Chapter-1

Introduction

1.1 Stress degradation

The stability of a drug substance or a drug product is a critical parameter which may affect purity, potency and safety. Changes in drug stability can risk patient safety by formation of a toxic degradation product(s) or deliver a lower dose than expected. Therefore, it is essential to know the purity profile and behavior of a drug substance under various environmental conditions.

Forced degradation is synonymous with stress testing and purposeful degradation. Purposeful degradation can be a useful tool to predict the stability of a drug substance or a drug product with effects on purity, potency, and safety (Steven *et al.*, 2011; John, 2002; Keith *et al.*, 2002; Srinivas *et al.*, 2014). It is imperative to know the impurity profile and behavior of a drug substance under various stress conditions. Forced degradation also plays an important role in the development of analytical methods, setting specifications, and design of formulations under the quality-by-design (QbD) paradigm (Brummer *et al.*, 2011; Kats, 2005; Maheswaran *et al.*, 2012; Ngwa, 2010; Singh *et al.*, 2010). The nature of the stress testing depends on the individual drug substance and the type of drug product (e.g., solid oral dosage, lyophilized powders and liquid formulations) involved. Benefits of stress degradations are summarized below:

a) To develop and validate a stability indicating method.
b) To determine degradation pathways of drug substances and drug products (e.g., during development phase).
c) To identify impurities related to drug substances or excipients.
d) To understand the drug molecule chemistry.
e) To generate more stable formulations.
f) To generate a degradation profile that mimics what would be observed in a formal stability study under ICH conditions.
g) To solve stability-related problems (e.g., mass balance).
   Based on the knowledge of different guidelines and research articles already published, standard approach of forced degradation studies are briefly discussed here.

Stress testing is the main tool that is used to predict stability problems, develop analytical methods, and identify degradation products and pathways. Since there are no detailed regulatory guidelines that describe how to carry out stress testing studies, stress testing has evolved into an artful science that is highly dependent on the experience of the company and of the individuals directing the studies.

Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.

1.2 Historical context of stress study

The terms stress testing and accelerated stability testing were often used interchangeably in the pharmaceutical industry. Usually these topics were discussed as part of an overall discussion of drug stability and/or prediction of shelf life (Kulschreshtha, 1976; Witthaus *et al.*, 1981; Carstensen *et al.*, 1995), although in some cases the focus was on degradation pathways or chemical reactivity/stabilization (Schou, 1960; Stewart *et al.*, 1984; Stewart *et al.*, 1985). In a classic article by Kennon, the effect of increasing temperature (from room temperature to 85°C) on the rates of degradation of pharmaceutical products was discussed in the context of predicting shelf life of pharmaceuticals. This article provided the basis for many articles that followed. The ''Joel Davis Rule,'' i.e., 3 months at 40°C/75% relative humidity is roughly equivalent to 24 months at room temperature (25°C) (Davis, 1991). Interestingly, Carstensen has pointed out that prior to the ''Joel Davis Rule,'' the historical ''rule-of-thumb'' had been that 5 weeks of storage at 42°C is equivalent to two years of storage at room temperature (Carstensen *et al.*, 1995). This rule had been derived from work done in 1948 on the stability of vitamin A and it assumes the same activation energy as found for vitamin A.

It is interesting to consider some of the conditions that have historically been employed in the stress testing of pharmaceuticals, documented both in the ''Analytical Profiles of Drug Substances'' (Florey *et al.*, 1998) and by Singh and Bakshi (Singh *et al.*, 2000). Acidic stress conditions can be found to vary from 0.1N HCl at 40°C for 1 week (with ''negligible

