Chapter 1 Introduction

1. INTRODUCTION

1.1 Novel analytical method development and validation

In pharmaceutical industry, analytical method development and validation are the constant and interrelated tasks associated with the research and development, quality control and quality assurance departments. [1] As, many novel drugs and combinations are introduced into the market, the analytical methods needed should also be novel, innovative and robust to analyze these novel formulations. Many pharmaceutical products are formulated as combination products to meet previously unmet patient needs by combining the therapeutic effects of multiple drugs in one product. These products can present dismaying challenges to the analyst responsible for the development and validation of analytical methods. [2] Novel analytical approaches for method development aid in overcoming these challenges. Analytical methods play a crucial role in risk assessment and management. They aid in laying scientific acceptance criteria for certain parameters as well deploying certain handling conditions based on stability of pharmaceuticals. [3] The chromatographic methods (HPLC, UPLC, GLC, GC-MS/MS, LC-NMR and Liquid chromatography– mass spectrometry are the available choices for assay involving sophisticated equipment, which are highly sensitive, accurate and consume very tiny amount of samples for analysis. [4] Drug analysis therefore reveals the identification, characterization & determination of the drugs in mixtures like dosage forms & also in biological fluids. [5] During manufacturing process and drug development the main purpose of analytical methods is to provide information about potency (which can be directly related to the requirement of a known dose), impurity (related to safety profile of the drug), bioavailability, stability and effect of manufacturing parameters to ensure that the production of drug products is consistent. [6]

Spectrophotometric along with multi-component analysis can be applied where spectra of drugs overlaps. Analysts frequently encounter a situation where concentration of one or more substances is required in samples which are potentially interfered with the assay of analyte.[7] Number of modifications to the simple spectrophotometric procedure are available which may eliminate certain sources of the interference and permit the accurate determination of one or all of the absorbing components. For the assay of substance in Multicomponent samples, some methods are routinely being used. [8] They are Simultaneous equation method (Vierodt), Absorbance ratio method (Q Ratio Method) and Absorbance correction method. Difficult Multicomponent combinations can be analyzed using Difference spectrophotometry, Derivative spectrophotometric method, Ratio Spectra derivative method, Mean centering of ratio spectra method, Dual wavelength method, Chemical derivatization method, Area under curve method, Multi component mode of analysis, Orthogonal analysis, Geometric correction, Chemometrics techniques like Classical least squares, inverse least squares, partial least squares, principal component regression etc.

However, most of the drugs in Multicomponent dosage forms can be easily analyzed by HPLC method because of its several advantages like rapidity, specificity, accuracy, precision and ease of automation in it. HPLC method generally eliminates tedious extraction and isolation procedures. There are different modes of separation in HPLC.

They are normal phase mode, reversed phase mode, reverse phase ion pair chromatography, affinity chromatography and size exclusion chromatograph (gel permeation and gel filtration chromatography). [8] Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used in LC studies include octa decyl silane (ODS) or C_{18}, C_8, C_4, etc., (in the order of increasing polarity of the stationary phase). [9]

The analytical technique are employed to assure the quality medicine, thus it is necessary that the outcomes are reliable. [10] Validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions. The objective of validation of analytical procedure is itself to demonstrate that it is suitable for its intended purpose. Validation is documented evidence, which provide a high degree of assurance for specific method. Any developed method may be influenced by variables like different elapsed assay times, different days, reagents lots, instruments, equipments, environmental conditions like temperature, etc so it is expected that after the method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests are reported. For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH), United States Food and Drug Administration (USFDA), American Association of Official Analytical Chemists (AOAC), United States Pharmacopoeia (USP), and International Union of Pure and Applied Chemists (IUPAC) provide a framework for performing such validations in a more efficient and productive manner. Typical validation characteristics which should be considered include Accuracy, Precision, Repeatability, Intermediate Precision, Specificity, Detection Limit, Quantitation Limit, Linearity and Range.

Along with novelty in analytical methods, analysts many times also come across with novel samples which would altogether require divergent thinking and methods for their estimation. Illustration can be taken of herbal formulations with a particular herbal marker of choice along with various other chemical constituents. In such cases for analytical method development, first the parameters to be taken needs screening. CNX approach acts as a best option for this purpose. Based on the parameters thus screened, then DOE can be applied on the selected factors.

For some life threatening ailments like cancer, AIDS, drug authorities often give super fast approval for the orphan drugs which may have low therapeutic window. In such cases, the reliability of assay and accuracy of the developed analytical method becomes super important. Thus for checking it total error approach along with % risk assessment

can be undertaken. Validated analytical methods are directly related to the finest optimal calibration curves gotten for the analyte. Thus, normal distribution of data in calibration curve is equally important. This can be achieved by conducting statistical analysis for checking normal distribution of data.

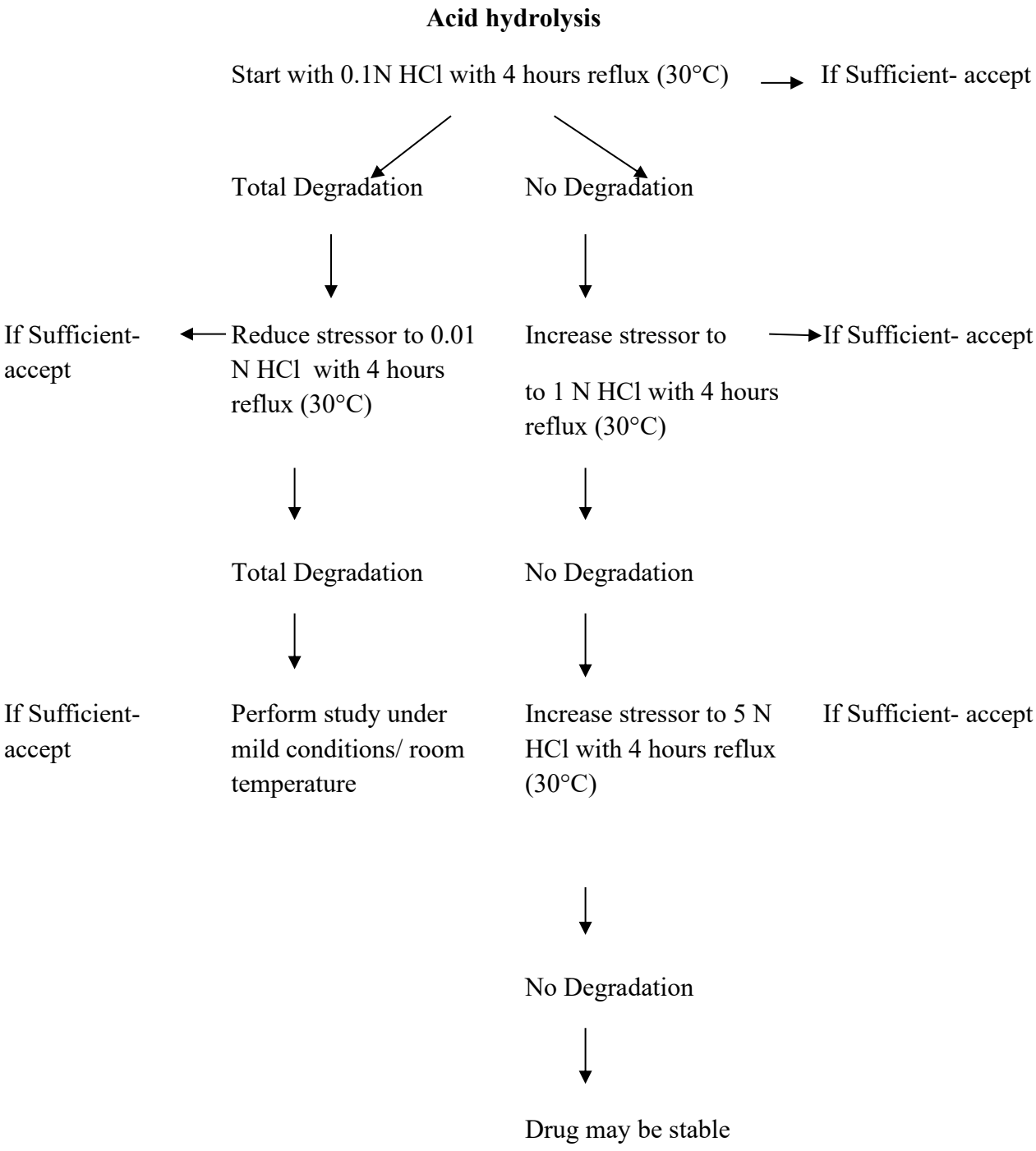
Utilizing the state of art chromatographic and spectroscopic techniques, counterfeiting as well as adulteration of pharmaceuticals can also be checked. [14] Even the elementary spectroscopic techniques like colorimetric on symbiosis with ultramodern sensors, a scope of new array of diagnostics as well as analytics can flourish. The sensors thus mentioned can be fabricated reckoning on numerous principles like with polymers modification, laser cutting as well as chromatography papers. Also the analysis of samples on such developed microfluidics can be based on numerous principles like electrochemical detectors, immunoassays as well as colorimetric assays. Thus novelty can explore in diverse directions. For pharmaceuticals also due to huge variations in the samples stumbled by analytical chemists, continuous upgrading prevails for development of newer and novel analytical methods. This task encompasses several principles and thus multidisciplinary focus is to be considered by the researchers to achieve optimized solution.

