antigen by antigen-specific antibody, called "capture antibody" that is immobilized directly on the surface of 96-well plate. Then, the antigen of interest is "sandwiched" between the capture and so called "detection" antibody or secondary, enzyme-coupled antibody. A chromogenic substrate, specific to the enzyme-associated antibody, yields a visible color change or fluorescence, indicating the presence of antigen. Since the fluorogenic substrates are with very high sensitivity, the levels of antigens can be very accurately measured by ELISA techniques. Quantitative or qualitative measures can be assessed based on such Hello colorimetric reading. There are several platforms of ELISA assays widely used not only in the medicine, as a diagnostic tool, but also in the industry mainly for research purposes.

Typically, ELISA is performed using 96-well plates which are able to passively bind different antibodies. Wash steps of the plates are always included in order to wash out the material that was nonspecifically bounded to the plate. After the first antibody was "coated" on the plate, a detection enzyme is be linked to the primary antibody or a secondary antibody can be introduced that specifically recognizes the primary ones. These detection enzymes are usually horse-radish peroxidase (HRP) or Alkaline Phosphatase (AP), (ThermoFisher Scientific, 2017). A corresponding substrate (TMB) is introduced in order the reaction to be visualized. The plate is read on a Plate Reader using appropriate validated and qualified software (SoftMax), OD values are measured and the concentration of the unknown analyte is determined, (ThermoFisher Scientific, 2017).

The most sensitive and robust format of ELISA is so called "sandwich" or "capture" ELISA format where the analyte (antigen), which will be measured, is quantified between both antibodies – capture and detection antibody. The analyte should have at least two antigen sites capable of binding to the corresponding antibodies. Thus it is considered as most commonly used format of ELISA because of it's highly efficiency in antigen detection. Moreover, many commercially available kits with pre-coated capture antibody (monoclonal or polyclonal ones) are manufactured following the same principle (CHO HCP ELISA kit, Protein a ELISA kit, Insulin ELISA kit). The monoclonal antibodies usually distinguish a single epitope and this permits quantification of small differences in antigen (analyte) contrasted with polyclonal which has the ability to pull down as much of the antigen as possible. The advantages of "sandwich" ELISA format are many, including high specificity, flexibility and sensitivity (the sample does not need to be purified before analysis) as well as suitability for more complex samples.

Steps in performing either ID or Binding ELISA are similar and can be summarized as follow:

Coating a 96-well polystyrene plate with capture antibody at a desired concentration, following by incubation step at either ambient temperature or 40C for established period of time.

Blocking the remaining protein-binding sites that left unbounded by addition of blocking buffer (commercially available or prepared in-house) for certain period of time. The incubation time with the blocking buffer of a choice depends on the protocol used and the nature of the molecule.

Addition of standards, quality controls, negative controls at concentrations, determined during the method development step. Spiked samples can be added in order to monitor the accuracy of the method performance.

Incubation with detection and secondary antibody at determined concentration/dilution. The most commonly used enzymes for detection are horse-radish peroxidase (HRP) and/or Alkaline Phosphatase (AP) which are being visualized using the substrates 3,3',5,5'-tetramethylbenzidine (TMB) and/or P-Nitrophenyl-phosphate (pNPP) respectively. The measurements (readings of optical density) are taken by a plate reader, which uses different wavelength of the spectrum. The wavelength used depends mainly on the substrate and its stability. For example, the readings can be taken at 450nm (using one wavelength) or it can be corrected by adding another wavelength such as 650nm.

The purpose of using the specific wavelength is to absorb the maximum from the samples/standards fluorochrome emission. However, this measure the non-specific emission from all the other materials in as well. Thus, the use of an irrelevant wavelength where the samples/standards will not give out signal is important to subtract signal that comes from these materials. Data analysis – using different platforms such as Excel, JMP, PLA.

Statistical evaluation of data is very important in order to show how accurate and reliable the method is. That is the reason why, a correct choice of Standard curve, appropriate assay parameters (diluent, blocking buffers, antibody), and sample/reagent concentration is very essential.