degradation'') (Bridle *et al.*, 1993), to 0.1N HCl at 65°C for 21 days (71.6% degradation) (Singh *et al.*, 2000), to 0.1N HCl at 105°C for 2 months (with considerable degradation'), to 4N HCl under refluxing conditions for 2 days (66% degradation) (Padmanabhan *et al.*, 1989), to 6.5N HCl at 108°C for 24 hr (50% degradation), to concentrated HCl at room temperature (56.5% degradation) (Groningsson *et al.*, 1985). Similar elevated temperatures, times, and base strength have been employed for basic stress conditions. For example, conditions can be found to vary from 0.1N NaOH at 40°C for 1 week (with negligible degradation) (Bridle *et al.*, 1993), to 0.1N NaOH at 65°C for 21 days (with 100% degradation) (Singh *et al.*, 2000), to 0.1N NaOH under refluxing conditions for 2 days (68% degradation) (Padmanabhan *et al.*, 1989), to 1N NaOH under boiling conditions for 3 days (7.2% degradation) , to 5N NaOH under refluxing conditions for 4 hr (100% degradation) (Muhtadi, 1988). In terms of oxidative degradation studies, hydrogen peroxide has been employed at strengths from 0.3% to 30%. Studies were often conducted at elevated temperatures, e.g., 37°C for 6 hr [3% hydrogen peroxide, 60% degradation (Nassar *et al.*, 1993)], 50°C for 72 hr (3% hydrogen peroxide, 6.6% degradation), and even refluxing conditions for 30 min (3% hydrogen peroxide, extensive degradation) (Muhtadi, 1988) or 6 hr (10% hydrogen peroxide, no significant degradation) (Johnson *et al.*, 1996).

As these examples illustrate, historically there has been tremendous variation in the conditions employed in acid/base and oxidative stress testing studies. There has also been tremendous variation in defining the appropriate endpoint of the stress testing studies, i.e., what length of time (and temperature) or amount of degradation is sufficient to end the stress exposure.

Perhaps the most dramatic variability in stress testing conditions is observed in the photostressing of drugs (Singh *et al.*, 2000), where the lamps and exposures range from short wavelength Hg arc lamps (254 nm, UVC range) to fluorescent light to artificial light to halogen lamps to xenon lamps. The variability of photo exposure during pharmaceutical photostability studies has also been documented by surveys of the pharmaceutical industry (Anderson *et al.*, 1991; Thoma, 1996; Thatcher *et al.*, 2001).

From the information provided above, it is apparent that stress-testing conditions have varied greatly from compound to compound and from investigator to investigator. Extremely harsh

conditions have been commonly used in the past to ensure degradation, even if the conditions far exceeded plausible exposures.

More recently, several articles relevant to stress testing have appeared in the pharmaceutical literature. A paper by Singh and Bakshi (Singh *et al.*, 2000) in 2000 provides the most thorough collection of references to various degradation studies of drug products, documenting the diversity of conditions and approaches to stress testing. This paper attempts to provide a classification system (Extremely labile, very labile, labile, and stable) based on a defined systematic approach. It is not clear from the article on what basis (scientific or otherwise) the classification system was devised; however, the paper does define ''endpoints'' to stressing (albeit, fairly harsh endpoints), allowing for the conclusion that a particular compound may be regarded as stable under a certain set of conditions.

In 1992 (and again in 1994), Boccardi provided some needed guidance on oxidative stress testing by asserting that most pharmaceutical oxidative degradation was the result of autoxidation and that hydrogen peroxide was not a very good reagent to mimic autoxidation processes. Boccardi was the first to describe the use of radical initiators such as azobisisobutyronitrile (AIBN) for oxidative pharmaceutical stress testing, and he provided a simple procedure with mild conditions he termed ''The AIBN Test.'' In 1996, Baertschi presented and discussed an approach to stress testing that had defined limits of harshness and exposure time. In 1998, Weiser (Weiser, 1998), in discussing the role of stress testing in analytical method development, suggested a set of conditions for performing stress testing that was arguably milder than many of the historical studies cited above. In 2001, Alsante *et al.* provided a guide to stress testing studies that suggested defined limits to the stress conditions. For example, for acidic and basic stressing, Alsante suggested conditions of 1N HCl and 1N NaOH for a maximum of 1 week at room temperature. In 2002, the views of the Pharmaceutical Research and Manufacturer's Association (PhRMA) were summarized in an article on forced degradation studies published in Pharmaceutical Technology (Reynolds *et al.*, 2002). The PhRMA article did not discuss specifics of conditions of stress, but rather focused more on what kinds of stress testing should be performed for drug substances and products and on the regulatory requirements.