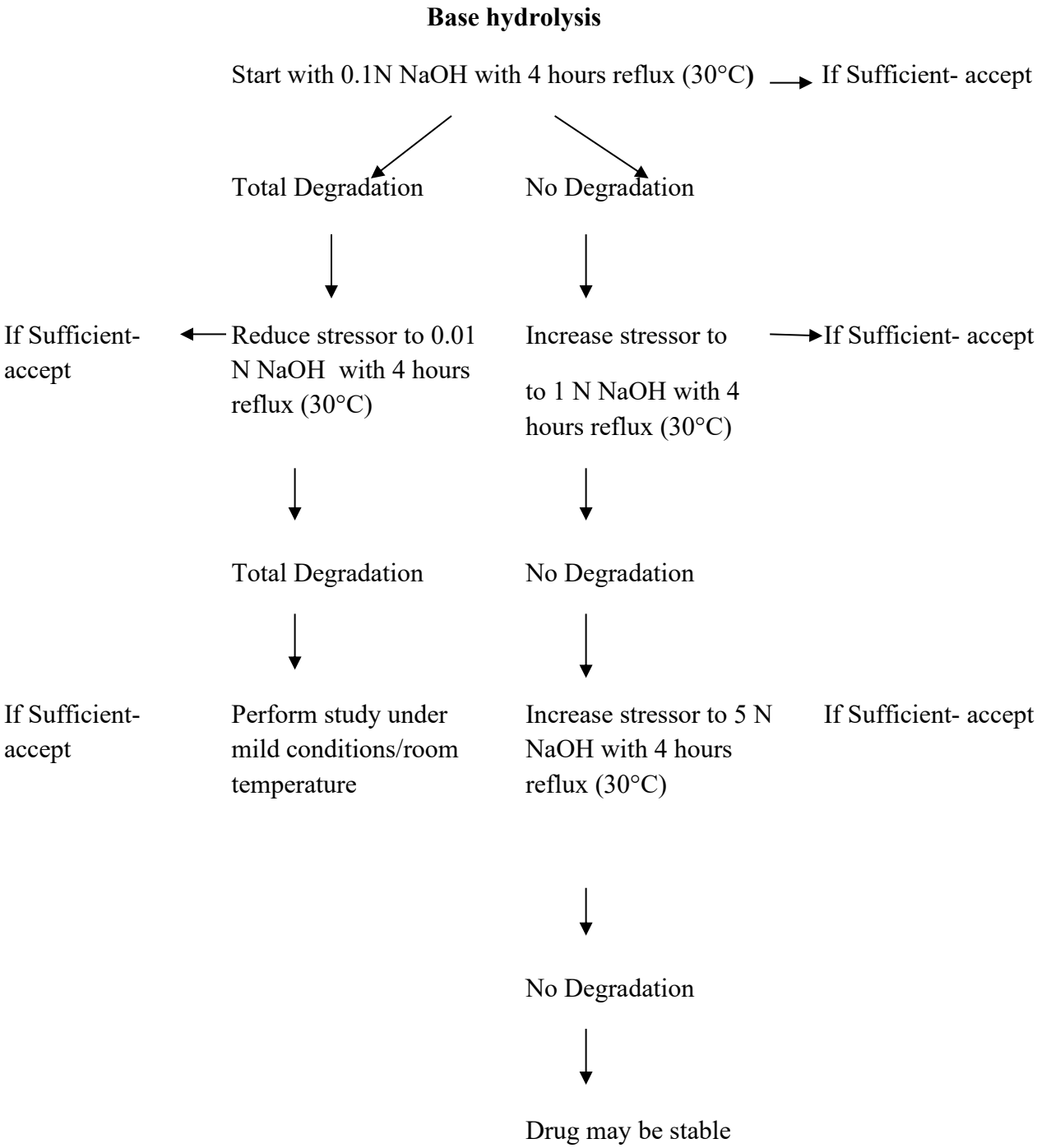
1.2 Stability indicating assay method

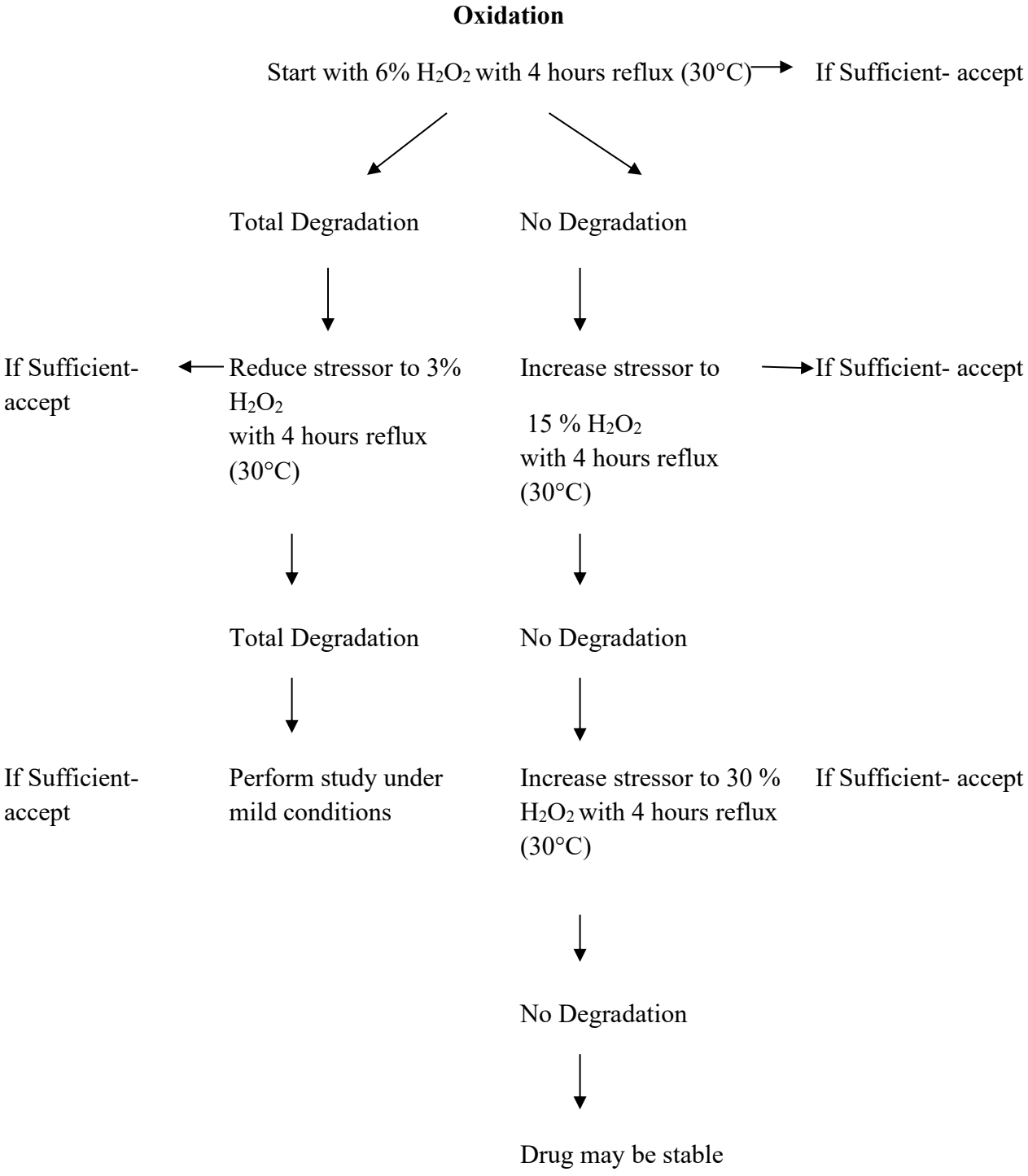
Chemical stability of drugs in their finished dosage form is of utmost importance for expected safety, efficacy and purity of medicines available to the patient. During its life cycle, drugs come across various chemical, environmental, storage conditions where chances of change in their potency are apparent. Right from the induction of synthesis, process related impurities have a chance to interfere with the purity of drugs. This process related impurities can be organic, inorganic or residual solvents. Since chemical reactions can catalyze in abundant directions, this impurities can be identified/unidentified, volatile/nonvolatile, traces of starting materials, intermediates, by-products, DPs, reagents etc. [15, 16] Environment and storage conditions go hand in hand because according to the effects of environment, storage conditions have to be decided. Due to globalization, import and export of pharmaceuticals have also attained a great upsurge and thus end-user of drugs may not