Concept of method development and method validation activities – steps and parameters evaluated

Preliminary DOE (design of experiment) should be set up in order to establish best parameters for the assay of desire. Method development is very long process which includes many steps and adjustments to the initially chosen parameters. In order the right parameters to be chosen, a Plackett-Burman factorial design could be used. It is the most commonly used design of 8 or 12 runs to evaluate between 5 to 11 factors. The design is applied usually in an early development stage where there is not sufficient knowledge about how the system work. The Plackett-Burman experimental factorial design is developed in 1946 by two statisticians – Robin Plackett and J. Burman and it is the main purpose is to find the active factors (variables) using as few experiments as possible. It is a design that screens out for the important factors (variables) that could potentially influence the output. Plackett-Burman factorial design should mainly be used when there is complete lack of knowledge about the factors (variables) and their interactions. Once the significant factors are available and a knowledge about the interactions is known, then multi factorial design is preferable, using SAS JMP software.

The main goal of each DOE strategy is to develop a robust potency assay with reduced number of experiments. Moreover, once the critical variables are found, DOE give us an advantage of making our assay robust enough. Some of the assay variables can be the coating concentration (concentration of the capture antibody); concentration of the detection antibody, pre-incubation and incubation time, blocking buffers used, and dilution buffers used. Once the variables are identified and experiments were completed, next step is optimization of DOE to find the optimal condition of the assay format. Last but not least, JMP analysis needs to be completed and overall prediction profiler should be applied. Profiling approach is needed in order to see what is going to happen if only one factor or many factors have changed. JMP provide a number of highly interactive cross-sectional views of any response, it gives an idea how the prediction model changes. The Profiler displays traces for each variable, it represents the predicted response as one variable is changed while the others are hold constant.

After the critical variables are set and the method is optimized based on data analysis, robustness experiments must be performed and data should be included. After all that steps are completed successfully, the validation of analytical method can be initiated.

Validation of analytical methods, including ELISA, is a confirmation and definite evidence that the exact requirements for the intended use are fulfilled. There are many publications, articles, books and guides which mainly focus on the topic of method validation, but there is no definite final protocol on how to perform this activity. One of the reasons is that the requirements for the different analytical methods are different on which are the essential parameters that need to be used. Most of the pharmaceutical companies have their own Standard Operating Procedures (SOPs) which describe step-by-step on how a validation of analytical method should be carried. Moreover, the same SOP (within the same laboratory, same pharmaceutical company) can provide altered instructions on how a validation should be held in different types of ELISA platforms such as ID, Binding and Impurity ELISAs.

Validation of analytical methods should follow certain criteria described in the Bioanalytical Method Validation (BMV) guidance. Most recently, an ICH M10, Concept paper final was published dated from 07Oct2016. This is a final endorsed concept paper draft which serve as a guide for Bioanalytical Method Validation which main idea is to resolve technical and scientific issues in BMV for method validation on chromatographic and ligand binding assays. This guidelines is still in development phase. The guideline provides recommendations on the regulatory requirements for bioanalytical methods and will provide a harmonization of current guidelines to support the drug development process. One of the issues pointed in this endorsed final concept paper is defining correctly each validation parameter

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needed (specificity, reproducibility, sensitivity, precision, recovery, range, dilution linearity, stability), clarification on what type of validation will be used (full, partial or cross-validation), establish requirements for reference standard and quality controls, critical reagents as well as combine scientific experience and advancement of equipment/technology. Documentation needed for the purpose of the validation and reports describing the study sample analysis will be also other aspect of the endorsed concept paper. The main idea is harmonization of requirements for bioanalytical method development and its application in accordance with the requirements for bioanalysis in non-clinical and clinical drug development.

So far, the main parameters included in each validation activity are as follow: specificity, accuracy, linearity, including sample linearity, precision, repeatability, Limit of Detection (LD), Quantitation Limit (QL), range. Not all parameters has been examined when a validation for ELISA methods is needed. Some of the runs can be performed such a way, so the data can serve for the purpose of evaluation of other parameters. For example, the data gathered from the accuracy run can be used for the precision, range and linearity. The data from accuracy run can be used for establishing the range and QL.

