Basic Principles and Applied GxP Regulations for ELISA Analytical Method Development of Drugs and Biologics and Subsequent Phase Appropriate Validations in FDA Driven Environment

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Abstract

Biological commodities are majorly molecular entities that are often bigger than many chemical commodities with an exception of peptides or synthetic oligonucleotides, living tissue, and complex cell metabolic companies that manufacture them. The required molecules should be separated from the biochemical compound comprising chemical elements relatively similar to the target commodities. Thus, it might be hard to completely remove impure substances generated from the host processes and systems. The purification process may include a variety of heterogeneous structural forms in which all or some may be very active. Unlike the normal pharmaceutical products, biochemical products are liable and unable to tolerate undue physical or chemical stress or high temperatures. The paper will examine various factors to consider when choosing appropriate analytical methodologies to evaluate the impurities, potency, purity, identity, concentration and the comparison of biotechnological commodities. Therefore, the focus of this proposed research is to evaluate the validation processes in ELISA methods of analysis, particularly in the development of drugs and biologics and subsequent phase appropriate validations in the FDA driven environment.

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CHAPTER ONE: Pharmaceutical Analysis

Introduction

Analytical methods are utilized in the biological research, development process, and chemical control functions. Every strategy used in the enzyme-linked immunosorbent assay (ELISA) have their own significant features, which must be taken into account (Black, 2009). Every step in the analytical method must be evaluated to determine the extent to which procedural, environment or matrix variables can influence the projection of the analyte in the matrix from collection time to the analysis time (Evans, 2014).

Pharmaceutical analysis calls for accurate and precise assay techniques to quantify drug substances either in biological or pharmaceutical samples. The assay methodology should be selective, reproducible, sensitive, and rugged (Fay & Proschan, 2010). The ELISA analytical method involves the quantitative and qualitative analysis of products in biological fluids such as urine and plasma. It plays a significant role in interpreting and evaluation of pharmacokinetic data.

The primary analytical phases include the usage of development method, validation method, and analysis of the sample (application method).

Pharmaceutical Analysis Purpose

- Validation method for FDA Guidelines
- New Drug and Biologics Development
- Research and Study in Pharmaceutical sciences
- Clinical Pharmacokinetic Research

When promising outcomes are acquired from the validation carried out at the development method phase, then only complete validation must be considered.

Assay of Drugs and Biologics and their Metabolites

Various analytical techniques used to differentiate metabolites from the biologics and drugs have been established drug or biologic metabolism reactions are subdivided into phase II or phase I categories. Phase I involves reduction, hydrolysis or oxidation of the reaction. On the other hand, phase II involves condensation and coupling of phase I metabolites or drugs with various body constituents such as glucuronic acid and sulfate. Apart from the processes of reduction, during most of phase II and I metabolites are generated; they are mostly polar, and thus, much more soluble than their parent biologics or drugs. Therefore, the assay must differentiate metabolites and their parent drugs.

Analysis of Biologics and Drugs from Samples

The samples usually used for analysis are urine and blood. Feces are also used, especially when the metabolite or drug is excreted in the bile or extensively not properly absorbed. Also in some circumstances breath, saliva and tissue are utilized. In the Food and Drug Administration (FDA) driven environment, manufacturers and producers of pharmaceutical goods are required to comply with FDA's Good Manufacture Practice (GMP). Specifically, the Food and Drug Administration has established appropriate techniques to monitor and control how various pharmaceutical companies follow guidelines and regulations of the Current Good Manufacturing Practice (CGMP) so as to produce high-quality drugs and biologics. The pharmaceuticals firms are required to comply and adhere to CGMP to enhance the purity and quality of drug substances by ensuring that the pharmaceutical enterprises sufficiently control the production and manufacturing operations.

