INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES
[ISSN: 0975-4725; CODEN(USA):IJPS00]
Journal Homepage: https://www.ijpsjournal.com

Review Article

An Industry Perspective: How To Develop Analytical Method For Related Substances Of Drug Product By Applying A QbD

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ARTICLE INFO

Received: 11 Feb 2024
Accepted: 15 Feb 2024
Published: 17 Feb 2024
Keywords:
Related substances, Impurities, Forced degradation.
DOI:
10.5281/zenodo.10673264

ABSTRACT

Related substances is one of the most important analytical test, which defines the quality, safety & efficacy of product by monitoring the growth of impurities in drug product. Knowledge about related substances at early phase of development helps to decide the storage condition and container closure system for finished product Development of robust and rugged analytical method for estimation of these impurities is challenging task for analytical scientist due to complex nature of formulation matrix. The main objective of this review article is to provide a systematic way to develop stability indicating analytical method for estimation of impurities from drug product for generic & NCE molecules.

INTRODUCTION

Related substances are structurally related to drug substances, which are identified, or unidentified or impurities arising from manufacturing process or during storage of a material [1,2]. These impurities are further divided into two parts: Process impurities & Process + Degradant. Process impurities are generated during synthesis of drug substance & which are controlled during synthesis process. Process impurities may be Starting materials, Reagent, ligands & catalysts, Intermediates, isomers [1]. Lamivudine carboxylic acid is process impurity of Lamivudine drug substance. Process + Degradant are process impurities which never generates during forced degradation conditions but sometimes in presence of excipients some of these impurities may get convert into degradation product due to an environmental condition. Degradation Products are the impurities which may generate either due to manufacturing process like wet granulation, curing of granules, coating of the tablet, etc. or generates during storage period due to environmental stress conditions like Hydrolysis, Temperature, Heat & Humidity, Humidity, and Oxidation. Degradation products are further categorized as

Specified Degradation Product:

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

A degradation product that is individually listed and limited with a specific acceptance criterion in the new drug product specification/ drug product monograph [3]. Specific acceptance criteria is the acceptance criteria other than that of calculated based on daily dose of drug product as per ICH guideline. Same drug product may have different acceptance criteria for degradation products depending on the regulatory market of product. These acceptance criteria may be either stringent limit or broad limit than the regulatory acceptance criteria based on stability & safety data of specific degradation product. A specified degradation product can be either identified or unidentified.

Identified degradation Product:

A degradation product for which a structural characterization has been achieved [3]. Drug product master file contains information about the all-possible identified degradation products.

Unknown/Unidentified Degradation Product:

A degradation product for which a structural characterization has not been achieved and that is defined solely by qualitative analytical properties (e.g., chromatographic retention time/ RRT, RRT-0.789, RRT-0.456) [3].

Unspecified Degradation Product:

A degradation product that is limited by a general acceptance criterion, but not individually listed with its own specific acceptance criterion, in the new drug product specification [3].

HOW TO SET SPECIFICATION LIMIT FOR INDIVIDUAL IMPURITY & TOTAL IMPURITIES IN DRUG PRODUCT?

Regulatory guidance documents provide the information regarding calculation of impurities. ICH guidelines have explained different terminologies to make process simple. Furthermore, rationale for the reporting & control of degradation products have been summarized in the guidance document. This article summarizes the different terminologies to report & control impurities in drug product as.

Reporting Threshold:

A limit above (>) which a degradation product should be reported [3]. In other words, reporting threshold is LOQ of analytical procedure. All the degradation products at & above this threshold should be reported and same should be considered in the calculation of total impurities. Some industries target LOQ lower than the Reporting threshold as a stringent criterion based on the stability data of development batches. Reporting threshold limits applies to the unknown degradation products only.

Identification Threshold:

A limit above (>) which a degradation product should be identified [3]. Identification is nothing but specification limit for unknown degradation products/ unknown impurities which may arise due to manufacturing process or during storage conditions due to environmental conditions. If any degradation product found above the identification threshold limit, it should be structurally identified by using hyphenated techniques like, IR, NMR & Mass spectroscopy. Further step is to predict degradation pathway, which helps to control growth of identified degradation product during manufacturing process as well as throughout the storage period.

Qualification Threshold:

A limit above (>) which a degradation product should be qualified [3, 4]. Qualification threshold is specification limit for known degradation products. If any degradation product found above the qualification threshold limit, it should be qualified in terms of safety. Toxicity data should be generated to prove that the limit of degradation product above the qualification threshold is safe and has no adverse effect in human body. Decision tree is available as a guidance for identification & qualification of a degradation product in ICH guideline.

- For generic molecule, if product monograph is available in Pharmacopeia, use the mentioned

limits as it is. For those products that (a) have USP monographs or (b) do not have USP monographs but have other compendia monographs and the monograph acceptance criteria are greater than the ICH Q3A(R2) or Q3B(R2) qualification thresholds, the impurity limits in those monographs may be acceptable as long as an apparent risk (e.g., structural alert) has not been identified [5]. If product monograph is not available in any pharmacopeia, refer ICH Q3B (R2) guideline to define specification limit.

