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Method Development and Validation- A Review

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ABSTRACT

Analytical method development followed by method validation is an important process in the drug discovery. Although the drug shows good potency, lack of validated analytical method will not allow the drug to enter into the market. This is to ensure the quality and safety of the drug. The main objective of this review is to give an idea about the old and novel techniques available for the analysis of drugs in their raw material and formulated forms, check the stability of the drugs in the presence of the excipients and other stress conditions experienced during their shelf life period. The review work puts a light on the hyphenated techniques for the analysis and impurity profiling of drugs like LC-MS-MS, LC-NMR-MS, GC-MS and LC –MS. This review also deals with the bioanalytical method development for the quantitative determination of the drugs in the various biological matrices. It also provides a means to determine the biological safety of the drugs by dealing with the SIAMs (stability indicating assay methods).

Keyword: Validation, Stability indicating, Impurity, bioanalytical, HPLC, HPTLC.

INTRODUCTION

Quality control and quality assurance are the major areas in the pharmaceutical industry dealing with the analysis of materials starting from the raw material, intermediate products, APIS and finished products. Now and then new techniques are being developed all over the world. As a result, classical methods have changed to instrumental methods and finally to hyphenated technique. Each technique is found to be superior to the pervious technique.

Enormous effort have been put the scientists to reach this stage of the analysis. Many methods are developed for a product or a raw material to finally reach their quantity and quality. Now it becomes necessary to find which method is the most suitable and is giving the exact result. Here comes the role of validation. Method validation is the repeated analysis of the materials by the developed method to confirm the accuracy and precision of the results. Once validated the method could be used in routine analysis of the drugs. In brief the objective of this review is to put a light on the various hyphenated techniques that could be adopted for the estimation of pharmaceuticals and their validation along with the degradation studies.

USING DIFFERENT METHODS

A. STABILITY – INDICATING METHOD

Critical issues related to development of SIAMs, such as separation of all degradation products, establishment of mass balance, stress testing of formulations, development of SIAMs for combination products, etc are also addressed. The stability – indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc and separation of drug from degradation products.

Unfortunately, none of the ICH guideline provides an exact definition of a stability-indicating methodology. However guidelines are provided in the United States-Food and drug Administration (US-FDA) stability guideline of 1987 (ICH) and the draft guideline of 1998 (FDA).

The major changes brought in the new guidelines are with respect to (i) introduction of the requirements of validation, and (ii) the requirements of analysis of degradation products and other components, apart from the active ingredients(s). The literature was found to be replete with publications on developments of stability-indicating assays of specific drugs. While the current requirement is of subjecting the drug substance to variety of stress condition and then separation of drug from all degradation products, many studies have just shown the separation of drug from known synthetic impurities and /or potential degradation products without subjecting it to any type of stress (Table-1). There are also reports in which drug has been decomposed by exposing it to one (Table-2), two (Table-3)

conditions among acidic, neutral or alkaline hydrolysis, photolysis, oxidation and thermal stress. They are some reports where directly the formulation, instead of the drug substance, has been subjected to stress studies for establishment, of the stability-indicating behaviour (Table-4).

Table 1: Selected reports of stability indicating methods where no stress testing has been done

Separations	Drugs	Methodology
Separations from process impurities	Benazepril HCL	HPLC
Separations from known / potential degradation products	Canrenone Phenylbutazone Homatropine methylbromide Felodipine	HPLC HPLC UV- spectrophotometry SFC
Separations from known / potential degradation products and process impurities	Benzodiazepine Piroxicam Fenclorac Azathioprine	HPLC HPTLC GLC CE

Table 2: Selected reports of 'Stability-Indicating' methods where only one stress condition have been employed

Stress conditions	Drugs	Methodology
Acid	Dyclonine HLC Lisinopril	HPLC UV spectrophotometry
Alkali	Allantoin Benzapril Carbachol	HPLC UV IR
Neutral	Physostigmine salicylate	HPLC
Oxidation	Nortriptyline HCL	UV
Light	Atenolol Nifedipine Ranitidine HCL	HPLC HPTLC Spectrodensitometric TLC

Table 3: Selected reports of 'stability indicating' methods where five (and additional) stress conditions have been employed

Stress conditions	Drugs	Methodology
Acid, alkali, oxidation, dry heat, light	Elanapril maleate	HPLC
Acid, alkali, oxidation, dry heat, light (separation from synthetic impurities also seen)	Sildenafil citrate	HPLC
Acid, neutral, alkali, oxidation, light	Nicardipine HCL	HPLC
Acid, alkali, oxidation, dry heat, wet heat, light dry, light wet	Paroxetine	HPLC
Acid, alkali, oxidation, dry heat, light, reduction	Cyproterone acetate	HPLC
Acid, alkali, oxidation, dry heat, light, moisture, sonication	Buspirone HCL	HPLC

On critical evaluation the literature reports that titrimetric, spectrophotometric and chromatographic techniques have been commonly employed in the analysis of stability samples. In these methods, usually the objective is the analysis of the drug of interest along in the matrix of excipients, additives, degradation products and impurities etc., and also other drugs in case of the combination products. Their advantage is the low cost and simplicity, though sometimes they are not sensitive.