A degradation study of haloperidol utilized 1M HCl and 1M NaOH (refluxed for 5 hr), and 30% hydrogen peroxide (70°C for 5 hr) for the most stressful conditions of the study (Trabelsi *et al.*, 2002). These conditions appear to have been chosen to enable production of known degradation products (six degradation products shown) to facilitate HPLC method validation efforts. A degradation study of ibuprofen produced 13 degradation products, several of which had never before been detected (Caviglioli *et al.*, 2002). In this study, oxidative studies were carried out utilizing potassium permanganate (0.05M) at room temperature up to 16 hr in 0.5M NaOH; up to 33% hydrogen peroxide at room temperature for 22 hr; and potassium dichromate (0.1 N) at room temperature up to 14 days in 0.5M HCl. Solid-state studies utilized 50°C up to 8 months and 100°C up to 16 hr to detect volatile degradation products. An NMR study of the aqueous degradation of isophosphoramide mustard was conducted in buffered aqueous solutions in the pH range of 1–13 (Breil *et al.*, 2001). The degradation of sumatriptan in 0.1N HCl, 0.1N NaOH, and in 3% hydrogen peroxide was studied using LC/MS and LC/MS/MS (44). The solutions were heated at 90°C for 30 min to 9 hr. Photostability was assessed by exposure to UV irradiation at 254 nm for 24 hr (no indication of irradiation intensity). A study of the major oxidative degradation products of SCH56592 was conducted by exposure of the drug substance in the solid state to 150°C for 12 days with identification of the major products using LC-MS and LC-NMR (Feng *et al.*, 2001). Singh et al. (Bakshi *et al.*, 2001) described stress degradation studies of ornidazole and prazosin, terazosin, and doxazosin (Ojha *et al.*, 2003) under conditions designed to be in alignment with the ICH Stability guideline (Q1AR). In the case of ornidazole, significant degradation was seen under acidic conditions of 0.1M HCl to 5M HCl at 80°C for 12–72 hr, although no degradation products were detected (presumably because of degradation to non-chromophoric products). Studies under basic conditions of 0.1M NaOH at both 80°C and 40°C revealed complete degradation at time zero. Milder studies were then conducted at pH 8 and 40°C. Oxidative studies involved 3% and 30% hydrogen peroxide at room temperature for 24 and 48 hr, with losses of 8% and 53% of the parent, respectively. Photodegradation studies utilized Option 2 of the ICH photostability guideline with exposures up to 30 days at 7000 lux (over 5 million lux-hr exposure). Similar conditions were employed for prazosin, terazosin, and doxazosin.

In these recent examples of stress testing studies, it is apparent that there is still a great diversity of conditions employed to induce degradation, although the diversity is arguably less than was observed prior to publication of the ICH guidances. This continued diversity of approach could be interpreted in a couple of ways. One interpretation is that stress-testing studies are inherently a research undertaking, and therefore flexibility and scientific judgment are required, leading to diverse conditions and approaches.

Another interpretation is that there is (appropriately or inappropriately) very little guidance (either regulatory or in the scientific literature) on the specifics of the conditions or appropriate endpoints of pharmaceutical stress testing. We assert that both interpretations are valid. The goal of my research is to provide additional scientific guidance to the researcher to enable sound, practical, and reasonably consistent approaches to pharmaceutical stress testing.

1.3 Regulatory context of stress study

The guidance does not explicitly require stress testing be performed or reported at the Phase 1–2 IND stages although it is encouraged to facilitate selection of stability indicating methods. Experience has shown, however, that regulatory authorities may still ask questions concerning results from stress testing as early as a Phase 1 IND, especially where potentially toxic degradation products are possible. The guidance does require stress testing for the Phase 3 IND for drug substances and suggests these studies be conducted on drug products. At Phase 3, the guidance strongly suggests, but does not always require, that degradation products detected above the ICH identification thresholds during formal stability trials should be identified. For an NDA, the guidance requires a summary of drug substance and drug product stress studies including elucidation of degradation pathways, demonstration of the stability indicating nature of analytical methods, and identification of significant degradation products. Stressing the drug substance under hydrolytic, oxidative, photolytic, and thermolytic conditions in solution and the solid state is required. The design of drug product studies is formulation dependent and is left to the discretion of the applicant.

Although not necessarily directly related to stress testing, the guidance also requires demonstration and/or a summary of an investigation of mass balance in degraded samples from

formal stability trials, an assessment of the drug's stereochemical stability, and distinguishing drug related and non-drug-related degradation products. However, these issues can often be addressed in stress studies fulfilling both scientific need and regulatory requirements. The predictive nature of well-conducted stress studies can forewarn of potential problems in these areas early-on facilitating appropriate and efficient changes in the development strategy if required.

The guidance suggests the analytical assumptions made when determining mass balance should be explained in the registration application. Failure to demonstrate mass balance may be acceptable provided a thorough investigation has been conducted to understand the chemistry of the molecule. Examining mass balance in stressed samples can reveal the need for better analytical methodology from the start.