- These guidance documents are generally not considered applicable for first-in-human (FIH) or early phase DS/DP specifications, and implementation of ICH acceptance criteria is usually not expected until later in development (i.e., during Phase 2b or pivotal Phase 3 clinical studies) [6].
- For non-mutagenic impurities in early phase clinical development specification can be set three times higher than ICH limits (i.e., 0.5% or 3 mg/day intake, whichever is lower, for a maximum daily dose ≤ 2 g/day) based upon (a) the limited patient exposure and low participant numbers and (b) risk to patient safety [6].
- For impurities known to be unusually potent, toxic, or have immunological, pharmacological, or clinical concerns, the proposed acceptance criteria based solely on ICH Q3A (R2) and Q3B (R2) qualification threshold are not sufficient and need to be adequately justified [5].
- The acceptance criterion for total impurities excluding significant human metabolites, generally, should not exceed the summation of acceptance criteria for individual specified (identified and unidentified) impurities [5].
- Acceptance criterion for individual impurities that are also significant human metabolites should be considered separately. The sum total of all impurity limits, including those for significant metabolites, should not exceed thresholds that may compromise product potency/assay through product expiry [5].

Common Observations of Fda For Related Substance Method [7]

Following are some common observation of USFDA about related substances method based on NDA & ANDA applications received from different pharma industries.

- The impurities in the drug product exceeds the limit for Total impurities permissible by ICH guideline
- "It is difficult to identify and estimate impurities" is insufficient rationale for not studying the impurities present in the drug product.
- The impurities in the drug product manufactured by you are not adequately identified and qualified.
- The API is sourced from two different vendors. However, the specific impurity profile for each source is not provided.
- Forced degradation studies have not been performed to indicate the possible degradants which can contribute to the impurities in the drug product.
- The method used for impurity testing is not stability indicating.
- Potential impurities arising from the starting materials, bye products and reagents are ignored.
- The method used for qualifying the individual impurity is not validated.
- List of Specified Impurities and Unspecified Impurities is not provided.
- Your HPLC method for impurity testing is unacceptable as it lacks description on Composition and pH of Mobile Phase ,Type width and length of HPLC Column ,Flow Rate of mobile Phase ,Column Temperature ,Type

and description of Detector ,Injection Volume ,Run Time ,Retention Time, Sample Preparation System Suitability etc.

- The evidence on the validation and suitability of analytical procedures used for the detection and assay of impurities is inadequate.
- You have failed to provide even RRT for unidentified impurities present in significantly high levels in your product.
- The Reporting Threshold (RT), Identification Threshold (IT), and Qualification Threshold (QT) of impurities do not match with the Maximum Daily Dose indicated on the label of your product.
- You are manufacturing the drug as per USP wherein no related substances are listed in the monograph. However, you have not referred other Pharmacopoeias such as EP and JP for likely impurities and related substances, which may be present in your product.
- Impurities reported as degradants are not covered in your analytical method.
- Mass balance not achieved.

Analytical Method Lifecycle

Development of any analytical method starts with goal/ATP setting which ends with the successful validation without any burden. In this review article, systematic approach has been discussed to develop robust analytical method for related substances of drug product (Fig. 1).

What is ATP & How to design it?

The ATP is a prospective description of the desired performance of an analytical procedure that is used to measure a quality attribute, and it defines the required quality of the reportable value produced by the procedure [8], aligned with the quality target product profile (QTPP). The ATP focuses the design goals for a new analytical procedure, serves as a basis for procedure qualification criteria, and provides a guide for monitoring of the procedure during its life cycle Understand your method requirement/ purpose to design ATP. Related substance method can be developed by using either of the below mentioned approach on the basis of feasibility & purpose of the method.

Impurities calculation against diluted standard:

This is most commonly & widely adopted approach by industries. The main purpose of use of diluted standard is to save the large amount of cost due to impurity reference standards. Another major reason is the saturated peak response of active due to high sample concentration of active that is a prerequisite for detection & quantification of all impurities at LOQ level. Now question comes to our mind that, how to prepare diluted standard? Generally, diluted standard is prepared at specification level of unknown impurity. Linearity of known impurities & active needs to be performed during development phase to calculate Relative Response Factor (RRF). Relative response factor of impurities is the response of impurities relative to active. Different impurities have different response due to differences in their chemical properties in terms of functional groups. Calculation of RRF is required to report actual/original values of impurities observed in drug product.

Impurities calculation against assay level standard (Assay/RS common method):

Now days, this approach is widely used by many industries. This approach can be used in two cases:

1. If response & spectra of active peak dose not saturate. For better clarity, Active peak response should not be above 1 Au at selected detection wavelength.
2. If run time of analytical method is short (≤ 30 minutes). Short run time gives the advantage of calculation of assay & impurities of drug product by using same analytical method and same sample preparation, which saves a lot of analysis time & solvent also.

Determination of RRF for impurities is required to report actual/original values of impurities observed in drug product in this approach also.

Impurities calculation against impurity standard

This approach can be used when an impurity shows the false / more RRF value relative to active (RRF<0.2 & >5.0). In such case, calculation of that specific impurity needs to be performed against that impurity standard. Impurity standard should be prepared at specification level of that impurity.

Example of ATP

To develop stability indicating analytical method for estimation of related substances of Lamivudine, Tenofovir disoproxil fumarate & Efavirenz in Lamivudine, Tenofovir disoproxil fumarate & Efavirenz tablets, 300 mg/300 mg/600 mg and must comply below mentioned criteria.

- **Specificity-**

Interference from blank & placebo not more than LOQ & purity angle should be less than purity threshold.

- **Stability Indicating Nature-**

5-20% degradation in stress conditions

- **Method operating range-**

LOQ to 150% of specification limit

- **S/N ratio-**

NLT 10 in LOQ preparation for all impurities & all three actives

- **Accuracy-**

70-130% recovery with RSD NMT 30% at LOQ level, 85-115% recovery with RSD NMT-15% at specification level & 95-105% recovery with RSD NMT 10% at 150% of specification level.

- **Linear response**

Of known & unknown impurities within the specified range with correlation coefficient of NLT 0.990, Y-intercept bias NMT 5% & RSD of response factor NMT 5%.