Chromatographic methods have taken precedence over the conventional methods of analysis. Other than separation of multiple components, the advantage of chromatographic methods is that these possess greater accuracy and sensitivity for even small quantities of degradation products produced.

HPLC has been very widely employed. It has gained popularity in stability studies due to high-resolution capacity, sensitivity and specificity. Various chromatographic methods that have been used are thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC) and gas chromatography (GC), HPLC and newer technique like capillary electrophoresis [1-2]. TLC is a simple technique that has been used in the past for developing SIAMs.

Table 4: Reports of 'stability indicating' methods on drug formulation

Stress conditions	Drugs	Dosage form	Methodology
Acid	Fluconazole	Admixtures	GC
	Flucytosine	Extemporaneous solutions	HPLC
Acid, alkali	Gancyclovir	Capsules	HPLC
Light, thermal	Sodium levothyroxine	Tablets	HPLC
Acid, alkali, oxidation	Pentoxifylline	Suspension	HPLC
Acid, alkali, oxidation, thermal	Chlorbutanol	Ointment	HPLC
Acid, alkali, oxidation, light	Fotemustine	5% dextrose	HPLC
	Efavirenz	Capsules	HPLC
Acid, light, oxidation, thermal	Fentanyl	Injection	HPLC
Acid, alkali, light, oxidation, thermal	Cyclosporine	Oral solution	HPLC
Acid, alkali, light, thermal, 45^0c /75% RH for two weeks	Aspirin and warfarin sodium	Tablets	HPLC
Aged samples (3 years at 40^0c and 75% RH	Losartan	Tablets	HPLC and LC-MS

This technique overcomes the shortcomings of TLC and is reliable. Moreover many samples can be run simultaneously using a small quantity of mobile phase thus minimising analysis time and cost per analysis.

Table 5: Validation criteria to be studied in pre-validation and validation steps

Criteria	Pre-Validation Step	Validation Step
Absolute recovery	+	+
Selectivity	(+)	(+)
Response function	(+)	+
Linearity	+	+
Accuracy	(+)	+
Precision	(+)	+
Limit of detection	+	+
Limit of quantification range		

(+): estimation of the criteria; +: validation of the criteria

GC is stability-indicating but it is not versatile as the drug substance may be non-volatile or thermally unstable. Further any attempt to increase the volatility of the drug and components by increasing the temperature may lead to degradation or racemization. Therefore, there are very few reports on GC for purpose of establishment of SIAMs. In comparison, HPLC has been very widely employed. It has gained popularity in stability studies due to high-resolution capacity, sensitivity and specificity. Non-volatile, thermally unstable or polar/ionic compounds can also be analysed by this technique. Therefore, most of the SIAMs have been established using HPLC.

A few studies have also reported the use of proton nuclear magnetic resonance (NMR) spectroscopy for the development of SIAMs. CE is the latest entry of the techniques for the development of SIAMs [3-6]. It has the advantage of high sensitivity, resolution and high efficiencies with high peak dispersion.

But the information on the basic steps to be followed for the development and validation of stability indicating methods is neither provided in the regulatory guidelines nor in pharmacopoeias. Therefore, the practical steps involved in the development of SIAMs are done by HPLC as it is found that 85-90% of the methods reported in literature.

I. Critical Study of the Drug Structure to Assess the Likely Decomposition Route (S)

Much information can simply be gained from the structure by study of functional groups and other key components. There are definite functional group categories like amides, esters, lactams, lactones that undergo hydrolysis [7], others like thiols, thio-ethers, etc. undergo oxidation [8] and compounds like olefins, aryl halo derivatives, aryl acetic acids and N-oxides undergo photodecomposition.

II. Collection of information on physiochemical properties

Before method development is taken up it is generally important to know various physiochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug in question. Knowledge of pKa is important as most of the pH- related changes in retention occur at pH values within ± 1.5 units of the PKa value. The ionisation value also helps in selecting the pH of the buffer to be used in the mobile phase.

III. Stress (forced degradation) studies

The next step in the development of SIAM is the conduct of forced degradation studies to generate degradation products of the drug. The ICH guideline Q1A suggests the following conditions to be employed: (i) 10 ^{0}C increments above the accelerated temperatures (e.g. 50 ^{0}C, 60 ^{0}C, etc.), (ii) humidity where appropriate (e.g. 75% or greater), (iii) hydrolysis across a wide range of pH values, (iv) oxidation and (v) photolysis. The hydrolytic degradation of a new drug in acidic and alkaline conditions can be studied by refluxing the drug in 0.1 N HCL/NaOH for 8h. In a similar manner, degradation under neutral conditions can be started by refluxing the drug in water for 12 hrs. Reflux time should be increased if no degradation is seen. If the drug is found to degrade completely, both time and temperature of study can be decreased. To test for oxidation, it is suggested to use hydrogen peroxide in the concentration range of 3-30%. The photolytic studies can be carried out by exposure to light, using either a combination of cool white and fluorescent lamps, or one among the xenon and metal halide lamps. Exposure energy should be minimum of 1.2 million lux h fluorescent light and 200W h/m^2 UV and if decomposition is not seen, the intensity should be increased by five times. In case no decomposition still takes place the drug can be declared as photo stable.