be in the similar zone where the drug was manufactured. During the carting of drugs, they may encounter various conditions like exposure to photolytic, thermal, oxidation or hydrolytic conditions which may have a significant impact on potency as well as safety of drug. Due to the above stated conditions, possibility of degradation of drugs becomes apparent. Also if impurities are formed due to chemical repercussions, their identification becomes crucial because they can be harmless or may be carcinogenic, genetoxic, teratogenic etc. Therefore checking the stability of pharmaceuticals initially becomes of primary importance. Before registering of dossier for new drugs molecule as per WHO, long term (12 months), accelerated (6 months) and intermediate stability studies are mandatory to be done. ICH has distinguished 4 climatic zones for the purpose of worldwide stability. The drug needs

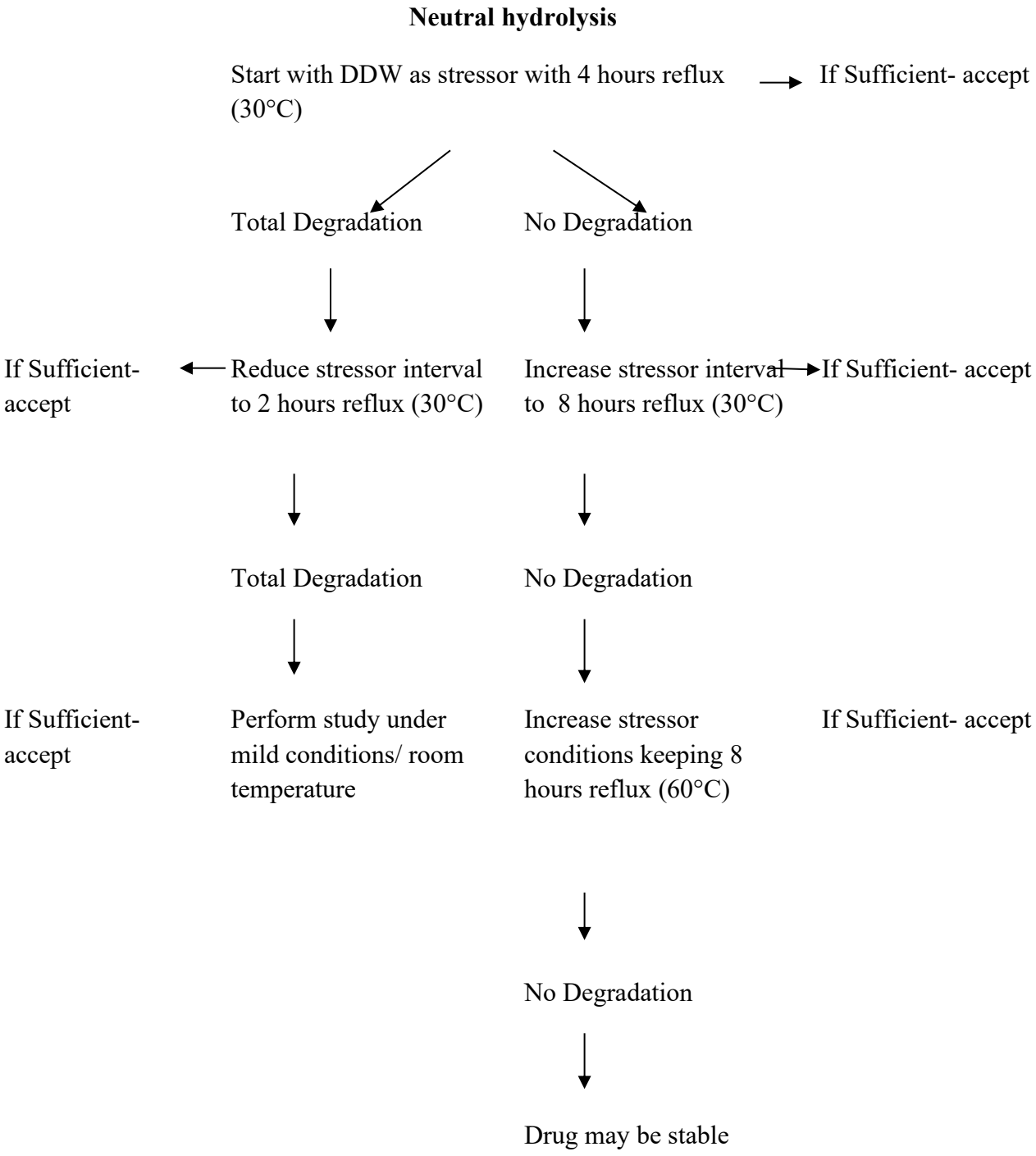
to qualify stability paradigm in all 4 climatic zones or atleast in the zone where drug's lifecycle would exist (viz., from its manufacture to transport and till the patient).