- **Resolution**

Between all impurities & active NLT 2.0

Understand your molecule

Method development is not possible without the information or chemistry of molecule for which method needs to be developed. Literature review is a crucial step, which creates a trajectory for method development. Now question comes to mind that which information is required to collect before the starting of method development. This review article provides the systematic guidance to make a perfect trajectory for successful development of analytical method for estimation of related substances

Understanding of Physicochemical Properties

- **pKa (Ionization constant):**

pKa is a number that describes the acidity of a particular molecule. Since most APIs have acidic and/or basic functionalities, their ionization state is controlled by both solution pH and acidic dissociation constants. These different chemical species (cationic, neutral, or anionic) often have vastly different properties with respect to water solubility, volatility, UV absorption, and reactivity with chemical oxidants [9]. The ionized form is usually more water soluble, while the neutral form is more lipophilic Molecule may have more than one pKa values depending on ionization site (Fig. 2). pKa of active helps in selection of mobile phase pH & selection diluent. Unionized form of molecule retains more in HPLC column, which in turn increases the run time, & ionized form retains less in HPLC column, which in turn decreases run time.

- **Solubility & Solution stability of molecule**

Solubility is the capacity of the solvent to dissolve a solute. Solubility of molecule describes its nature (Polar/Non polar) which helps in diluent selection. Solubility of ionizable acids & bases is pH dependent because the charged species have a higher affinity towards the aqueous environment than the neutral form [13]. Solubility plays major role in pharmaceutical formulations due to presence of number of excipients, which affects the solubility of active in selected solvent.

Solubility of drug molecule in selected solvent is not only requirement but it should remain stable in the solvent also. Solution stability information can be predicted from forced degradation data of drug substance. Drug master file of drug molecule contains the detailed information of solubility & solution stability of drug substance.

- **Isomerism**

Isomers by definition are the molecules of identical atomic compositions, but with different bonding arrangements of atoms or orientations of their atoms in space i.e., isomers are two or more different substances with the same molecular formula [14]. Some molecules may not exhibit isomers but their degradation products may show the isomerism & it should be separated & quantifiable in the developed analytical method for estimation of related substances. Isomers of molecules & / or impurities helps in selection of HPLC column, mobile phase & diluent due to the differences in chemical properties of these isomers. Some isomers may easily be quantified by using RP-HPLC method. Budesonide has two epimers- Epimer-A & Epimer-B that has been quantified by RP-HPLC method [15]. Risperidone does not show isomerism but its degradant impurity has two isomers- Cis-N-oxide & Trans-N-oxide & it has been analyzed using RP-HPLC method [16, 17]. Some isomers may require normal phase chromatography mode for quantification.

- **Polymorphism**

Pharmaceuticals may exist in various solid forms. In materials science, polymorphism describes the existence of a solid material in more than one form or crystal structure. Polymorphism is a form of isomerism. Polymorphic forms of a drug substance can have different chemical and physical properties, including melting point, chemical reactivity, apparent solubility, dissolution rate, optical and mechanical properties, vapor pressure, and density [18]. Some polymorphic form are more stable & shows less degradation that helps in method development (Table 1) [19-24].

- **Analytical Methods in Pharmacopeia**

For generic molecule, it is required to check the analytical method availability in pharmacopeias. This information gives the idea about all possible impurities available in drug substance & drug product, which helps to decide the strategy for method development.

- **Impurity information**

Information of impurities of drug substance helps in method development. Refer DMF for impurities related to drug substance because impurities may change based on route of synthesis of drug substance. Search all possible degradation products for drug substance through literature review from online sources. Below are the critical information, which can help in method development (Table 2) [25]

- **Type & origin of impurity helps to design ATP.**

Degradation pathway

Provides the information about generation of degradation products, which can help to control degradation products either by controlling the manufacturing process or by container closure system.

- **pka of impurities**

Helps in selection of mobile phase & diluent to resolve all impurities.

- **Knowledge about chromatographic requirement**

Knowledge about chromatographic requirement plays a major role in development of robust analytical method & reduces the burden on analytical scientist because it reduces the number of experimental trials. General chromatographic parameters includes,

- **Selection of Detection Wavelength:**

For many samples, good analytical results will be obtained only by careful selection of the wavelength used for detection. This choice

requires a knowledge of the UV spectra of individual sample components [26]. Detection wavelength selection should be based on optimum responses of all impurities & active (Fig. 3). Some analytical scientist prefer wavelength at isobestic point that is wrong because there may be chances of lower response at isobestic point. If we select wavelength of isobestic point, it will make major differences in RRF & sometimes may get RRF > 2, which is wrong. Another important criteria for wavelength selection is the UV cutoff of selected solvent in mobile phase & diluent. Each solvent has their own UV Cutoff, which shows absorbance at that wavelength. Ideal detection wavelength should be 20 nm far away from the solvent UV Cut off value to avoid interference. Never select wavelength at or below solvent UV Cut off value.

Selection of HPLC Column

HPLC columns plays major role in separation of impurities in related substance method but how to select a right column? First, you will need to establish if you are performing normal-phase or reversed-phase HPLC. In normal-phase HPLC, the stationary phase is hydrophilic while the mobile phase is hydrophobic. However, reversed-phase HPLC is far more common. In reversed-phase HPLC, the mobile phase is hydrophilic while the stationary phase is hydrophobic. A number of factors influence the properties of silica-based RPs. The nature of the silica is characterized by the particle diameter, specific surface area, pore diameter, pore volume, chemical purity and acidity [27]. That means that molecules are eluted by decreasing polarity with an organic solvent. Selection of HPLC column is not limited to type of chromatographic requirement only but there are number of factors which plays major role for separation of compounds as mentioned below.