The Food and Drug Administration (2011) defines process of 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" (p. 4). The process entails a series of activities that take place during a product's lifecycle. FDA guide on the general principles for process validation describes the activities in three key stages: design, qualification, and continued process verification. It is important to point out that there has been a recent inquest as to whether the CGMPs require three validation batches before a new API or a final drug product is released for distribution (Food and Drug Administration, 2017d). In response, the FDA asserts that neither its policy nor the CGMP regulations specify a minimum number of batches to validate a manufacturing process (Food and Drug Administration, 2017d). Moreover, the existing FDA guidance on APIs also does not stipulate the number of batches for any process validation. Nonetheless, the FDA recommends that manufactures should expand their testing based on the traditional validation protocol so as to provide an additional guarantee that a batch satisfies all the proper and established standards before API is used in the final drug product (Food and Drug Administration, 2017d).

Therefore, the 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 ones, and to other biological matrices, for example, skin and tissue samples. Moreover, the guidance can as well be used in enzyme-linked immunosorbent assay (ELISA) tests. The aim of this study is to evaluate the validation processes in ELISA methods of analysis, particularly in the development of drugs and biologics and subsequent phase appropriate validations in the FDA driven environment.

CHAPTER TWO: Review of the Literature

The enzyme-linked immunosorbent assay (ELISA), according to Garber (2008) and Wiederschain (2009), is an important test that uses color change and antibodies to identify a substance. Fundamentally, this biochemistry assay utilizes a solid-phase enzyme immunoassay (EIA) to identify the presence of a substance, often an antigen in a wet or liquid sample (Farzan, Friendship, & Dewey, 2007). ELISA is extensively used as a diagnostic tool in plant pathology and medicine (Belák, 2007; Stapel et al., 2008; Adams, 2009; Miller, Reed, & Harmon, 2009). The biological assay is also used as a quality-control (QC) tool in various industries (Leng et al., 2008). The central advantage of the ELISA technique is that its results are often quantifiable (Laing et al., 2010). The other advantage of this important biochemical assay is that it does not involve the use of radioactive materials which have been used on a large scale at the start of molecular biology as a science. Instead, the technique uses enzymes which react with antibodies and subsequently form colored products. It is important to point out that the development of color in this assay is an indicative of a positive result (Crowther, 2001; Kolosova et al., 2007; Hossain et al., 2012; Gan, & Patel, 2013, Ge et al., 2014). According to Hnasko (2016), the ELISA technique has two main variations: direct and indirect detection methods. The author explains that in the direct detection method, the ELISA technique is used to identify the presence of antigens that are recognized by an antibody. In other words, the direct detection method entails the use of a labeled primary antibody that is known to react with the antigen. Hnasko further elucidates that, on the other hand, the indirect detection method of the ELISA technique is one that is used to test the antibodies that recognize an antigen (2016).

Subsequently, in the recent years, multiple studies have focused on determining the validity of ELISA techniques when applied in different protocols and procedures, including diagnosing of diseases in medicine. For example, Adela Valero et al. (2012) evaluate the diagnostic accuracy of an ELISA with *F. asciola* antigen obtained from an adult liver fluke to detect the IgG class antibodies against *F. hepatica* in the human serum. The findings of the study reveal that the respective sensitivity and specificity of the ELISA were 95.3% and 95.7%. Moreover, Adela Valero et al. (2012) observe that there is no correlation between the egg output and amniotic fluid delta optical density 450 (OD450) values of the *F. hepatica* IgG ELISA test. Therefore, the researchers conclude that the ELISA assay is a valid diagnostic test when used in combination with other techniques. In a different study, Levy et al. (2013) concluded that the Haptoglobin (Hp) ELISA is a user-friendly, fast and highly accurate diagnostic tool that can be used in determining Hp phenotypes. Thus, Levy et al.'s (2013) inference confirm that the ELISA test is a valid method that can greatly help in typing of thousands of samples in the current studies. Although Adela Valero et al. (2012) and Levy et al. (2013), including several other studies, confirm that ELISA is a valid biochemistry technique that can be applied in different contexts, it is clear that its methods do have some differences: methods using commercially available kits and the methods developed using

specific platforms. As a result, there is a great chance that the validation activities of the various ELISA tests might be quite different.