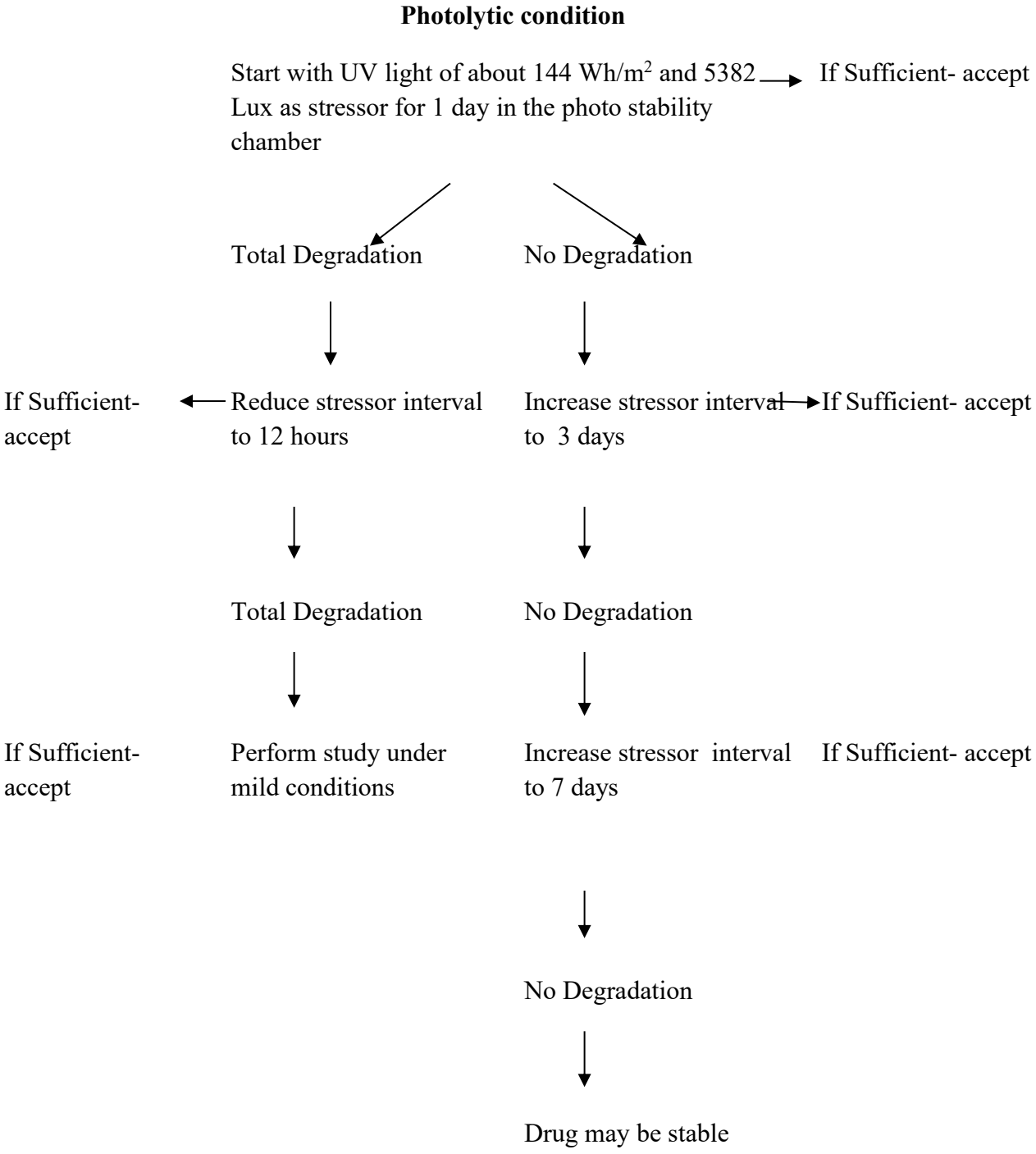
Whereas in forced degradation studies, within few days or week the stability studies can be accomplished. [17] The drug is exposed to extreme degradation conditions so that the degradation products are formed and then it is checked if the degradation products formed are safe or not. This procedure requires 3 crucial steps which are 1) Selection of appropriate forced degradation condition 2) HPLC method development and optimization for drug with impurities and 3) Characterization of impurities. [18] As selection of appropriate forced degradation condition is very important, the flowchart represented in Figure 1.1 [19-21] would explain in gist about the methodology to be employed for it. Hydrolysis stress conditions inculcate acid, base as well neutral hydrolysis studies. For acid hydrolysis generally HCl is used as stressor reagent. For alkali hydrolysis condition, generally NaOH is used as stressor reagent. For neutral hydrolysis double distilled water is used as stressor. For oxidation condition, hydrogen peroxide is used as stressor reagent. Photolytic studies are done using photo-stability chamber with specifications prescribed in the ICH guideline Q1B and dry heat degradation study is done in oven. For application of hydrolysis and oxidation stress conditions sample is kept for refluxing in precision water/oil baths equipped with temperature controller. Initially study is done at environmental temperature (viz., 30°C or as per the zone where drug is manufactured) and then if found stable forced degradation with elevated temperature proceeds. During the stress degradation study as mentioned in Figure 1.1, whenever sufficient degradation is achieved due to stress condition, it is selected as optimum for proceeding towards next steps of method optimization. For stability indicating assay method 5-20% degradation in considered optimum. [22] Thus for forced degradation study parameters like temperature, concentration of stressor reagents as well as time for application for stressor reagents can be optimized for selection of most appropriate stress condition for development of stability indicating assay method. Different forced degradation conditions can result into different degradation products; also there are chances that due to different stress conditions the parent moiety breaks only into a particular degradation product irrespective of degradation condition applied. For instance there are chances that drug A degrades into 3 different degradation products DP1, DP2, DP3 on stress conditions like hydrolysis, oxidation , photolytic whereas also there is possibility that only DP1 is formed on application of 3 different stress conditions (viz., hydrolysis, oxidation , photolytic). If more than one degradation products are formed in the study then the next steps of HPLC method development becomes more challenging as sufficient resolution along with symmetric peak shape needs to be maintained for drug along with as many DP's are formed in the stress degradation study.