- Column length, Column end capping, Carbon loading, Bonded phase, Pore size, Column pH range & Column temperature range

Selection of Mobile Phase

Mobile phase is a mixture of buffer & solvent to retain analyte of interest. Solvent strength or % organic solvent content in the mobile phase controls the retention time of the analyte [28]. Many drugs have either acidic or basic functional groups and can exist in solutions in ionized or non-ionized forms. Buffers are commonly used to control the pH of the mobile phase for the separation of acidic or basic analytes. Table 3 summarizes the common buffers for HPLC and their respective pKa [29]. Acid above or below its pKa value becomes >99% ionized or unionized, respectively [30]. Bases are ionized below their pKa and non-ionized above their pKa. The non-ionized form becomes less polar and thus more strongly retains in a reversed-phase system. Thus, at low pH, acids retains more, whereas bases retains at high pH. If we keep mobile phase pH near pKa of drug molecule, molecule will remain in partially ionized & unionized state, which affects the reproducibility in retention time [30]. Mobile phase pH should (ideally) be at least 2 pH units below or above the sample pKa. For the most effective buffering, a buffer should be used within ±1 pH unit of the buffer's pKa. For LC-MS applications, the buffer must be volatile, so the choice of buffers is more limited.

Selection of instrument parameters

Flow rate:

Based on separation need, analysis time & column length.

Column temperature:

Based on column backpressure & need of resolution.

Sample cooler:

Based on stability of standard & sample solution.

Injection volume: Based on response of analyte and column loading.

Establishment of System suitability Parameters

The system suitability tests are the integral part of gas & liquid chromatographic methods [31]. These tests ensures that the chromatographic system is

suitable for the analysis purpose & supports in risk assessment of the developed analytical method. Specification limits for system suitability parameters can be set based on the development data. Table 4 summarizes the system suitability parameters & general acceptance criteria [32]. Broad limit for % RSD can be set based on the concentration of standard preparation using Horwitz equation. In 1980 Horwitz, Kamps, and Boyer pointed out that: "an examination of the results of over 50 interlaboratory collaborative studies conducted by AOAC on various commodities for numerous analytes shows a relationship between the mean coefficient of variation (CV), expressed as powers of 2, with the mean concentration measured, expressed as powers of 10, independent of the determinative method." [33]

$$RSD_R\% = 2^{(1-0.5\,log\,C)}$$

Standard and sample preparation

Standard Preparation:

Based on the method development approach design standard preparation. If you are developing RS method by calculation against diluted standard then you need to prepare the standard at specification level of unknown impurity & if you have decided to develop common Assay/RS method then prepare standard at assay level concentration because calculation of impurities is by area normalization.

LOQ Preparation:

LOQ preparation may be designed from the ICH Q3B (R2) guideline. Generally, you can keep reporting threshold concentration as a LOQ preparation, which is calculated from daily dose. There is no thumb rule to select that concentration as a LOQ preparation, you can use lower concentration also for LOQ preparation based on the response & signal to noise ratio of active & all impurities. Industry practice is to keep 0.05% of sample concentration as a LOQ preparation.

Sample Preparation:

Sample preparation is a critical part of method development because it affects the estimation of impurities. The main challenge is how to set concentration. Most of the scientist do major mistake, first they select the sample concentration rather than to set LOQ concentration. This approach misleads the analytical scientist & have to rework on sample preparation just because of S/N ratio is not achieved. Ideal way to decide sample concentration is based on the LOQ preparation using below mentioned equation.

$$Sample\ Conc.\left(\frac{\mu g}{mL}\right) = \frac{Conc.of\ LOQ\ in\ \frac{\mu g}{mL}}{Conc.of\ LOQ\ in\ \%} \times 100$$

Diluent selection plays a major role in sample preparation. The ideal sample diluent should have the following attributes [34].

- Dissolves the major analyte, impurities and degradation products
- is conducive to acceptable peak shape & does not interfere with analyte response
- Prevents analyte interaction with container surfaces, and does not promote analyte degradation.
- Solvent selected for diluent should not be stronger than mobile phase.

Different extraction techniques are used for complete dissolution & recovery of active & impurities like, sonication, wrist action shaking, orbital shaking & magnetic stirring. All these techniques work on different principles, so during method development it is necessary to evaluate the requirement of combination of shaking & sonication technique. Time duration of sonication & shaking plays major role in complete recovery of active & impurities in presence of excipients.

Method Evaluation Using Validation Parameters

Method should be evaluated at the early stage of development using some of the validation parameters. Full validation is not required but question comes to mind that what level of validation is required to evaluate method. The

answer is very simple, all critical validation parameters should be evaluated except robustness because you are going to use the developed method for routine analysis of development batch samples. Verification requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied [35]. Testing of batches is required to support the design of quality-finished product and to fulfill this requirement partial validation is required at early stage of development. This review article covers the systematic approach regarding method evaluation using validation parameters.

Filter compatibility Study

Filtration step is required to avoid any blockage in HPLC system & column by removing undissolved particles of excipients from sample preparation [36]. The main purpose of filter selection is to select filter, which is compatible with sample preparation. Sometimes analyte of interest may get absorbed somewhat in filter if wrong filter is selected. Filter study should be performed on spiked sample with all known impurities at specification level and placebo preparation. To choose the appropriate syringe filter for your application, you need to determine the amount of filtration required or the pore size needed [37].