Moreover, the validation of most of the ELISA methods strongly depends on the changes that are needed to support particular studies (Abbott et al., 2010). It is arguable that the validation could be full when a novel bioanalytical method will be developed and implemented, or a partial one, when only the modifications are represented to the already validated system (Pfister et al., 2009; Asensio et al., 2010). In particular, that could include a change of the matrix system, change of instrument software, modifications in samples processing procedures and more. A Cross-Validation can be performed as well, which includes a comparison of various validation parameters between same studies or across different studies. According to Food and Drug Administration (2001), cross-validation involves the comparison of validation factors when two and more bioanalytical methods are used to produce data across different readings or within the same reading. A good illustration of cross-validation is where an original validated bioanalytical method is used as the reference and meanwhile, the revised bioanalytical method is used as the comparator; in cross-validation, the comparisons have to be done in both ways (Food and Drug Administration, 2001).

Moreover, when sample analyses within one study are carried out in more than one laboratory or at more than a single site, it is vital for cross-validation with spiked subject examples and matrix standards to be conducted in each laboratory or site so as to determine interlaboratory reliability. According to the Food and Drug Administration (2001), cross-validation should as well be considered when data is produced with the usage of different analytical techniques, for example, ELISA and LC-MS-MS. It is critical to ensure that all the modifications are evaluated to establish the recommended validation degree (Food and Drug Administration, 2001). The analytical laboratory carrying out toxicology or pharmacology as well as other pre-clinical studies for purposes of making regulatory submissions are expected to comply with FDA's Good Laboratory Practices (GLPs) and sound quality assurance principles throughout the testing process. According to the Food and Drug Administration (2001), it is a mandatory requirement for an analytical laboratory to have a documented set of its standard operating procedures (SOPs) so as to ensure a wholesome system of quality control and assurance. In particular, the set of the SOPs that an analytical laboratory has made should address all the analysis aspects from the time the sample is collected and reaches the laboratory until the findings of the analysis are reported (Food and Drug Administration, 2001). The SOPs should have accountability systems that guarantee the integrity of the test articles, for instance, record keeping, security, and chain of sample custody. The Food and Drug Administration (2001) adds that the SOPs should also have sample preparation and analytical tools such as reagents, instrumentation, equipment, methods, and the procedures for verifying results and quality control.

The validation includes many parameters such as Accuracy, Precision, Specificity, Reproducibility, Linearity, Determination of limit of quantitation (QL), Detection limit (DL), and system Suitability parameters to evidence that the system works according to the performance expectations and set criteria (Pourvasei et al., 2013). The successful completion of all the validation activities is critical for an effective manner of clinical and non-clinical studies. In order to guarantee the reliability of analytical measurements, a whole system of quality measures or quality assurance measures including the use of validated methods, control charts, reference materials, and participation in proficiency testing schemes must be effected by the competent laboratory (Picó, 2012). The author acknowledges that method validation is one of the critical components of this sophisticated system to provide reliable analytical findings that can easily be replicated among different laboratories. Therefore, method validation of the ELISA tests is a quality assurance that focuses on establishing the reliability of its analytical results that are comparable in various competent laboratories. Typically, there are several major reasons that justify method validation of ELISA-based methods

(Popping, Diaz-Amigo, & Hoenicke, 2010). First, validation is used to ensure that the method is perfect for a particular purpose. Second, method validation is used to demonstrate that the method performs appropriately even in the hands of different users. Third, method validation provides impartial data with respect to method performance under specified conditions. Finally, method validation is essential for comparing the performance of methods.

Subsequently, it is important to note that the choice of method of analysis to be used is dependent on the needs of the user; ELISA method must be capable of effectively addressing or solving the user's analytical problem. It is against this background that the proposed thesis seeks to evaluate and study the validation processes in ELISA methods of analysis. Specifically, the proposed thesis shall place considerable emphasis on examining and estimating all the parameters in an ELISA method validation process because of their significance in yielding good performance. According to Crowther (2001), validation entails all the process that influences the performance of an assay to achieve a particular set of objectives. The author argues that it is only when actual data obtained can the test parameters, be evaluated and the confidence in findings be assigned in a statistical sense. Fundamentally, method validation is a continuous process (Crowther, 2001). Therefore, this means that knowledge concerning an assay is gained every time a validation process is carried out. Crowther (2001) further explains that the continuous process of method validation also involves the data that is obtained when the test in question is performed hitherto untried situations. Since the majority of the assays start in the research field, the use of validated tests in the form of approved kits by a broad range of scientists in the laboratories varying extensively in equipment, climatic conditions, and expertise can cause problems. Hence, the primary objective of any method validation is to define a specific assay in terms of parameters that can be quantified statistically with measured confidence (Shah et al., 2000; Scortichini et al., 2005; González & Herrador, 2007).