The guidance recommends treating chiral impurities as though they were achiral impurities with the caveat that the ICH identification and qualification thresholds may not apply for analytical reasons. Experimental demonstration that stereoisomers of the drug substance and its degradation products do not form during stress studies can obviate the need for testing for these potential impurities during formal stability trials. Experience has shown that merely arguing a particular chiral center is unlikely to invert on strictly theoretical grounds is unacceptable to the FDA.

Differentiation between drug-related and non-drug-related degradation products can be achieved with stress studies of the drug substance, drug product, and placebo. These studies should allow discrimination between synthetic process impurities, excipients, degradation products derived from excipients alone and drug-related degradation products including drug excipient combinations.

The guidance suggests the potential for reactions between active ingredients in combination products should be investigated. For a triple combination tablet formulation, the FDA suggested stressing the three actives together under conditions usually applied to a single drug substance. These studies were conducted and reported in the NDA.

The guidance specifies identification thresholds for degradation products observed in formal stability samples of the drug substance and product that depend upon the dosage. Consideration for not identifying degradation products which are detected at the threshold levels is given for degradation products which are unstable. In those cases, a summary of the efforts to isolate and identify the unstable degradation product may suffice.

1.4 Interpretation of the results of stress testing

One of the more interesting and challenging aspects of stress testing involves interpreting the results of stress testing such that the data becomes meaningful to the development process. For example, what levels of instability are indicative of problems for analytical handling, manufacturing/processing, formulation, patient in use, and storage and distribution? Can stresstesting results be easily interpreted to conclude whether or not a compound will have high-, medium-, or low-stability concerns for the development process? These questions are difficult as there are no (as yet) scientifically derived criteria for predicting the develop ability of a new drug entity. Such interpretive criteria would be very useful for the pharmaceutical development process.

Attempts have been made to use stress-testing results to classify compounds as extremely labile, very labile, labile, and stable (Singh *et al.*, 2000). The basis for this classification system was the personal experience of the authors.

Interpretation of stress-testing results has several different facets. An obvious consideration is the degradation rate under a particular condition. The rates of degradation are critically important for determination of shelf life (although stress-testing results should not be used for shelf-life determination) and for handling considerations during manufacturing, formulation, and analysis. In addition to the kinetic interpretation, it is important to understand what the stress-testing results indicate about mechanism of degradation (e.g., from the structures of degradation products and the degradation pathways involved). Such information is important in designing the appropriate degradation-control strategies (Olsen *et al.*, 2003) (e.g., developing a stable formulation, appropriate packaging and storage conditions, and relevant analytical methodologies).

1.4.1 Solid state

There are three main stress conditions evaluated during stress testing in the solid state: temperature, humidity, and photostability. The stress of elevated temperature is perhaps the stress condition that lends itself most directly to predictive interpretation. This assumes, of course, that Arrhenius kinetics is observed within the range of long-term storage temperature to the stressed temperature(s). Assuming Arrhenius kinetics and reasonable energies of activation, predictions can be made to relate the amount of degradation and increases of individual degradation products at the stress temperature to the long-term storage temperature.

The solid-state stress of elevated humidity provides information related to the need for protection from exposure to high or low humidity. Such information is generally considered to be only semiquantitative in nature, that is, whether or not the stability of the compound is best at high or low humidity. Quantitative correlations (e.g., is there a critical relative humidity threshold?) are difficult and would require additional experiments (e.g., statistically designed experiments).

The solid-state stress of exposure to light (i.e., ultraviolet and visible radiation) is guided by the ICH guideline on photostability. The ICH guideline is primarily directed toward confirmatory photostability testing, which outlines photoexposure levels for the purpose of identifying potential photostability problems that may be encountered during storage and distribution of the marketed product. Most of these types of problems can be addressed by modifications of packaging, labeling, and/or formulation (with some associated expense).

The forced degradation studies should be designed to provide suitable information to develop and validate test methods for the confirmatory studies. These test methods should be capable of resolving and detecting photolytic degradants that appear during the confirmatory studies. When evaluating the results of these studies, it is important to recognize that they form part of the stress testing and are not therefore designed to establish qualitative or quantitative limits for change.

Thus, photostressing studies are primarily useful for developing an understanding of the photochemistry of the drug and for developing approtopic.