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present [38]. Typically, these might include impurities, degradants, matrix, etc. To prove that developed method is specific, it should be free from interference due to blank & placebo peaks at the retention time of active & all impurities in spiked sample preparation & in all stress condition samples. Sample preparation spiked with all impurities at specification level should be injected along with blank preparation & placebo preparation. Peak purity parameters are used to prove spectral uniqueness of compound. Peak purity of main component and known impurities in control and degradation samples should be established using PDA (Photo diode array) detector. Forced degradation is a part of specificity study. This article elaborates different stress conditions & purpose of forced degradation study.

Purpose of Forced degradation [39, 41]

- Regulatory requirement of stability indicating methods.
- To identify impurities related to drug substance or excipients that helps in formulation design (DEC study).
- To understand the drug molecule chemistry which helps in selection of product packaging.
- To generate more stable formulations.
- To generate a degradation profile that mimics what would be observed in a formal stability study under ICH conditions.

Stress conditions & study Design

For the related substances method to be stability indicating, the stressed samples should be analyzed using related substances method conditions. In a typical study, relevant stress conditions are light, heat, humidity, hydrolysis (acid / base influence) and oxidation or even a combination of described parameters [40]. Desired degradation level can be achieved by selecting suitable concentration of acid, base and oxidizing agent, applying combination stress (e.g., degradation media+temperature) and exposure time [40]. Generally, 5-10% degradation is optimum to prove stability-indicating nature because excessive degradation will generate secondary degradation products that will never generate during real time storage of finished product and may mislead mass balance results because of difference in response factor of unknown impurities. Now question comes to mind that, 5-10% degradation limit comes from where as it is not mentioned in any regulatory guideline. So answer is that it comes from the assay

specification limit, specification limit for generic product is 95%-105% & for NCE molecule it is 90%-110%. Table-10 depicts the stress study design.

Acid/Base Hydrolysis

Hydrolysis reaction takes place between excipients & drug substance in formulation (Citric acid, Stearic acid, Alginic acid, HPC, HPMC, CMC, etc). For acid hydrolysis hydrochloric acid or sulfuric acids and for base hydrolysis sodium hydroxide or potassium hydroxide are commonly used reagents [41]. Stress study should be initiated at room temperature & then further increase temperature. Selection of temperature should be based on boiling point of selected solvent in diluent. In case of limited solubility of drug substance, stress solutions can be prepared in organic solvent. (Methanolic HCl & NaOH).

Neutral/Water Hydrolysis

Hydrolysis reaction of water present in formulation reacts with drug substance (MCC is hygroscopic & has water content of about 5%). Neutral hydrolysis should be performed using spiking of water in formulation during sample preparation [42]. Neutral hydrolysis should be initiated at room temperature & then further increase temperature.

Oxidation

Peroxide mediated reactions are most common oxidation mechanism, where a drug reacts with hydrogen peroxide [43]. Peroxides are present in commonly used excipients, and, because of this, these reactions occur in most formulations with drug substances that are susceptible to oxidation. Hydrogen peroxide reacts with secondary and tertiary amines, thioethers, and olefins. Hydrogen peroxide can react with both secondary & tertiary amines, but the reaction is more favorable with tertiary amines. Oxidation reaction takes place due to disproportionation of salt form of drugs, which happens during processing like wet granulation, drying, tableting or during storage, because of excipients in the formulation. Another mechanism for oxidation is autoxidation. The term autoxidation classifies the oxidation of a substrate by molecular oxygen. Autoxidation can start a chain process when the oxidized substrate generates a reactive species that subsequently attacks additional substrate molecules [43]. This mechanism is also known as a radical chain reaction, where the addition of oxygen gives rise to hydro peroxides and their associated peroxy radicals. The initiation of oxidation reactions can involve the abstraction of H-atoms from various moieties of the drug substance by the impurity-derived radicals [43]. These can result from the reaction between hydro peroxides and trace amounts of iron or copper ions. Hydrogen peroxide is generally used for oxidation stress & AIBN, $FeCl_3/CuSO_4$ is used to mimic free radical oxidation [43].

Thermal Stress

Thermal degradation test should be performed on active pharmaceutical ingredient and doses form with or without humidity [44]. There are two ways to perform thermal stress on finished product, one is in dry condition that is only heat stress at elevated temperature & another one is in wet condition that is combine stress of heat & humidity to mimic real time stability. Thermal stress result of dry condition helps to control generation of heat sensitive degradation products by modifying manufacturing process. Curing temperature during drying process of granules in wet granulation technique plays major role to generate heat sensitive degradation products. Some degradation products may generate due to combine effect of heat & humidity. Hydrolytic reactions takes place in wet condition & results of this study helps to decide packaging material/ container to control growth of degradation products.

Humidity Stress

Some drug substances are moisture sensitive & generate degradation products [43]. Generally,

oxidation & hydrolysis reaction takes place in moisture sensitive products because addition of oxygen takes place. To control moisture uptake in formulation from atmosphere formulator may either select Blister pack, which reduces the headspace, & another way is to use desiccant / oxygen scavengers in HDPE bottle. Saturated salt solutions of potassium chloride & potassium nitrate are used to produce 85%-90% of relative humidity environment in desiccator [45].

Photo stability

This is very important degradation step for light sensitive molecules, but irrespective to light sensitivity, we should evaluate all molecules to identify any unacceptable change due to light exposure. Some recommendations are described in ICH guidelines Q1B Photo stability Testing of New Drug Substances and Products. samples should be exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200-watt hours/square meter to allow direct comparisons to be made between the drug substance and drug product [46].