Acid hydrolysis
Start with 0.1N HCl with 4 hours reflux (30°C)
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Reduce stressor to 0.01 N HCl with 4 hours reflux (30°C)
Increase stressor to to 1 N HCl with 4 hours reflux (30°C)
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Perform study under mild conditions/ room temperature
Increase stressor to 5 N HCl with 4 hours reflux (30°C)
If Sufficient- accept
No Degradation
Drug may be stable


Base hydrolysis
Start with 0.1N NaOH with 4 hours reflux (30°C)
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Reduce stressor to 0.01 N NaOH with 4 hours reflux (30°C)
Increase stressor to to 1 N NaOH with 4 hours reflux (30°C)
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Perform study under mild conditions/room temperature
Increase stressor to 5 N NaOH with 4 hours reflux (30°C)
If Sufficient- accept
No Degradation
Drug may be stable


Oxidation
Start with 6% H_2O_2 with 4 hours reflux (30°C)
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Reduce stressor to 3% H_2O_2 with 4 hours reflux (30°C)
Increase stressor to 15 % H_2O_2 with 4 hours reflux (30°C)
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Perform study under mild conditions
Increase stressor to 30 % H_2O_2 with 4 hours reflux (30°C)
If Sufficient- accept
No Degradation
Drug may be stable


Neutral hydrolysis
Start with DDW as stressor with 4 hours reflux (30°C)
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Reduce stressor interval to 2 hours reflux (30°C)
Increase stressor interval to 8 hours reflux (30°C)
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Perform study under mild conditions/ room temperature
Increase stressor conditions keeping 8 hours reflux (60°C)
If Sufficient- accept
No Degradation
Drug may be stable


Photolytic condition
Start with UV light of about 144 Wh/m2 and 5382 Lux as stressor for 1 day in the photo stability chamber
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Reduce stressor interval to 12 hours
Increase stressor interval to 3 days
If Sufficient- accept
Total Degradation
No Degradation
If Sufficient- accept
Perform study under mild conditions
Increase stressor interval to 7 days
If Sufficient- accept
No Degradation
Drug may be stable

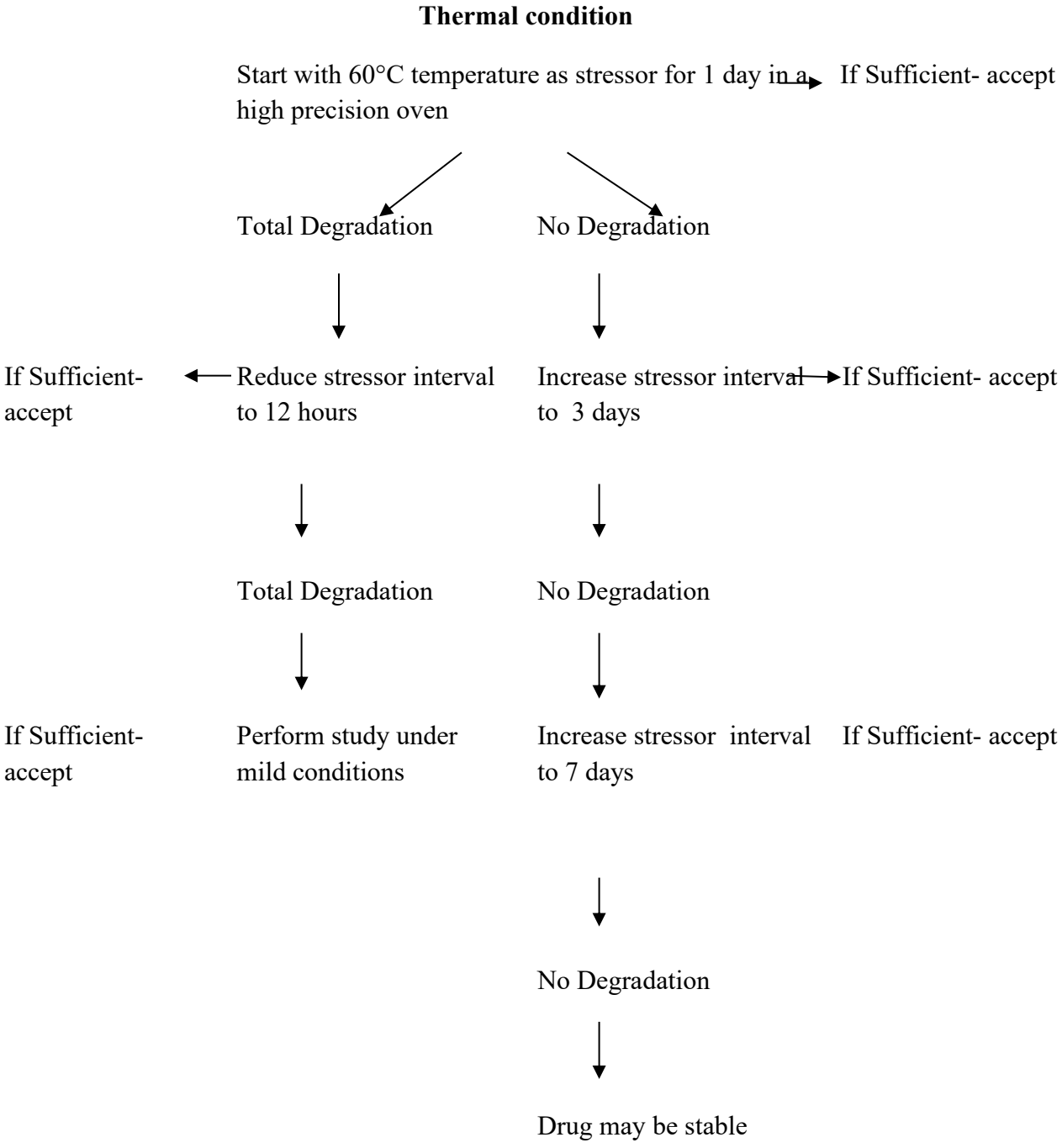


Figure 1.1 Flow chart for stress conditions [19-21]

1.3 QBD (Quality by design) and statistical analysis for normal distribution of data

QBD as defined in the International Conference on Harmonization guidance Q8 on pharmaceutical development is as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management."[23] For drug discovery and development ICH Q11 has prescribed guidelines for implementation of QBD. [24] Other than that for formulation development also it is used to a substantial extent. [25] QBD for analytical method development is known as AQBD. It is included in ICH Q13 and ICH Q14 guidelines. AQBD helps to understand the critical process parameters affecting the performance of the method and is also widely used which ensures a controlled risk-based development of analytical method where quality assurance will be guaranteed. AQbD life cycle encases various tools such as Analytical Target Profile, Critical quality attributes, Method operable design region, Control Strategy and Risk Assessment, method optimization using Design of experiments, Continuous Method Monitoring and Validation for gaining robustness and understanding about the developed method. [26-30]

The preliminary step for AQbD as per USFDA is target measurement based on QTPP (Quality target product profile) and CQA (Critical quality attributes). Then appropriate analytical technique is to be selected based on method performance criteria. Then risk assessment for method and sample variables is to be done using risk assessment tools like FMEA (Failure mode and effect analysis) model or Ishikawa diagram. Method optimization using DOE approach to be done for understanding and for assuring robustness of method. For reaching particular performance criteria, control strategy and system suitability are to be defined. Then further as technology evolves continuous improvement can be followed on monitoring method performance. These steps have a two way flow. Continual review of method dictates validated and optimized method furnishing reliable method outflow. [31] Right from selection of appropriate parameters to be included for analytical HPLC method, QBD plays a vital role.

Potential failure modes for HPLC method development can be deselected manually by discussing with experienced analyst or by using fishbone or Ishikawa diagram. Then on eliminating the factors not interfering with method optimization, CNX (Control, noise and experimentation) approach can be applied for further elucidating the key points affecting the analytical method. The key variables which can affect the reliability of any analytical method include people (analyst), instrument, measurement, materials and environment. Each key variable have copious motifs. The failure modes for analyst may be variation in mobile phase preparation procedure, calculation error, sample preparation procedure etc. The failure modes for instrument may be detector type, wavelength inaccuracy, HPLC model etc. The failure modes for measurement may be sampling rate as well as misidentification of peaks. The failure modes for materials may be organic modifier concentration, organic phase concentration, sample stability, column variability etc. The failure modes for environment may be column temperature, sample temperature etc.