The blank subjected to stress in the same manner as the drug solution, zero time sample containing the drug which under normal conditions and drug solution subjected to stress treatment. The comparison of results of these provides real assessment of changes.

IV. Preliminary separation studies on stressed samples

The samples so obtained are subjected to preliminary analyses to study the number and types of degradation products formed. For doing so, the simplest way is to start with a reversed-phase octadecyl column. It should be preferred to use water-methanol or water-acetonitrile as the mobile in initial stage. The solvent can be changed if the peak shape or separation problems are seen. Initially water : organic modifier ratio can be fixed at 50 : 50 ratio or can be suitably modified so as to obtain the capacity factor of around 5-10 for the drug. As degradation products from the drugs are generally polar in nature (of course with exceptions) pushing the drug peak to say ~ 15 min or somewhat more in a 25 cm column, can result in separation of even several degradation products, when formed. Normally, the total run time should be 2.5 times more than the drug peak. The detection wavelength can be set based on the study of spectral behaviour of degraded samples.

V. Final method development and optimization

Subsequent to preliminary chromatographic studies, the retention time and relative retention times (RRT) of all products formed should be tabulated for each retention condition. Special attention is then paid to those components whose RT or RRT is very close. It was later established that the drug was almost instantly converted when brought into contact with alkali and the product was formed quantitatively. Therefore, if PDA or LC-MS results suggest that any of the products are different but are co-eluting then suitable identification should be done in the chromatographic method to achieve a satisfactory resolution. To separate close or co-eluted peaks the method is optimised by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type and the column and its type.

VI. Identification and characterization of degradation products and preparation of standards

Before moving to the validation of a SIAM it is necessary to identify the drug degradation products and arrange for their standards. These are required to establish

specificity/selectivity of the method. To identify the resolved products a conventional way is to isolate them and determine the structure through spectral (MS, NMR, IR, etc.) and elemental analysis. The modern approach is to use hyphenated LC techniques coupled with spectrometry. This strategy integrates in a single instrument approach, analytical HPLC, UC detection, full scan mass spectrometry (LC-MS) and provides a fair idea on identity of resolving components. To isolate a product the best way is to identify a reaction condition where it is formed selectively. If the product precipitates or crystallizes on its own on completion of the reaction it can be recovered simply. Otherwise the reaction mixture can be lyophilized directly. If freeze drying is done directly after neutralization of the reaction mixture the product can be recovered by extraction with dry methanol or any other dry solvent. The recovery can also be made by selective extraction with an organic solvent after acidification, neutralization or basification of the solution, depending upon the initial pH. Subsequently the extraction can be evaporated to recover the product.

VII. Validation of SIAMS

There are two stages in the validation of a SIAM. First stage is early in the development cycle when drug substance is subjected to forced degradation studies and the SIAM is established based on the knowledge of drug degradation behaviour. The main focus of validation at this stage is on establishment of specificity / selectivity, followed by other parameters like accuracy, precision, linearity, range and robustness etc, the limits of detection and quantitation are also determined for degradation products to help in establishment of the mass balance. In the second stage, when the SIAM so developed is extended to formulations or other matrices, the emphasis gets limited to just prove the pertinence of the established validation parameters in the presence of excipients or other formulation constituents. Here only selectivity, accuracy and precision are revalidated. If the SIAM is being developed directly for a formulation, without involving the bulk drug route, then all validation parameters are necessary to be established. However, a better method of determining accuracy of SIAM is by spiking the drug in the mixture of degraded solutions. The linearity for SIAMs should be established initially in the range of 0-100%, as the drug may fall to very low concentrations during forced decomposition studies. The range may be 50-120% in case of injections or other formulations where the drug is prone to degradation. Validation range for degradation products during stability studies usually should vary from 0-20%.

B. Impurity studies – method [9-10]

Drugs play a vital role in the progress of human civilization by curing diseases. Today a majority of the drugs used are of synthetic origin. These are produced in bulk and used for their therapeutic effects in pharmaceutical formulations. These are biologically active chemical substances generally formulated into convenient dosage forms such as tablets, capsules, suspensions, ointments and injectable. These formulations deliver the drug substance in a stable, non-toxic and acceptable form, ensuring its bioavailability and therapeutic activity. Safety and efficacy of pharmaceutical are two fundamental issues of importance in drug therapy. The safety of a drug is determined by its pharmacological – toxicological profile as well as the adverse effects caused by the impurities in bulk and dosage forms. The impurities in drugs often possess unwanted pharmacological or toxicological effects by which any benefits from their administration may be outweighed. The quality and safety of a drug is generally assured by monitoring and controlling the impurities effectively. Impurity control is more important. In the beginning of 20th century, drug products were produced and sold having no imposed control. The impurities to be considered for new drugs are listed in regulatory documents of the food and drug administration, International Conference on the Harmonization of the technical requirements for registration of pharmaceuticals for human uses and United States of Pharmacopoeia. The USP and National Formulary (NF) are the recognised standards for potency and purity of new drugs. The ICH which took place in Yokohama, Japan in 1995 has released new guidelines on impurities in new drug products. These guidelines have a number of advantages, both for the industry and the regulators. The most critical aspect of the elaboration of the guidelines was the identification and qualification. Analytical procedures should be able to separate all the impurities from each other and the method should be optimized to separate and quantify them in the dosage forms. Such methods are to be validated demonstrating the accuracy, precision, specificity, limit of detection, quantification, linearity, ranges and interferences. The ICH has harmonized the requirements in two guidelines. The first one summarizes and defines the validation characteristics needed for various types of test procedures, the second one extends the previous text to include the experimental data required and some statistical interpretation.