Moreover, there are many differences on how the validation is lead when different ELISA platforms are available. Impurity ELISA methods often include the sample linearity runs where samples of interests are spiked into a matrix (know standard with known concentration) and then different dilution are prepared and analyzed. This way, the proper dilution factor for the sample of interest is established and same is used during the entire validation process. Furthermore, feasibility study is also important, often performed during the development stage.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. In order to ensure that the impurity/analyte is specifically determined by the specific test method and that the recovery is not affected by other compounds or sample matrix, spiked samples (samples of interest, spiked with certain amount of the impurity stock) and formulation buffers (buffer without the active ingredient) as well as assay diluent must be evaluated. Usually, the formulation buffer and the assay diluent are treated the same way as the sample, spiked at the same amount of the stock of interest (for impurities it could be either Insulin, rProtein A, CHO HCP or Glucan). If the amount of recovered impurity for all samples, formulation buffer and or diluent are same, the conclusion could be that there is no interference, meaning that the impurity is not affected by the sample matrix, assay diluent or ingredients in the buffer. For regular ELISA formats such as Binding or ID methods, spikes are not required, but another not-specific compound may be included to the run to ensure that there is no specific binding occurred. This would confirm the specificity of the method when only the analyte of interest specifically bounds to the antibodies on the plate.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. When using a combined experimental approach to obtain results for Linearity, Precision, Accuracy and Quantitation Limit, samples are usually spiked at different concentrations and analysis is repeated between couple of analysts. The recoveries of all spiked concentrations for each one of the samples is calculated and the %Coefficient of Variation (CV) of the mean value is calculated. Acceptance criteria for all experiment should be set up before the validation has started.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Usually two different types of linearity are assessed in many Impurity methods - sample linearity, where samples are spiked at different concentrations that falls within the method range, % Recovery is calculated and then plotted

against the nominal "target" concentration. Another approach is evaluation of assay linearity, where recoveries from all used standards from the standard curve are taken and analyzed. For Binding and ID ELISA formats, performing only assay linearity could be acceptable.

Precision

Under precision part there are major parameters - repeatability, intermediate precision and reproducibility. The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision/validity. Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. and it is referred as an external precision/validity. Precision may be expressed as the relative standard deviation (RSD) or the percent coefficient of variation (%CV).

Reproducibility is a measure of inter-laboratory variation; the use of the analytical method in different laboratories (i.e., a collaborative study).

The evaluation of reproducibility is not required for qualification of an analytical method.

Range

The range of an analytical method is the interval between, and including, the upper and lower levels of analyte that has been demonstrated to have a suitable level of precision, accuracy, and linearity.

QL – The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

DL – The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Usually the robustness part is completed during the method development phase, when all essential parameters are already set. The concept of robustness study will be described later in this thesis.

After all acceptance criteria is met, and a proper documentation (Validation Protocol that summarize all experimental data) is on board, the validation is considered completed. In some cases, part of the Precision/ Intermediate Precision could be given to another lab, receiving lab that will eventually own the validated method and will carry all the responsible for any future executions, corrections, and technical reviews. This is important step in a process called tech-transfer or transfer of already set, validated and scientifically sound method which will be described later.

Moreover, all validation activities need to be in accordance with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH harmonized tripartite guideline, and Validation of Analytical Procedures Text and Methodology Q2 (R1), November, 2005.

Concept of robustness studies

Robustness can be defined as the capacity to replicate the (analytical) method in different laboratories or under different conditions without the existence of unanticipated changes in the obtained result(s), and a robustness test as an investigational set-up to assess the robustness of a method. A synonym of robustness is ruggedness.

The terminology robustness is now widely applied in the pharmaceutical world and is given by the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH).

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Robustness experiments are executed with already established assay conditions. The concentrations of all antibodies used (capture, detection), the standard curve fit parameters, all dilution factors, diluents and blocking buffers as well as the incubation steps for each step of the method are well- known. Moreover, the system suitability criteria in terms of appropriate ranges of adjusted results, %Recovery, %StDev and %CV of each replicate analyzed should be established as well.

Even though the assessment of robustness study is not required by the ICH, the actual execution of the study would allow us to monitor the ability to reproduce the analytical method in different laboratories or under different conditions without any unexpected differences in results.

Different factors from the operating procedure are carefully chosen in order to observe the possible source of variability. Those factors are examined in a certain known range that slightly exceeds the variations that are expected from the analysts, performing the method in different laboratories (intra-variables). In such a way, those factors that disrupt the good method performance are being discovered and they must be strictly measured during the implementation of the method.