Moreover, González and Herrador (2007) contend that an assay can only be validated when it has been defined on the basis of its capacity to categorize samples with respect to the presence or absence of a specific analyte. According to the researchers, method validation depends on the evaluation of as many parameters as possible. The implication of it is that at any evaluation stage, it is imperative that the quantifiable parameters are defined by describing the test. Additionally, the mechanisms for carrying out a re-evaluation of the quantifiable parameters must be put in place. Therefore, a validated assay depends on its design characteristics that ensure the results. According to Crowther (2001), this usually results in a robust assay that is not readily affected by physical factors, or the geographical location where the samples are acquired or used. Crowther (2001) explicates that with respect to ELISA, the development as well as the validation of an assay is often made using a limited number of tests, on samples that have been selected from a group or groups of patients or animals, and carried out over a short period.

The affectability of discovery relies upon enhancement of the signal amid the diagnostic responses (Gómez-Morales et al., 2008). Since chemical responses are exceptionally notable, the signal is created by catalysts which are connected to the recognition reagents in settled extents to permit precise evaluation – hence the name "compound connected." The analyte is additionally called the ligand since it will tie or ligate to a discovery reagent; along these lines ELISA falls under the greater classification of ligand restricting measures (Gómez-Morales et al., 2008). The ligand-particular restricting reagent is "immobilized," i.e., normally covered and dried.

Warmth stun protein 90 (Hsp90) is an atomic chaperone that assumes a key part in the conformational development of oncogenic signaling proteins including HER2, AKT, BCR-ABL and mutant p53 (Gómez-Morales et al., 2008). Hsp90 inhibitors prompt proteasomal debasement of Hsp90 customer proteins and are being tried in phase I/II clinical trials for oncology signs. Current strategies to identify bioactivity of Hsp90 inhibitor mixes include a western smear examine from

tumor biopsies or hPBMCs, a strategy that is relentless and semi-quantitative. While trying to create more quantitative and strong strategies that would at last help to decide ideal natural measurement and encourage beginning dosage determination for Hsp90 inhibitor stage II clinical trials, we assessed elective ELISA based techniques for recognition and quantitation of discharged and intracellular types of Hsp70 in hPBMC treated with the Hsp90 inhibitors ex vivo (Gómez-Morales et al., 2008).

Human PBMCs and SCLC cell lines were refined with various dosages of HSP90 inhibitor drugs. Cell pellets and supernatants were gathered following 24, 48, and 72 hours. Intracellular HSP70 levels were evaluated by Western blotch and an exceptionally delicate ELISA strategy of an electrochemiluminescent (ECL) stage. Emitted Hsp70 levels were examined by ECL based ELISA. The ideal opportunity for maximal discovery of intracellular Hsp70 was in the vicinity of 24h and 48h. Intracellular Hsp70 levels recognized by ELISA in sedate treated cells were far higher than the Western blotch test (Gómez-Morales et al., 2008). Furthermore, identification by ELISA design offered an unrivaled dynamic range, was more quantitative and delicate than the Western smear measure. Besides, around 30-overlay less aggregate cell protein was required to measure Hsp70 levels by ELISA contrasted and Western smearing. Late examinations have shown that Hsp70 is additionally emitted into the serum of disease patients by travel by means of an endolysosomal compartment. Utilizing the ECL based ELISA framework to measure emitted Hsp70 we recognized a 10 to 25-overlap increment in the emission of Hsp70 by hPBMC and tumor lines treated with different Hsp90 inhibitors (Gómez-Morales et al., 2008).