Qualification of the impurities is the process of acquiring and evaluating data that establishes biological safety of an individual impurity. Identification of impurities is done by

variety of Chromatographic and Spectroscopic techniques, either alone or in combination with other techniques. There are different methods for detecting and characterizing impurities like TLC, HPLC, HPTLC and AAS etc. Conventional liquid chromatography, particularly HPLC, has been exploited widely in the field of impurity profiling; the wide range of detectors and stationary phases along with its sensitivity and cost-effective separation have attributed to its varied applications. TLC is the most commonly used separation technique for isolation of impurities due to its ease of operation and low cost compared to HPLC. An advancement of thin layer chromatography-HPTLC is a well-known technique for the impurity isolation. Number of articles have stated guidelines and designed approaches for isolation and identification of process-related impurities and degradation products, using Mass spectrometry (MS), Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography (HPLC), Fourier Transform Ion Cyclotron Resonance Mass Spectrometry [11-13].Impurity profile is the description of identified and unidentified impurities present in new drug substances.

Origin of impurities

Impurities in drugs are originated from various sources and phases of the synthetic process and preparation of pharmaceutical dosage forms. A sharp difference between the process related impurities and degradation products is always not possible. The majority of the impurities are characteristic of the synthetic route of the manufacturing process. Since there are several possibilities of synthesizing a drug, it is possible that the same product of different sources may give rise to different impurities.

Types of impurities

The United States Pharmacopoeia (USP) classifies impurities in various sections;

1).Organic impurities (process and drug related) 2).Inorganic impurities 3).Residual solvents.

Organic Impurity

Organic impurities may arise during the manufacturing process or storage of the drug substance may be identified or unidentified, volatile or non-volatile, and may include; 1. Starting materials or intermediates 2. By-products 3. Degradation products.

100% yield is very rare; there is always a chance of having by-products. Impurities can also be formed by degradation of the end product during manufacturing of the bulk drugs.

Inorganic Impurity [14]

Inorganic impurities may also be delivered from the manufacturing processes used for bulk drugs. They are normally known and identified and include the following

Reagents, Ligands and Catalysts

The chances of having these impurities are rare; however in some processes, these could create a problem, unless the manufactures take proper care during production.

Heavy metals

The main sources of heavy metals are the water used in the processes and the reactors where acidification or acid hydrolysis takes place. These impurities of heavy metals can easily be avoided using demineralised water and glass-lined reaction.

In process production Impurities

Impurities can originate from several sources, such as, a) Crystallization-related impurities b)Stereochemistry-related impurities c) Synthetic intermediates and by-products d) Formulation-related impurities e) Impurities arising during storage f) Method related impurity g) Mutual interaction amongst ingredients h) Functional group-related typical degradation.

(a) Crystallization-related impurities

Based on the realization that the nature of structure adopted by a given compound upon crystallization could exert a profound effect on the solid-state properties of that system, the pharmaceutical industry is required to take a strong interest in polymorphism and solvato-morphism. Polymorphism is the term used to indicate crystal system where substances can exist in different crystal packing arrangements, all of which have the same elemental composition [15].

(b) Stereochemistry-related impurities

It is of paramount importance to look for stereochemistry related compounds; that is, those compounds that have similar chemical structure but different spatial orientation, these compounds can be considered as impurities in the API's. Chiral molecules are frequently called enantiomers. The single enantiomeric form of chiral drug is now considered as an improved chemical entity that may offer a better pharmacological profile and an increased therapeutic index with a more favorable adverse reaction profile.

(c) Synthetic intermediates and by-products

Impurities in pharmaceutical compounds or a new chemical entity (NCE) can originate during the synthetic process from raw materials, intermediates and /or by-products. Impurity profiling of tablets by GC-MS and MDMA (3,4-Methylene dioxy methamphetamine) samples produced impurities in the intermediates via the reductive amination route [16].

(d) Formulation-related impurities

Many impurities in a drug product can originate from excipients used to formulate a drug marginal product, unacceptable for reliability. Solutions and suspensions are inherently prone substance. If the source is from an excipient, variability from lot-lot may make a to degradation due to hydrolysis or solvolysis [17]. In general, liquid dosage forms are susceptible to both degradation and microbiological contamination. Microbiological growth resulting from the growth of bacteria, fungi, and yeast in a humid and warm environment may result in unsuitability of an oral liquid product for safe human consumption.

(e) Impurities arising during storage A number of impurities can originate during storage or shipment of drug products. It is essential to carry out stability studies to predict, evaluate, and ensure drug product safety [18].

(f) Method related impurity

A known impurity, 1-(2, 6-dichlorophenyl) indolin-2-one is formed in the production of a parenteral dosage form of diclofenac sodium if it is terminally sterilized by autoclave [19]. The conditions of the autoclave method (i.e., 123 + 2^{o}C) enforce the intramolecular cyclic

reaction of diclofenac sodium forming an indolinone derivative and sodium hydroxide. The formation of this impurity has been found to depend on initial pH of the formulation.