Mass balance

Now a days FDA strictly asks the mass balance results of forced degradation study of related substance method to prove stability-indicating nature of analytical method [41]. There is no guideline or industry-accepted practice for mass balance limit for forced degradation studies but we need to demonstrate the mass balance of the method, whatever is achieved if all other validation parameters are OK. Generally, 95 % to 105 % mass balance is well accepted. Mass balance is always challenging to evaluate accurately. A common formula used by pharma industries for mass balance is. Mass balance is always challenging to evaluate accurately. Figure-5 summarizes the all-possible reasons of imbalance of mass [46].

Mass balance (%)=((% Assay of stress sample+% Total impurities of stress sample)/((% Assay of control sample+% Total impurities of control sample)×100

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample [38]. Linearity should be evaluated for all known degradant impurities & active from LOQ level to 150% of specification limit. The correlation coefficient, y-intercept, slope of the regression line, residual sum of squares & % RSD of response factor should be submitted Relative response factor can be calculated from the slope values of impurities & active using below mentioned formula.

RRF=(Slope of impurity)
(Slope of active)

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted as either a conventional true value or an accepted reference value and the value found [38]. Accuracy should be evaluated for all known degradant impurities & active from LOQ level to 150% of specification limit (Table 5).

LOD & LOQ Determination

The detection limit is a lowest amount of analyte that can be detected by analytical method but not necessarily quantify the exact amount [38]. The quantitation limit is the lowest amount of analyte that can be accurately & precisely quantified by analytical method. Many industries set 0.05% of sample concentration as a LOQ & one-third concentration of LOQ as a LOD. Precision at LOQ level should be assessed because quantification of impurities at LOQ level is prerequisite of related substance method.

Risk Assessment of Method

Risk assessment should be done for all analytical methods at early phase of method development to avoid challenges during method validation & transfer activity. Risk assessment at early stage of development phase helps to avoid challenges during validation study. Risk assessment involves the identification of probable route cases that may affect the quality & reproducibility of analytical method [48]. Risk assessment can be done by using risk matrix, decision tree, failure modes and effects analysis (FMEA), and bowtie model. Table 6 summarizes the risk assessment of related substance method.

Risk priority number can be calculated using below formula,

$$RPN=P\times S\times D$$

P= Probability, S= Severity & D= Detection, RPN= Risk Priority Number

Monitoring of System suitability parameters in Routine analysis

Monitoring of predefined system suitability parameters helps to ensure that the system can be used for the analysis purpose using the selected analytical method. Based on the development result you can use any stress condition solution as a resolution mixture for critical pair of impurities or even you can use impurity marker solution for identification purpose. System suitability reduces the risk of failure of analysis & provides the trend of analytical method.

Method Validation

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics [38]. Full validation of related substance method includes Specificity, Linearity, Accuracy, Precision, LOD & LOQ determination & Robustness study. Frequently, validated analytical methods do not perform as expected. For example, chromatographic peaks are not separated as the method predicts. This may happen when:'

- A method is transferred from a development laboratory to a routine lab or between routine laboratories.
- Different instrument models with different characteristics either from the same or different vendors are used, such as HPLCs with different delay volumes.
- The column performance changes over its lifetime.
- New column batches with different characteristics are used.

Conclusion

Related substance is an important test, which ensures the product safety & quality by quantifying impurities available in formulation. To fulfil this requirement, robust analytical method is required to develop & this article provides the right trajectory starting from setting of specification limits for impurities, QbD based method development, method evaluation at early phase of development, risk assessment of method to validation.

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HOW TO CITE: P. Patel, S. Solanki, B. Vihol, An Industry Perspective: How To Develop Analytical Method For Related Substances Of Drug Product By Applying A QbD, Int. J. of Pharm. Sci., 2024, Vol 2, Issue 2, 360-379. https://doi.org/10.5281/zenodo.10673264

Table 1: Significance of Polymorphic Forms

Nane of API	No. of Polymorphic form	Bioavailable form	Significance
Cortisone acetate	5	I	Stable
Methyl prednisolone	2	I	Higher dissolution
Tolbutamide	4	II & IV	Higher solubility, dissolution & oral absorption
Furosemide	2	I	Photochemical stability
Carbamazepine	3	I & III	Less degradation
Phenobarbital	6	A, B & F	More stability

Table 2: Impurities of Emtricitabine Drug Substance

Name of active/impurity	Type of impurity	Origin of impurity
Emtricitabine	Active	NA
S-oxide impurity	Degradant	It arises due to oxidation
Desamino impurity	Degradant	It arises due to base hydrolysis
Lamivudine impurity	Process	Intermediate

Table 3: Common HPLC Buffers & Their Respective pKa

Buffer	PKa	pH range
Phosphoric Acid	2.15	1.1-3.1
Mono/dihydrogen phosphate buffer	7.20	6.2-8.2
	12.33	11.3-13.3
TFA	0.3	Up to 1.5
Formic acid	3.75	2.8-4.8
Acetate buffer	4.76	3.8-5.8
Citrate	3.13	2.1-4.1
	4.76	3.7-5.7
	6.40	4.4-6.4
Triethylamine	10.72	9.8-11.8

Table 4: Evaluation of System Suitability Parameters

Parameter	Purpose	Acceptance Criteria
% RSD of standard preparation	To check injection repeatability	For Assay level standard (% RSD of 5 replicate injections NMT 2.0%)
		For diluted standard (% RSD of 6 replicate injections NMT 5%
Signal to noise ratio	To check method sensitivity at LOQ level for the accurate quantification of impurities at LOQ level	NLT 10
Tailing factor	To check the symmetry of peak	NMT 2.0
Resolution	To check separation efficiency of analytical method for critical pair of peaks.	NLT 2.0

Table 5: Accuracy Sample Preparation

Accuracy of Known impurities			
Level	**Type of sample**	**What should be spiked?**	**Spiking into which?**
LOQ	Placebo	All degradant impurities	Sample stock preparation
100% & 150%	API+ Placebo		
Accuracy of Unknown impurities			