For all this factors risk assessment can be done using CNX approach where the factors which needs to controlled, the factors which cause noise and the factors which should be optimized by experimentation can be defined. Then using FMEA (Failure mode and effect analysis) approach, rank for denoting the risk in order of 1 to 5 can be given. Rank 1 denotes minimum risk and vice versa. Risk is calculated as product of severity, probability and detectibility. Further, considering the key points which can affect the analytical method, QBD is applied for screening the optimal parameters. Various DOE (Design of experiments) methods like Plackett Burman design, Box Behnken design, D-optimal screening design etc are used for this purpose. Then on screening optimum factors DOE is further applied for obtaining the optimized method with optimal design space. Full factorial design, general factorial design Central composite design etc are used for this purpose. Further variations in design space can be done in future if required as per variation in product portfolio.

Three types of normality tests are common to verify if statistical procedure follows normal distribution or not. They are
1) Anderson-Darling Test
2) Ryan-Joiner Test
3) Kolmogorov-Smirnov Test.
Anderson Darling normality test is more sensitive test than other two test as it a modification of above two tests. Anderson Darling normality test calculates critical values using data of specific distribution [32-34]

1.4 Degradation kinetics study and total error approach

Degradation kinetics aids in more effective understanding about the drug behavior and thus can be fruitful for deciding the storage conditions for drug, formulation development of drug as well for selecting appropriate analytical methods which would not hamper the potency of drug. Extrapolating stress degradation studies and considering similar parameters like temperature, time and concentration of stressor to drug, we can have a full fledge understanding of how the chemical reaction associated with degradation of drug in various stressor condition progresses. Generally for pharmaceuticals degradation reactions are based on chemical reactions undergoing due to stressor conditions and are finite in nature. The rate and order of reaction are based on principle of mass action. [37] Solvent used for dilution, reagents concentration, temperature, pH maintained for the sample and solution, time applied for the stressor condition, additional reactants if added are the crucial factors which can affect these reactions.

Reactant concentration defines the order of reaction. Most pharmaceuticals degrade by zero order, first order, second order, and pseudo first order. Exceptions may exist where it degrades by complex mechanism. [35] From degradation kinetics study, rate constants values for the chemical reaction can be obtained from slope of the graphs. For zero order reaction, %degradation vs. time graphs are to be plotted, for first order reaction, log% degradation vs. time graphs are to be plotted and for second order reaction,

1/%degradation vs. time graphs are to be plotted. Rate constant values can be utilized for calculation of half-life and shelf-life. To analyze the effect of temperature on the rates of chemical reactions, Arrhenius plots can be plotted. Logarithm of K values vs. 1/Temperature applied as stressor conditions consist as X and Y axis in graphs of Arrhenius plots. From the slope of Arrhenius plots, values of activation energy for the chemical reaction can be calculated.

Accuracy of analytical method defines the quality of medicines; thus reliability of product to meet the quality specifications depends entirely on validation of analytical methods. Uncertainties in results of pharmaceutical products should be in specific limits for assurance of quality products. After issuance of ISO 17025 for calibration and testing laboratories, uncertainty calculation in pharmaceutical results gained importance. Its calculation aids in confidence for the finished product results. It can be applied for accuracy results in HPLC method validation program. Generally it is calculated using total error approach where calculation is based on 90% confidence interval and 66.7% beta expectation tolerance limit. [36] Based on % recovery data obtained from accuracy studies, % bias is calculated. Based on the % bias obtained, correction factor is introduced. Then computations like two sided tolerance interval, uncertainty and expanded uncertainty, upper and lower uncertainty limits as well as upper and lower limits of tolerance are undertaken.

1.5 Impurity profiling

Impurity profiling is the representation of identified as well as unidentified impurities present in drug molecule. Also as mentioned in ICH Q6 specification, substance other than drug and excipients found in the finished drug comes under the definition of impurity. [41, 47] Like purity profiling of drugs is mandatory, impurity profiling of drugs have also gained tremendous importance in the last decade. Due to escalation in concern for impurities identification and characterization, ICH prescribed maximum daily dose for impurities which qualifies the threshold criteria to be < 2g/day 0.1 % or 1 mg per day intake (whichever is lower) >2g/day 0.05%. [38, 39] Forced degradation studies outlines the degradation products which can form in different stress conditions. Further their identification comes under the paradigm of impurity profiling. For identification various methods can be employed like 1) Reference standard method 2) Spectroscopic method 3) Hyphenated methods.

1) Reference standards for impurities prone to be observed in certain drug substances or which are reported to have any specific toxic effects, are available in regulatory agencies. Like reference standards for drug substances they act as benchmarks for impurities as well as related substances found in the drug product.

2) For spectroscopic method

a) Total degradation method: If stability indicating assay is well established for a particular drug, initially LC/MS/MS study can be undertaken. If in that particular stressor condition, only one degradation product is formed, than this method can be applied. Then based on m/z ratio of drug and degradation product, the probable degradation pathway

can be elucidated. From degradation kinetics study relationship between % degradation and stressor conditions can be obtained. If the relationship is direct proportional then, extreme stressor conditions can be applied for total degradation of drug and obtaining impurity. Further its crystallization and purification needs to be done.

b) Isolation method: If total degradation is not possible for particular pharmaceutical, isolation of impurity stays the only option. Isolation of impurity can be done in number of ways. To name a few, preparative HPLC remains one of the best option for isolation of impurities as validated HPLC method for the drug is already available and thus only with few modifications in the optimized method, enough impurity can be obtained. Further its characterization can be done using spectroscopic techniques like LC/MS, NMR, FTIR, DSC etc. Mass spectroscopy can be undertaken using various ionization sources viz., ESI and MALDI etc. Also the ionization methods can be APCI, CI, EI, ESI, FAB, FD / FI, MALDI, and TSP. In NMR also various techniques like Proton NMR, C_{13} NMR, D_2O exchange NMR, DEPT NMR, HMBC- NMR HSQC- NMR gives more detailed information about the characterization of impurity. Also newly introduced EPR (Electron paramagnetic resonance) is used nowadays other than NMR (Nuclear magnetic resonance) which detects and quantifies species with unpaired electrons. These species include free radicals, transition metals and defects in materials. Thus impurities which lead to formation of free radicals can be easily identified by this technique. Other isolation techniques which can be employed include thin layer chromatography, solid phase extraction, liquid liquid extraction, column chromatography, flash chromatography, capillary electrophoresis, supercritical fluid extraction column chromatography, gravimetric technique etc. After successful isolation of impurity with above stated techniques its crystallization and purification needs to be done.

3) Hyphenated methods

If purpose of study is not getting solved with one technique, coupling of chromatographic as well as spectroscopic techniques can be employed. Coupling of techniques for impurity profiling have been reported in various ways as pointed below: [42-46]

- HPLC-DAD-MS
- HPLC-DAD-NMR-MS
- GC-MS
- HPLC-TLC
- HPLC-CE
- HPLC-MS
- HPLC-NMR
- LC-MS-MS
- Capillary Electro-chromatography
- FTICR- Fourier Transform Ion Cyclotron Resonance
- Hydrophilic Interaction Liquid Chromatography

Regulatory authorities obligate impurity profiling with two different frames 1) Analytical aspect and 2) Clinical aspect. Analytical aspect is as what we have at length discussed above. For clinical aspect the safety data sheet for the impurity needs to be produced even if the impurity is below the accepted limits as specified by ICH. As discussed above for any unspecified impurity, 0.1% works as acceptance limit but if any impurity is reported to have untoward toxic or adverse effect, acceptance criteria even below 0.1% can be specified for that particular impurity. Total impurities: organic, inorganic as well as residual solvents should stay below 0.1% of limit. For residual solvents, acceptance criteria for most commonly used solvents like methanol; acetone, acetonitrile, isopropyl alcohol, butanol etc are given in ICH Q3C guidelines which have to be strictly followed. [40] For their analysis, GC-MS method is generally used.