(g) Mutual interaction amongst ingredients

Most vitamins are very labile and on aging they create a problem of instability in different dosage forms especially in liquid dosage forms. Degradation of vitamins does not give toxic impurities; however potency of active ingredients drops below Pharmacopoeial specifications. Because of mutual interaction, the presence of nicotinamide in a formulation containing four vitamins (nicotinamide, pyridoxine, riboflavin, and thiamine) can cause the degradation of thiamine to a sub-standard level within a one year shelf life of vitamin B-complex injections.

(h) Functional group-related typical degradation

Ester hydrolysis can be explained with a few drugs like aspirin, benzocaine, cefotaxime, ethyl paraben and cefpodoxime proxetil [20-21]. Hydrolysis is the common phenomenon for ester type of drugs, especially in liquid dosage forms like benzyl penicillin, oxazepam and lincomycin.

Oxidative degradation of drugs like hydrocortisone, methotrexate, hydroxyl group directly bonded to an aromatic ring (like phenol derivatives such as catecholamines and morphine), conjugated dienes (like vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (especially flavorings) are all susceptible to oxidative degradation.

Photolytic cleavage includes example of pharmaceutical products that are exposed to light while being manufactured as solid or solution, packaged, or when being stored in pharmacy shops or hospitals for use by consumers. In ciprofloxacin eye drop preparation (0.3%), sunlight induces photo cleavage reaction producing ethylenediamine analog of ciprofloxacin. As seen earlier, impurities in drug products can come from the drug or from excipients or can be brought into the system through an in process step by contact with the packaging material. For most drugs, the reactive species consist of, Water- that can hydrolyze some drugs or affect the dosage form performance, Small electrophiles- like aldehydes and carboxylic acid derivatives, Peroxides- that can oxidize some drugs, Metals-

which can catalyze oxidation of drugs and the degradation pathway and leachable or extractable- can come from glass, rubber stoppers, and plastic packaging materials. Metal oxides such as NaO_2, SiO_2, CaO, MgO, are the major components leached/extracted from glass [22]. Generally most synthetic materials contain leachable oligomers/monomers, vulcanizing agents, accelerators, plasticizers, and antioxidants [23].

Residual solvents

Residual solvents are organic volatile chemicals used during the manufacturing process or generated during the production. Some solvents that are known to cause toxicity should be avoided in the production of bulk drugs. Depending on the possible risk to human health, residual solvents are divided into three classes [24]. Especially solvents in Class I like benzene (2 ppm limit), carbon tetrachloride (4 ppm limit), 1,2-dichloroethane (5 ppm), 1,1-dichloroethane (8 ppm) and 1,1,1-trichloroethane (1500 ppm) should be avoided. In Class II, solvents like N, N-dimethylformamide (880 ppm), acetonitrile (410 ppm) etc should be limited. Class III solvents, like acetic acid, ethanol and acetone have permitted daily exposure of 50 mg or less per day as per the ICH guidelines and are classified as solvents with low toxic potential. Using this method, the main contaminants of each organic solvent can be quantified. Moreover, the developed method allows the simultaneous determination of ethanol, isopropanol, chloroform, benzene, acetone, dichloromethane, methanol and toluene with propionitrile as the internal standard.

ICH limits for Impurities

According to ICH guidelines on impurities in new drug products, identification of impurities below 0.1% level is not considered to be necessary, unless potential impurities are expected to be unusually potent or toxic. According to ICH, the maximum daily dose qualification threshold to be considered is as follows; < 2g/day 0.1 % or 1 mg per day intake (whichever is lower) >2g/day 0.05%.

Analytical method Development

New drug development requires meaningful and reliable analytical data to be produced at various stages of the development [25-26].

a) Sample set selection for analytical method development

b) Screening of Chromatographic conditions and Phases, typically using the linear-solvent-strength model of gradient elution.
c) Optimization of the method to fine-tune parameters related to ruggedness and robustness

The impurities can be identified predominantly by following methods are: 1.Reference standard method 2.Spectroscopic method 3.Separation method 4.Isolation method and Characterization method

Reference standard method

Reference standard serves as the basis of evaluation of both process and product performance and is the benchmark for assessment of drug safety for patient consumption. These standards are needed, not only for the active ingredients in dosage forms but also for impurities, degradation products, starting materials, process intermediates, and excipients.

Spectroscopic methods

The UV, IR, MS, NMR and Raman spectroscopic methods are routinely being used for characterizing impurities.

Separation methods

The Capillary electrophoresis (CE), Chiral Separations, Gas Chromatography (GC), Supercritical Fluid Chromatography (SFC), TLC, HPTLC and HPLC are regularly being used for separation of impurities and degradation products.

Isolation methods

It is often necessary to isolate impurities. But if the instrumental methods are used isolation of impurities is avoided as it directly characterizes the impurities. Methods that can be used for isolation of impurities are Solid-phase extraction method, Liquid-liquid extraction method, Accelerated solvent extraction method, Supercritical fluid extraction, Column chromatography, Flash chromatography, TLC, GC, HPLC, HPTLC, Capillary electrophoresis (CE) and Supercritical fluid chromatography (SFC). High performance chromatography and the chromatographic reactor approach, with solution phase hydrolysis kinetics can be used

for an Aprepitant prodrug and fosaprepitant dimeglumine. In loratidine the impurity was found to be ofloratidine and other examples include celecixib and amikacin [27-30].