Concept of stability of drug product and critical reagents and their subsequent qualification

Critical reagents are crucial to the assay performance due to their unique characteristics. They can be binding reagents such as binding proteins, capture/detection antibodies, conjugated antibodies, and biotinylated probes, antibodies that are used as quality controls, such as positive and negative controls. Typically, reagents are produced via biological processes and most of them prone to lot to lot variability. This is the reason why a qualification/validation of them is needed in order to monitor long-term stability as well as quality.

Moreover, finding out different environmental factors such as exposure to light, temperature, low/high humidity, or chemical factors such as acidic, basic or oxidative media that could possibly influence the stability of the critical reagents and/or drugs is important, thus should be included in every process of validating a bioanalytical method. Stability study could possibly give a clear picture on how critical reagents and drug products can be held, what would be the desirable shelf life, and how quality changes under certain circumstances i.e. changing the pH of the media, exposure to UV light, hi/low pH etc. This is essential in order to provide information about re-test date and storage conditions. Short-term stability as well as long term stability of critical reagents and drug products is essential and despite the lack of more detailed information about the stability study in the FDA and EMA Bioanalytical Method Validation Guidance for Industry, an indication about characterization and qualification shall be done for the intended purpose even though the degree of required characterization varies considerably.

Stability study should be execute as per ICH guidelines. ICH Q1B - photo stability studies for New Drug Substances and Products as well as following Q1A (R2) stability testing. The finalized stability guideline gives a direction on the basic testing protocol necessary to assess the light sensitivity and stability of new drugs and products as well as references on stability testing procedures including temperature, humidity and duration in climatic Zone I and II. The method validation qualifies all reagents used, but further usage of them needs more detailed analysis and characterization in terms of long-term stability testing. Accelerated stability study can be determine as well, but the choice depends on the product's nature. All pharmaceutical companies have different approaches in qualifying critical reagents/ drug products. Moreover, corresponding SOPs (standard operating procedures) should be written and strictly followed. Qualification of critical reagents or drug products can be executed differently depending on the status of the evaluated compound. If a critical reagent/drug product has change in terms of lot number (batch) or formulation, a simple comparison between the original and the new lot or old formulation and the new formulation can be done. Acceptance criteria for the method of interest as well as the suitability criteria is compared and evaluated.

When a previous lot/formulation is not available and a new lot/formulation has to be introduced, more detailed qualification is needed and it strictly depends on the procedures outlined. A single-point stability study may be desirable.

A re-qualification of a critical reagent (same reagent over time, reagent in regular use) is performed in order to establish the new re-test date of that reagent following same method and passing all acceptance criteria for the assay. A ±3STD calculations are included in order to set a range of variability

as well as to monitor the performance of the method over time. Trend analysis must be presented and documented in order to monitor the performance over time.

Concept of calibration/qualification of GxP instruments

IQ/OQ/PQ should be performed prior initializing validation of methods. IQ stands for Installation Qualification, OQ for Operational Qualification and PQ is Performance Qualification. A basic requirement of good bioanalytical method validation is that analytical instruments used for the intended purpose must be appropriately installed, calibrated and maintained. IQ is a process of proving if the installation of the instrument is correct, ensuring that all components meet the approved specification. Moreover, all recommendations as per manufacturer should be fulfilled.

OQ is the process when a testing of the instrument is done in order to ensure that the system functions well, meets certain criteria as well as to check how the result of testing is recorded. PQ also called process qualification has the goal to ensure that the specified criteria can be achieved on a reliable basis over a long period of time. It is important in order to demonstrate the instrument performs according to specification, appropriate to its routine use. Usually the IQ/OQ/PQ of an instrument is performed by a vendor and regulated by the SOPs specific for each pharmaceutical company.

Conclusions

FDA had not established specific regulations on specific method validation. Instead, the FDA has only developed guidelines that can help pharmaceutical companies in developing and implementing a validation method for an analytic process such as the ELISA. Consequently, various pharmaceutical companies have developed different method validation processes for their analytical methods. Since the majority of the pharmaceutical companies have differences in handling entire validation process, the emphasis of this research proposal is to show some basic concepts of validation for ELISA. For this purpose, validation parameters should be evaluated for the purpose of a robust and scientifically sound method. All Validation activities must be in accordance and compliant with the International Conference on Harmonization (ICH) Q2 (R1) Guidelines for the Validation of Analytical.

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