LOQ	Placebo	Active	Sample stock preparation
100% & 150%	Placebo		

TABLE 6: RISK ASSESSMENT OF RELATED SUBSTANCE METHOD

Potential Route cause	Failure Effect	Risk Mitigation	P	S	D	RPN
Water source	Changes to baseline & ghost peaks	Use HPLC grade water & filtered through 0.22 µm membrane filter	2	1	1	2
Reagent Grade	Changes to baseline noise	Use HPLC grade of ammonium acetate	2	1	1	2
Vials	Leaching from vial generates more impurity	Use waters vials only	2	3	3	18
HPLC	Manufacturer of HPLC changes in resolution of impurities	Use Agilent & Waters HPLC only for analysis	3	2	2	12
Sampling rate	Changes to peak tailing	Use 5 points/sec sampling rate	1	2	3	7
Mobile phase stability	Changes to chromatography & generates more backpressure due to precipitation	Do not use mobile phase more than two days	1	3	2	6

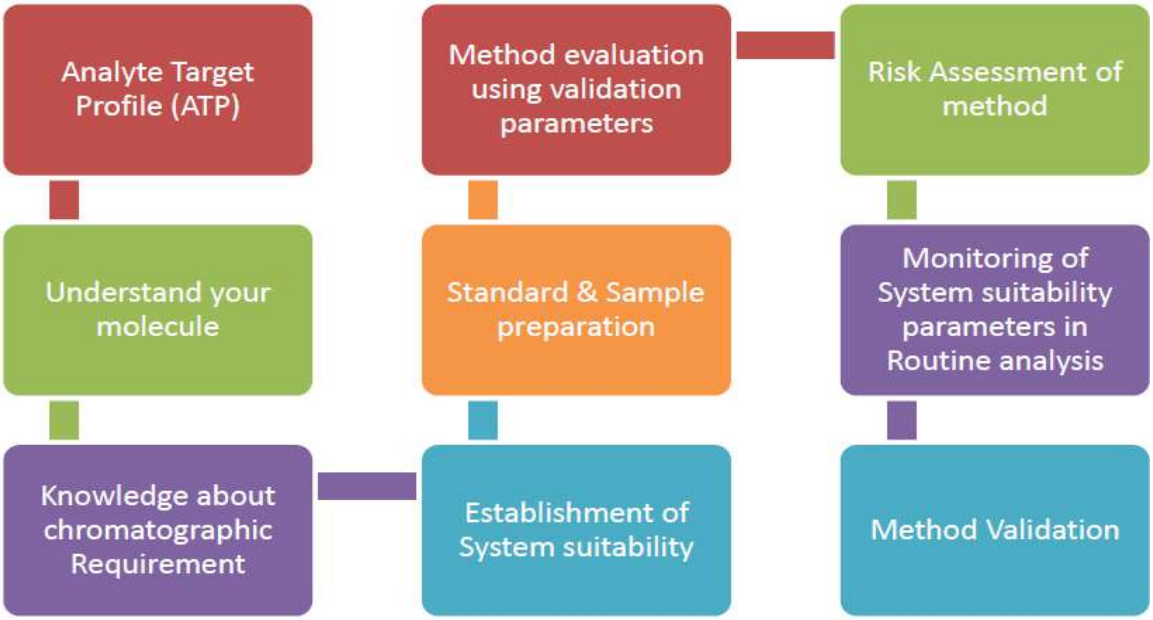

Fig. 1: Analytical method lifecycle

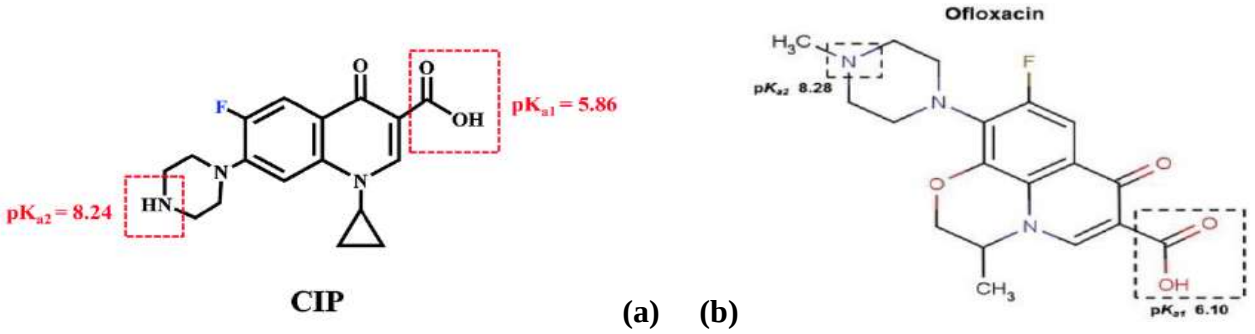

Fig. 2: Chemical structure with pka of a) Ciprofloxacin & b) Ofloxacin

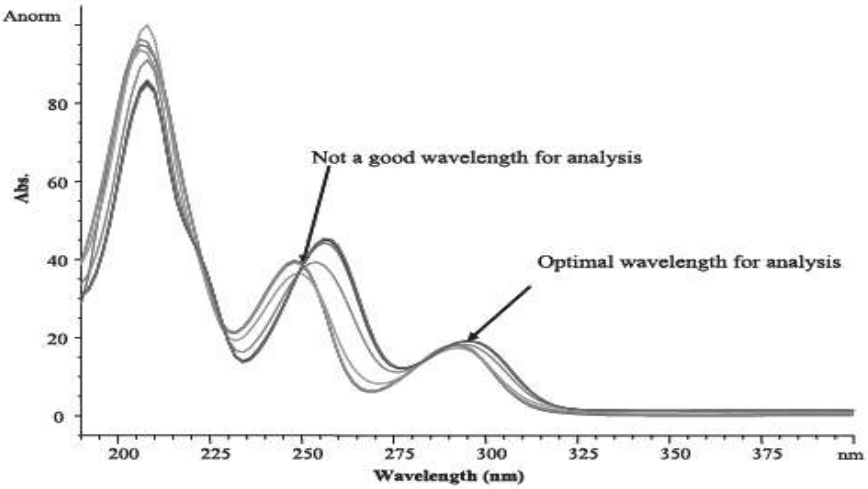

Fig. 3: Wavelength selection based on optimum responses

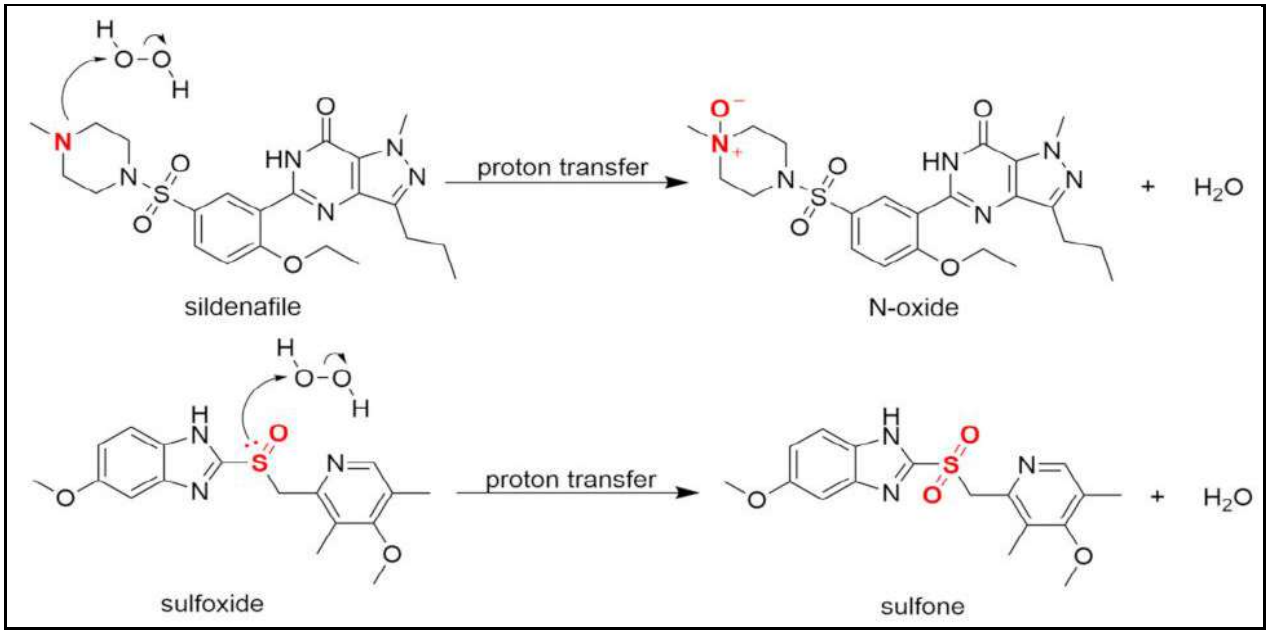

Fig. 4: Peroxide mediated oxidation

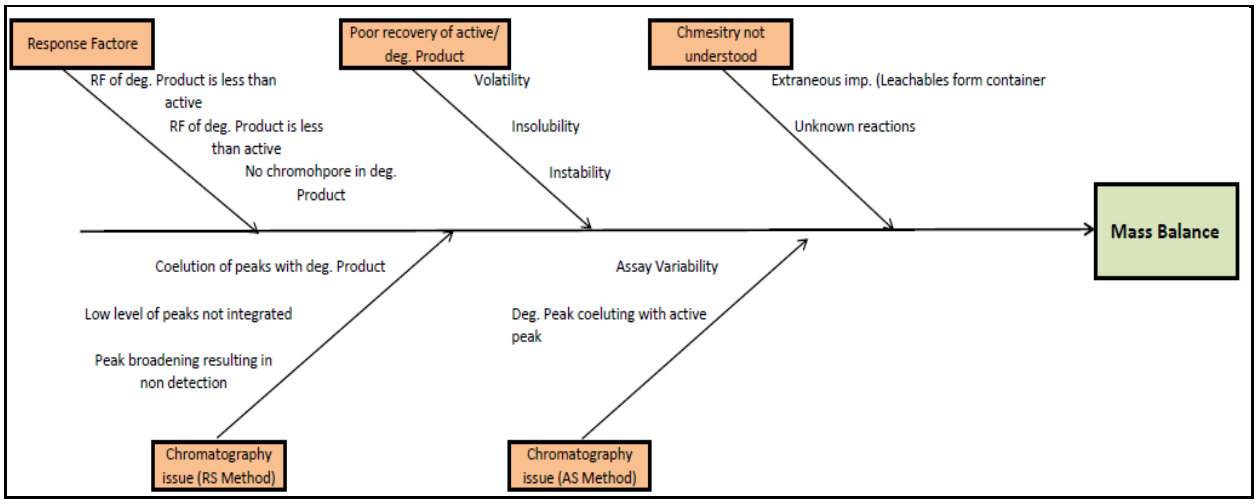

Fig. 5: Fishbone diagram for major causes of mass balance