Characterization methods

Highly sophisticated instrumentation, such as MS attached to a GC or HPLC, are inevitable tools in the identification of minor components (drugs, impurities, degradation products, metabolites) in various matrices. For characterization of impurities, different techniques are used; which are as follows;

NMR Spectroscopy

The ability of NMR to provide information regarding the specific bonding structure and stereochemistry of molecules of pharmaceutical interest has made it a powerful analytical instrument for structural elucidation. The ability of NMR-based diffusion coefficient determination to distinguish between monomeric and dimeric substances was validated using a standard mixture of authentic materials containing both monomers and dimers[31]. Unfortunately, NMR has traditionally been used as a less sensitive method compared to other analytical techniques. Conventional sample requirements for NMR are on the order of 10 mg as compared with MS which requires less than 1 mg [31].

Mass spectroscopy

It has an increasingly significant impact on the pharmaceutical development process over the past several decades. Advances in the design and efficiency of the interfaces that directly connect separation techniques with Mass Spectrometers have afforded new opportunities for monitoring, characterizing and quantification of drug-related substances in active pharmaceutical ingredients and pharmaceutical formulations. If single method fails to provide the necessary selectivity, orthogonal coupling of chromatographic techniques such as HPLC-TLC and HPLC-CE, or coupling of chromatographic separations with information rich spectroscopic methods such as HPLC-MS or HPLC-NMR may need to be contemplated but hopefully only as a development tool rather than a tool for routine QC use.

1. HPLC-DAD-MS
2. HPLC-DAD-NMR-MS
3. GC-MS
4. LC-MS

An example of reverse-phase LC-MS analysis in gradient elution with two distinct soft ionization techniques is the Atmospheric pressure ionization with electrospray source (API-ESI). A common goal for investigation of both process and product degradation-related impurities is to determine which of the many potential impurities are, in fact, produced in the manufacturing process and which occur under a given set of storage conditions.

Applications

Numerous applications have been sought in the areas of drug designing and in monitoring quality, stability and safety of pharmaceutical compounds, whether produced synthetically extracted from natural products or produced by recombinant methods. The applications include alkaloids, amines, amino acids, analgesics, antibacterial, anticonvulsants, antidepressant, tranquilizers, antineoplastic agents, local anesthetics, macromolecules, steroids and miscellaneous.

C. Bio-Analytical method

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites are crucial for the successful conduct of preclinical and/or biopharmaceuticals and clinical pharmacology studies. Bio analytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine is reliable and reproducible for the intended use.

The first bioanalytical method workshop was conducted in 1990. It dedicated to investigating and harmonizing procedures varied in method validation. The 1st workshop clearly identified 2 distinct phases of bioanalytical method validation [32].

1. Analytical method development (pre study validation) where the appropriate bio analytical method with its various parameters is developed and the assay is defined; and 2) application of the bio analytical to actual analysis of samples from bioavailability, bio equivalence and pharmacokinetic studies. One of the most important outcomes of the first workshop was that it defined the acceptance criteria for a run. The first workshop was well received within the global pharmacokinetic community. They needed to validate bio analytical methods and the development and acceptance of general standards for their

conduct, brought about significant improvement in the quality of bio analytical methods and in the submission of pharmacokinetic, bioavailability and bio equivalence studies to .the workshop report was not an official document of the FDA. Therefore the agent decided to develop and publish draft guidance in 1999.

The IInd workshop, cosponsored by AAPS and the FDA, was held in Jan 2000, one year after the publication of the draft guidance by the agency. The workshop focused on discussing the advances in analytical technology that had occurred over the part dread and reconfirmed and updated the principles of bio analytical method validation. The second workshop discussed the advances in hyphenated mass spectrometry and ligand binding assays and it also discussed different categories of validation. There are partial validation, cross validation and full validation. The workshop reemphasized that it is not necessary to have 100% recovery where using an extraction procedure. The following principles of bioanalytical method validation provide steps for the development of a new method or for establishing an existing method. The parameters essential to ensure the acceptability of the performance of a bio analytical method are accuracy, precision, selectivity, sensitivity, reproducibility and stability. The guidance provides information for determining the parameters. The guidance also establishes requirements for a standard curve. The matrix based standard curve should consists of a maximum of five standard points, excluding blanks, using single or replicate samples, and should cover the entire range of expected concentration. All these parameters used to be defined during the full validation of a bio analytical method. The (LLOQ) should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection (LOD). There are two distinct phases of bio analytical method validation. (a).the bioanalytical method development phase in which the assay is defined and validated. (b). the application to actual analysis of samples from pharmacokinetic, bioavailability, bioequivalence and drug interaction studies.