1.6 Bio analytical method development and validation

If the analytical method developed is utilized for estimation of analytes (viz., drugs, metabolites, biomarkers etc) in biological samples (viz., serum, plasma, urine, blood, saliva, semen, breath etc) then it can be called as a bioanalytical method. The purpose for development of bioanalytical method includes pharmacokinetic studies, toxic kinetic studies as well as bioequivalence studies.

Bioanalytical method development concise of mainly 3 steps procedure to be dealt with sample for its identification and quantification.

1) Sample collection
2) Sample preparation
3) Sample analysis.

Criticality in **sample collection** depends on the subject from which it is to be collected and for what purpose. For example, blood collection from animals like rat, mice or guinea pig which do not require anesthesia can be done from sephaneous vein or dorsal pedal vein whereas for blood collection requiring general/local anesthesia can be done by tail vein. Also terminal procedure is needed to be done for some complications like cardiac puncture. [48]

Sample preparation persists to be to the more crucial step for bioanalytical method development. The conventional methods used for this purpose include, protein precipitation, liquid extraction and solid phase extraction.

Protein precipitation method: The drug entrapped in the biological medium can be extracted using simple protein precipitation method where the biological medium consisting of proteins is precipitated, furnishing clean sample for further chromatographic analysis. Generally organic solvents like acetonitrile, methanol, acetone etc are used as precipitating agents along with acid like trichloroacetic acid, perchloric acid which increase the hydrophobic effect of these precipitating agents.

Liquid liquid extraction: It is also one of the potential techniques used for this purpose. The analyte partitions between two solvents in preoptimized ratio. Various steps like

mixing, centrifuging as well sonication are to be undertaken for partition of sample. After successful partitioning of sample in organic layer, aqueous layer can be discarded manually or can evaporated using nitrogen cylinder. The sample thus reconstituted is ready for further chromatographic analysis. The solvent system generally consists in a particular ratio of organic and aqueous phase. The different solvents which are used in this technique include chloroform, n-pentane, n-hexane, MTBE (methyl tert-butyl ether), ethyl acetate, phosphate buffer, double distilled water, ammonium formate buffer etc.

Solid phase extraction: More sophisticated method which can be utilized for sample preparation includes solid phase extraction. Intermolecular interaction between the sample and stationary phase lead to sample extraction. Normal phase as well reverse phase cartridges are available for sample extraction using this technique. For pharmaceuticals generally reverse phase cartridges are used. The 5 steps procedure is needed to be followed for development of optimized SPE method using reverse phase mechanism.

[i] *Sample pretreatment* is to be done so that it does not block the SPE cartridges.
[ii] Then *conditioning* of cartridges is done using organic solvents like acetonitrile, methanol etc.
[iii] Then as per the size of cartridge *sample* is to be *loaded*.
[iv] Next step of *washing* is very important steps in this procedure and is generally accomplished using double distilled water or buffer solution.
[v] Finally *elution* is carried out using suitable solvent.

Generally alumina and silica consists of the bonded phase of cartridges. Though silica bonded reverse phase like LC-18, LC-8, LC-4 remains most preferred used stationary phase for cartridge. [49] Also much recent advancement are done for sample extraction techniques like molecularly imprinted polymer SPE, stir bar sorptive extraction, dispersive SPE, salting out LLE, PP filter/tubes etc. [50]

Sample analysis: For analytical method development of nonvolatile samples, HPLC-PDA, HPLC-MS remains the method of choice whereas for volatile samples GC-MS instrumentation can be applied. [51]

For certain specific samples like for breath test, also instruments based on Infrared spectroscopy are used. Validation of bioanalytical method is to be done similar to normal analytical method validation summarized in ICH Q2 (R1) guidelines with consideration of few extra points to be taken care of like selectivity, matrix effect, dilution integrity as well as stability.

Selectivity and specificity generally go hand in hand for any method. Here, selectivity signifies the analysis of analyte of interest unequivocally even in presence of other interfering counterparts in the sample. For checking the selectivity of drug for pharmacokinetic studies, internal standard is generally added along with the drug on interest whose pharmacokinetic study is undertaken. Keeping the concentration of internal standard constant, calibration curve of drug is plotted. Linearity in values of peak ratio signifies development of validated analytical bioanalytical method.

Matrix effect of biological sample is very much apparent to interfere in development of robust analytical method. Sample clean up procedure selected for the study proves to have a huge impact on this factor. Better the sample clean up, lower the matrix effect to be observed in the chromatogram. Even after utilizing the best sample pretreatment procedure, matrix effects are still apparent, changes in optimization of analytical method can be done so as that the matrix does not interfere with the analyte as well internal standard detection. Also to demonstrate the authenticity of method, chromatogram of blank matrix can be displayed to avoid the confusion arising due to extra interference in the chromatogram.

Dilution of samples for bioanalytical method development becomes very tricky due to 2 reasons. 1) The concentration of samples generally ranges in nanometer range. After dosing as per daily dosage recommended by FDA, drug goes into systemic circulation. After the predefined lag time during the sample collection, it generally reaches to this low range. 2) Most of times sample preparation procedure envisages many steps for proper sample clean up. The requirement of maintaining 1:10 ratio between drug and biological fluid, inclusion of many solvents for clean up using various methods, demands very attentive focus on dilution factor inducted during the sample preparations step.

The *stability* is to be determined under five different paradigms [52]

1) Bench top stability: This study is to be taken at laboratory conditions for minimum period of 24 hours.

2) Extract stability: After application of sample pretreatment procedure, the sample stability should exist. Chromatograms of standard calibrators are to be compared with extracted samples.

3) Freeze thaw stability: For study of freeze thaw stability, minimum 3 cycles at -20°C for 12 hours followed by 12 hours at room temperature are to be carried out and then the chromatograms taken with the extracted sample. If expected results are achieved, then the stability can be established.

4) Long term stability: For this study the period from sample preparation to sample analysis is to be kept in mind, also the sample storage is done at -20° is to be considered. 3 cycles of removal from -20°C to room temperature can be undertaken and then also fruitful results are needed to be achieved.

5) Stock solution stability: It should also be validated as per the storage conditions laid for the stock solution. If stock solution is stored at room temperature, atleast 24 hours stability at room temperature should be checked, similar stability needs to be established for freeze stored samples.

1.7 Chemometric assisted analytical method development for checking adulteration in drugs

The World Health Organization (WHO) defines counterfeit medicines as "One which is deliberately and fraudulently mislabeled with respect to identity and/or source. [53] Counterfeit drugs have emerged to a tremendous extent in the last decade. It poses serious concern for healthcare industry. This adulteration of pharmaceuticals generally takes place in developing countries rather than developed countries due overlook on regulatory regulations in developing countries. WHO has estimated almost 30% drugs to be counterfeit summing up from Asia and South Africa. [54] The categories of drugs mostly consist of life threatening diseases like cancer and AIDS as their costs are not affordable for the patients in those countries. Albeit the cases of counterfeit drugs were also recorded in developed countries like USA up to 1% mostly in life style medications like for aphrodisiacs, anorectics etc. [55, 56] Prescription as well as generic medicines can be counterfeited.