The ideal opportunity for the maximal location of emitted Hsp70 was in the vicinity of 48h and 72h. Moreover, a great relationship (R2 > 0.9) was found in the levels of intracellular and discharged types of Hsp70 following treatment of cell lines with various dosages of Hsp90 inhibitors. Moreover, utilizing this stage, it was possible to show the capability to identify basal levels (2-5 ng/mL) of Hsp70 in the serum from tumor patients and typical benefactors (Gómez-Morales et al., 2008). This strategy for the identification of emitted Hsp70 was approved for clinical use by evaluating for exactness, accuracy, network impacts and vigorousness. We report advancement and approval of an exceptionally delicate, quantitative and powerful strategy for the precise assurance of intracellular and emitted types of Hsp70. The improved measures ought to be valuable in dosage determination and observing the natural impacts of investigational Hsp90 inhibitor treatment for growth patients. Besides, the capacity to measure Hsp70 in serum is a decent contrasting option to the right now utilized intracellular Hsp70 evaluations as it is trying to actualize the last convention in a clinical setting.

For human parasitic ailments, no indicative test or reference materials have been institutionalized, with the exception of human serum of hostile to Toxoplasma IgG. As per the research center accreditation procedure of ISO/IEC 17025:2005, it is first important to approve a serological test, and after that continue to the institutionalization procedure (Gómez-Morales et al., 2008). In the event that the test demonstrates a satisfactory execution as far as affectability, specificity, precision, and reproducibility, the test outcomes could be consolidated with other research facility discoveries and with clinical and epidemiological information to make the last determination. With particular respect to trichinellosis, albeit a few strategies have been utilized for serological determination, ELISA has and keeps on being the most regularly utilized strategy in light of its high affectability (Gómez-Morales et al., 2008). Nonetheless, to the best of our insight, no serological test has been approved using a sufficiently substantial board of serum tests from sound people, those with affirmed trichinellosis, and the ones with wellbeing issues other than trichinellosis (Gómez-Morales et al., 2008).

In approving a serological test, it is crucial that the cutoff is characterized. Subsequently, the specimen measure must be sufficiently substantial to limit the stochastic vulnerability in the

cutoff determination (Kawano et al. 2007; Frost, 2009; Rola-?uszczak et al., 2013). To choose a positive and a negative reference populace, the highest quality level must be accessible, yet on account of trichinellosis, no such quality level has been observed. To this end, the researchers carried out 1,159 serum tests on apparently solid people, and 367 on people with trichinellosis affirmed on the premise of the calculation proposed by Dupouy-Camet and Bruschi. In light of the ROC examination, the cutoff esteems were set at 11.8% for IE and 0.233 for OD; in view of these qualities, the affectability and specificity were 98.7% and 98.4% respectively.

In the preceding discussion, it was pointed out that the FDA regulations such as the GLP, GMP, and other quality standards require that analytical methods should be evaluated before and during regular use. Moreover, there are no particular regulations on method validation. Nevertheless, the FDA, other government agencies, and industry task forces have developed guidelines for validating methods. Therefore, this means that as long as pharmaceutical companies evaluate analytical methods before and during regular use, they are not under any statutory obligation to comply with the FDA guidelines on method validation, including ELISA. It is incumbent upon these companies to either consider using the recommended FDA guidelines on method validation or to develop and implement a method validation that guarantees the security, efficacy, and safety of the drug product as provided under the FDA guidelines. Hence, pharmaceutical companies can, in fact, develop and adopt an ELISA test validation method that best suits their drug product. Since the focus of this proposed research will be on evaluating the validation processes in ELISA methods of analysis, particularly in the development of drugs and biologics and subsequent phase appropriate validations in the FDA driven environment, and that there are no specific regulations on method validation, the study will examine both the FDA guidelines on bioanalytical method validation and the existing empirical evidence on ELISA validation process.

Immunoassays, for example, ELISA, are used when there is a need to quantify an unknown analyte concentration within a sample (Cox et al., 2014). The researchers explain that in order to obtain the most precise determination of the unknown analyte concentration, it is important for the immunoassay to be developed on the basis of not only the common development criteria but also based on how accurate the immunoassay can predict the value of the unknown sample.