As bioanalytical tools and techniques have continued to evolve, significant scientific and regulatory experience has been gained since the last workshop in 2000 and issuance of the guidance in 2001.The evolution and expansion of bioanalytical tools require a critical review of the scope, applicability, and success of the presently employed bioanalytical guiding principles. The third work shop in this series was held in May 2000 in Arlington USA. The purpose of this 3rd AAPS/FDA Bioanalytical Workshop was to identify, review and

evaluate the existing practices, white papers, and articles clarify the FDA Guidance. The workshop addressed quantitative bioanalytical methods validation and their use in sample analysis, focusing on both chromatographic and ligand-binding assays.

The purpose of the 3rd AAPS/FDA Bioanalytical Workshop was to [33]

1. Review the scope and applicability and bioanalytical principles and procedures for the quantitative analysis of samples from bioequivalence, pharmacokinetic and comparability studies in both human and non human subjects.
2. Review current practice for scientific excellence and regulating compliance, suggesting classification and improvements where needed.
3. Review and evaluate validation implementation requirements for chromatographic ligand bound. Quantitative bio analytical assays, covering all types (sizes) of molecules.
4. Review recent advances in technology, automation regulatory and scientific requirements and data achieving on the performance and exporting of quantitative bio analytical work and discuss current based approaches for the conduct of quantitative bio analytical work regardless of the size of the molecule analysed.

In these guidelines are used to various essential development and validation characteristics for bio analytical methodology have been discussed with a view to improve the standard and acceptance in this area of research. Characterization of the stability of analytes in biological samples collected during clinical studies together with that of critical assay reagents including analyte stock solutions is recognized as an important component of bio analytical assay validation. The information in this guideline generally applies to bio analytical procedures such as [34-39] Gas chromatography, High- pressure liquid chromatography, Combined GC and LC mass spectrometric, LC-MS, LC-MS-MS and GC-MS
These procedures are generally performed for the quantitative determination of drugs and/or metabolites in biological matrices such as blood, serum, plasma and urine. This guideline also applies to other bio analytical methods, such as immunological and microbiological procedures and to other biological matrices, such as tissue and skin samples. This guideline provides general recommendations for bio analytical method validation. It is essential to employ well characterized and fully validated bio analytical methods to yield reliable results that can be satisfactorily interpreted. It is also important to emphasize that each bio analytical technique has its own characteristics which will vary

from analyte to analyte in these instances, specific validation criteria may need to be developed for each analyte. When sample analysis for a given study is conducted at more than one site, it is necessary to validate the bio analytical method at each site and provide appropriate validation information for different sites to establish inter laboratory reliability. For quantitative bioanalytical method validation procedure and requirements, there was a relatively good agreement between chromatographic assays and ligand-binding assays. It was realized that the quantitative and qualitative aspects of bioanalytical method validation should be reviewed and applied appropriately.

Bio analytical method development and validation

The process by which a specific bio analytical method is developed, validated and used in routine sample analysis can be divided into 1.Reference standard 2.Bio analytical method development and establishment of assay procedure 3.Application of validated bio analytical method to routine drug analysis and acceptance criteria for the analytical run and/or batch.

Reference standard

Analysis of drugs and their metabolites in a biological matrix is carried out using samples spiked with calibration standards and using quality control samples.

Three types of reference standards are usually used. They are:

1. Certified reference standards
2. Commercially supplied reference standard applied from a reputable commercial source
3. Other materials of documented purity custom-synthesized by an analytical laboratory.

Bio analytical method development

Typical method development and establishment for a bio analytical method include determination of selectivity, sensitivity, accuracy, precision, stability of analyte in spiked samples.

A) Selectivity

It is the analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analysis of blank samples of the appropriate

biological matrix should be obtained from at least six sources. Each blank sample should be tested for interference and selectivity, should be ensured at the lower limit of quantification.

B) Accuracy, precision, and recovery[40]

Accuracy

Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured by minimum of five determinations per concentration. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as a measure of accuracy.

Precision

It should be measured using a minimum of five determinations per concentration. The precision determined at each concentration level should not exceed 15% of the coefficient of variation except for LLOQ where it should not exceed 20% of the CV.

Recovery

The recovery of analyte in the assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix compared to the detector response obtained from the true concentration of the pure authentic standard. Recovery of the analyte need not to be 100%, but the extent of recovery of an analyte and of the internal standard should be precise and reproducible.

Calibration/ standard curve

A calibration curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte.

Concentrations of the standard should be chosen on the basis of concentration range expected in a particular study. A calibration curve should consist of i) A blank sample ii) A zero sample iii) Six to eight non-zero samples covering the expected range, including LLOQ.

Lower limit of quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met 1).The analyte response at the LLOQ should be at least 5 times the response compared to blank response.11).Analyte peak should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80-120%

Calibration curve/standard curve-concentration-response.

The simplest model that adequately describes the concentration-response relationship should be used. The following conditions should be met in developing a calibration curve 1). 20% deviation of the LLOQ from normal concentration. 2).15% deviation of the standard other than LLOQ from normal concentration.

Stability in a biological fluid

The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long term (frozen at the intended storage temperature) and short term (bench top, room temperature) storage, after going through freeze and thaw cycles and the analytical process. All stability determinations should use asset of samples prepared from a freshly made stock solution of the analyte in the appropriate –analyte free, interference free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

Freeze and Thaw stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24hrs and thawed unassisted at room temperature. When completely thawed the samples should be re-frozen for 12-24hrs under the same conditions. The freeze-thaw cycle should be repeated two more times then analysed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70^{0}c during the three freeze and thaw cycles.