Counterfeiting can be done in number of ways as per WHO. [57]

[i] The drugs without the presence of active ingredients

[ii] Having fallacious amount of active pharmaceuticals ingredient

[iii] Substitution of active pharmaceutical ingredient with other chemical constituent. In this case substitutions with chemical substituent are generally done of similar category pharmaceutical which is cheap and less potent. Also the worst case scenario can exist here is substitution with a chemical ingredient having clinical indications (side-effects) similar to parent drug but itself the chemical ingredient be toxic. This type of scenario prevailed in recent past where 15 women died in Chhattisgarh due to intake of fraudantly labeled medicines according to the consulting doctor.

[iv] Fake packaging had prevailed to a great extent not only in pharmaceutical, in almost all commercial products. Extracting the root suppliers and manufacturers in very difficult nowadays due to online marketing and pharmacies. The premises mentioned on the packaging not even exist.

[v] The formula for pharmaceutical product although be similar but without authentic packaging also comes under the paradigm of counterfeits.

[vi] Authentic product with enormous amounts of impurities and contaminants also comes under the definition of counterfeit sample.

Due to upsurge for leading healthy lifestyles in the last decade, many people are diverting towards phytopharmaceutical instead of rampant use of allopathic drugs by self medication. Thereby many allopathic drug manufacturers also have plunged into herbal products manufacturing. But the intrinsic truth about Ayurvedic medicines is extended period of time for curbing of ailment. To exquisite this drawback some greedy drug manufacturers add synthetic drug analogues of herbal medication in the phytopharmaceutical. [58] This type of adulteration causes a serious concern on the

patients exposed to synthetic drugs in lieu of Ayurvedic medicines because Ayurvedic medicines generally owns a very broad therapeutic window ret assuring the chances of side effects to nil whereas synthetic drugs have narrow therapeutic window and thus if taken by patients in excess can prove to be life threatening.

For checking the adulteration of pharmaceuticals, chemometrics is used to a great extent. [59, 60] With the alliance of mathematical and statistical models, chemo metrics aids in understanding of chemical correlation for inspection of quality of pharmaceuticals.

Various methods of chemo metrics like Hierchial cluster analysis, Principal component analysis, nonlinear iterative partial least squares algorithm, Partial least square-Discriminant analysis etc are utilized for it. The statistical analysis is done of basis of Eigen values and explained variance. The spectroscopic techniques like Near Infra red, Raman spectroscopy as well as FTIR are utilized as base techniques. For smoothing and differentiation of data, Savitzky-Golay is used by least-squares technique. It provides simple equivalent convolution as improvement to lengthy least square calculations. It provides (2m + 1 point) least-squares fit across the data. [61, 62]

Also chromatographic methods have been used to a great extent for checking counterfeiting of pharmaceuticals. Methods like HPLC-PDA [64], UPLC, LC-MS-MS [65], GC-MS [63, 66], and XRF [67] have been reported in last decade for this purpose.

Chemometric methods are one type of multivariate analysis i.e. considering more than one variable at a time. When applied to UV spectrophotometric, many wavelengths are taken as variable and absorbance at each wavelength is considered. Least square approach involves mathematical modeling by which the square of residual (difference between actual and predicted concentration) is minimized to lowest level. Four different Chemometric methods are used which are

1. Classical Least Squares
2. Inverse Least Squares
3. Principal Component Regression
4. Partial Least Squares *or* Projection to Latent Structures

These methods first calibrate the mathematical model by using absorbance data of calibration standards with known concentration and then predict the concentration of unknown samples from their absorbance data. If there are *m* number of calibration standards and *l* chemical components (drugs) and *n* is the number of wavelengths considered all methods involve presentation of absorbance data as a matrix with *m* rows and *n* columns, concentration data as a matrix with *m* row and *l* columns.

1.8 Microfluidic devices for analytical and diagnostic approaches

Microfluidic diagnostic devices deal with science governing capillary movement of fluids (generally in pico and nano liters) through channels (generally milli or micrometers) leading to results encompassing multiple detecting principles. (Electochemical, colorimetric, Immunoassay etc) Collective utilization of principles from disciplines of chemistry, physics, and biology as well mathematics are required for its development. First conceptualized in 1980's its development has flourished many folds in the last two decades. Material of construction for development of microfluidic devices can vary from glass, polymer, silicon to ceramic [70, 71]. Fabrication of microfluidic devices have been materialized in various ways. Widely used methods include 3D printing, jet printing, laser printing [68], hot embossing [73]. Above stated techniques employ polymer as primary material of construction. In that also PDMS (poly dimethyl siloxane) ranks in priority due to its various favorable material specifications making microfluidic device fabrication easy and precise.

Paper based micro fluidics have also flourished to a great extent due to their low cost of manufacture, raw material, disposable characteristics along with accuracy in parallel to its complementary techniques. Whatman chromatographic filter papers have been used in most cases for its development. As a matter of fact, paper consists of hydrophilic layer. For preparation of micro channels, hydrophobic layer is needed to be laid on paper through which the liquid in volumes of pico/nano/micro liters can flow through capillary action on porous membrane. For preparation of this hydrophilic/hydrophobic membrane, various techniques are available in literature. Printed circuit technology [72], wax printing [69], inkjet printing, photolithography, flexographic printing [75], plasma treatment [76], laser treatment, wet etching, screen-printing [74] etc. A simple and easy method for fabrication of paper microfluidic devices was reported by E.M. Dunfield in 2012. [77] The underlying principle for paper microfluidic devices lies in capillary action of fluids through the reagent impregnated micro channels for detection through specific reaction with the of analyte of interest in the fluid. This specific reaction can be based on electrochemical, immunoassay consummating ELISA, or colorimetric principles.
The majority of microfluidic available in market rely on ELISA technique where antibodies and specific enzymes consist as the reagents impregnated into the micro channels. [78]
For electrochemical based micro fluidics, electrodes consist of the circuit impregnated on to the paper for specific electrochemical detection. Most of times, a readout system of analog to digital is also attached with this systems for easy readout by the end user. [79]
For colorimetric based microfluidic, the reagent/ reagents having specific reaction with the analyte of interest are impregnated in to the micro channels. [80] Precaution here

needs to taken to preassure the reagents don't have chemical reaction with each other instantaneous as well on storage.

Diagnostic kits are available abundant in market. To illustrate a few, pregnancy kit which detects human chorionic glycoprotein as a specific biomarker being released during pregnancy is developed underlying the principle of ELISA [81]. Similarly microfluidic kits for diagnosis of alpha fetoprotein [83], C-reactive protein [82], glucose for monitoring of diabetes [84] all come under the similar paradigm of ELISA based microfluidic kits.

For certain neurological disorders, frequent monitoring of dopamine levels is needed. Microfluidic kit based on principle of electrochemical detection was developed in 2014 aiding in easy accomplishment of this task. [85]

Similarly due to more people diverting towards vegetarian lifestyle, cases of pernicious anemia due to Vitamin B_{12} deficiency have also emerged to a great extent. Thereby its diagnosis based on paper microfluidic kit can also be undertaken.

The problem of counterfeiting is discussed at length above. For checking the counterfeiting of pharmaceuticals also microfluidic kit was developed. Counterfeiting of anti malarial drug artesunate was checked with the use of that kit. [80] Similarly for other pharmaceuticals prone to be counterfeited like aphrodisiacs, anorectics also paper microfluidic kit development can be undertaken.

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