Short-Term Temperature stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4-24 hrs (based on the expected duration that samples will be maintained at room temperature in the intended study) and analysed.

Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long term stability testing.

Stock solution stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hrs. If the stock solutions are refrigerated or frozen for the relevant period, the ability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

Post-preparative stability

The stability of processed samples, including the resident time in the auto-sampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards. Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of analyte stability in a biological matrix can be used. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

Bioanalytical method Validation [41]

Pre-validation study

Before going for the actual validation process so called pre-validation is done. In pre-validation study large numbers of replicate spiked matrix samples are analyzed in single batch before a method validation is started. The utility of this pre-validation approach is illustrated using actual laboratory data. The process of interpreting the results and drawing conclusions about assay viability is demonstrated. The resulting conclusions provide sufficient background information to indicate if an assay is ready to enter the validation process. Following validation criteria is studied in pre-validation and validation steps. (Table-5)

Full validation

1. Full validation is important when developing and implementing a bio analytical method for the first time.
2. Full validation is important for a new drug entity.
3. A full validation of the revised assay is important if metabolites are added to an existing assay for quantification.

Partial validation

Partial validations are modifications of already validated bio analytical methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation. Typical bio analytical method changes that fall into this category include, but are not limited to: 1.Bio analytical method transfers between laboratories or analysts 2.Change in anticoagulant in harvesting biological fluid.3.Change in analytical methodology (e.g., change in detection systems).4.Change in matrix within species (e.g., human plasma to human urine).5.Change in sampling processing procedures.6. Change in species within matrix (e.g., rat plasma to mouse plasma) 7. Change in relevant concentration range 8. Changes in instruments and/ or software platforms 9. Limited sample volume (e.g., pediatric study) 10. Selectivity demonstration of an analyte in the presence of specific metabolites.

Cross-validation

Cross-validation is a comparison of validation parameters when two or more bio analytical methods are used to generate data within the same study or across different studies. An example of cross validation would be a situation where an original validated bio analytical method serves as the reference and the revised bio analytical method is the comparator. The comparisons should be done both ways. When sample analyses within in a single study are conducted at more than one site or more than one laboratory, cross validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter laboratory reliability. Cross validation should also be considered when data generated using different analytical techniques (e.g., LC-MS-MS vs. ELISA) in different studies are included in a regulatory submission.

Documentation

The validation of an analytical method should be established and verified by laboratory studies, and documentation of successful completion of such studies should be provided in the assay validation report. General and specific SOPs and good record keeping are an essential part of a validated analytical method. The data generated for bio analytical method establishment and the QCs should be documented and available for data audit and inspection. Documentation for submission to the agency should include:

1. Summary information
2. Method development and establishment
3. Bio analytical reports of the application of methods to routine sample analysis
4. Other information applicable to method development and establishment and/ or to routine sample analysis.

Documentation for method establishment

Documentation for method development and establishment should include:

1. An operational description of the analytical method 2.Evidence of purity and identity of drug standards, metabolite standards, and internal standards used in validation experiments 3.A description of stability studies and supporting data 4.A description of experiments conducted to determine accuracy, precision, recovery, selectivity, limit of quantification, calibration curve (equations and weighing functions used, if any) and

relevant data obtained from these studies 5.Documentation of intra- and inter-assay precision and accuracy.

Application of validated method to routine drug analysis

1. Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability data are available. In general, biological samples are analysed with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defined by validation data. The following recommendations should be noted in applying a bio-analytical method to routine drug analysis
2. Response Function: Typically, the same curve fitting, weighing, and goodness, of fit determined during restudy validation should be used for the standard curve within the study. Response function is determined by appropriate statistical tests based on actual standard points during each run in the validation. Changes in the response function relationship between pre-study validation and routine run validation indicate potential problems.
3. The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
4. System Suitability
5. Based on the analyte and technique, a specific SOP (or sample) should be identified to ensure optimum operation of the system used.

CONCLUSION

Analytical methodology provides to an analyst the required data for a given analytical problem, sensitivity, accuracy, range of analysis, precision i.e. the minimum requirements which essentially are the specifications of the method for the intended purpose to be able to analyse the desired analyte in different matrices with surety and certainty. Analytical methods need to be validated before their introduction into routine use; whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix); and whenever the method is changed, the change is outside the original scope of the method. The stability indicating assays have been developed for a large number of drugs but most of them fail to meet current regulatory requirements of separation and analysis of individual degradation

products. So the discussion provided would be general and of wide use. Nowadays, it is a mandatory requirement in various pharmacopoeias to know the impurities present in API's.

Isolation and characterization of impurities are required for acquiring and evaluating data that establishes biological safety which reveals the need and scope of impurity profiling of drugs in pharmaceutical research. The aim of this article is to provide a simple way to use approaches with a correct scientific background to improve the quality of the bioanalytical method development and validation. Applications of bio analytical method in routine drug analysis are also taken into consideration in this article. Method development involves a series of simple steps. All the conditions are optimized as required for the purpose of the separation and the method is validated using ICH guidelines. The validated method and data can then be documented.

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