One possible use of photostability/photostressing studies is assessment of the potential for problems in manufacturing and analytical handling. It has been recommended by EFPIA that 100,000 lux-hr is a reasonable amount of light exposure to determine whether or not special precautions should be considered during manufacture (Anderson, 1996). This level of light exposure should also provide a reasonable estimation of problems that might be encountered during analytical and formulation development. Of course, the potential for exposure to UV light may also need to be assessed depending on the lighting conditions of the analytical laboratories and the manufacturing facility. The best way to assess the potential light exposure would be to make actual measurements in the actual laboratories and manufacturing facilities.

1.4.2 Solution: Acid/base stress-testing results

These results will indicate whether or not the drug molecule has a particular instability in aqueous conditions as a function of pH. An approximate pH profile can sometimes be constructed from the data, but caution should be exercised because it is not uncommon for a drug compound to be relatively insoluble in some pH ranges, and some samples may require cosolvents in order to achieve dissolution. If precise information on either kinetics or the precise pH range of maximum stability is desired (e.g., for a compound that will have a liquid formulation), further studies in which the solvents, the ionic strength, the buffer type, and the concentration are carefully controlled should be conducted.

Stress testing information can be relevant for ADME concerns. Critical evaluation of the pH 1–2 degradation rate data can help assess the potential need for enteric coating of drugs to be administered orally. The pH of gastric fluid (worst case) can be roughly simulated using 0.1N HC1 (pH1). While transit times in the stomach are highly variable (dependent on variables such as %liquids/solids ingested, amount ingested, fasting level), most literature values fall within the time of 30 min to 4 hr (Dressman, 1986). Assuming a worst case transit time (4 hr exposure to the acidic environment in the stomach), it seems reasonable to consider the potential need for an enteric-coated formulation if a loss of potency of~10–20% or greater is observed after 4 hr at 40°C in 0.1N HC1; assuming first-order kinetics of degradation, this degradation rate would correspond to a half-life of ~26 hr. Consideration of the potential need for enteric coating would involve many factors. For example, do the acidic degradation products raise questions of safety

or efficacy? How rapid is the absorption? What is the half-life of the compound in the body? What are the pharmacological needs for efficacy of the parent? These questions need to be addressed on a case-by-case basis in an interdisciplinary manner (e.g., scientists from ADME, pharmacokinetics, analytical, formulations, and medical/clinical areas).

Evaluation of solution stability under neutral to moderately basic (e.g., pH 6–9) can help in assessing the potential for nonenzymatic break down of the compound under physiologically relevant conditions (e.g., pH 7.5). Such information can be useful not only for metabolism studies, but also for pharmacokinetic concerns (i.e., understanding how long a compound might remain intact in vivo) and what degradation products might form under such conditions. Thus, for example, if a compound degrades at pH 7.5 at a rate significantly faster than the half-life of the compound, the nonenzymatically formed degradation products will likely contribute to the metabolic profile of the compound.

Evaluation of degradation rates under different pH conditions also provides needed information related to analytical concerns. For example, how much degradation might occur during the analytical workup under specific pH conditions? Can samples be prepared and held at room temperature for a specified length of time without appreciable degradation? Do samples need to be refrigerated in order to maintain stability during analysis? Answers to these questions are fairly straightforward if the analytical constraints are defined.

1.4.3 Solution: Oxidative stress-testing results

It can be difficult to translate oxidative stress-testing results into accurate predictions of the susceptibility of a compound to oxidation. This is partially because oxidative mechanisms can be quite diverse and complex and oxidative degradation often does not follow typical Arrhenius kinetic models.

Boccardi suggests that the two most relevant tests for prediction of oxidative susceptibility of a drug compound involves the use of radical initiators (such as AIBN) for testing susceptibility to autoxidation and the use of dilute hydrogen peroxide for testing susceptibility to oxidation by peroxides (e.g., from excipients). Boccardi asserts that, assuming a test involving use of AIBN in

an equimolar basis with the drug, after48 hr at 40°C, compounds that are sensitive to autoxidation will likely be degraded >10% whereas compounds that are relatively stable to autoxidation will likely be degraded no more than a few percent. Harmon (Harmon, 2004) has recently developed an oxidative susceptibility stress test for ranking of compounds with respect to their potential for oxidizing in typical oral dosage formulations. The predictability of this promising new system remains to be documented in the literature with examples. A scientifically sound approach to quantitative assessment of the oxidative susceptibility of pharmaceuticals would be a valuable contribution to the future of pharmaceutical development.

In the case of hydrogen peroxide, it is difficult to associate a percent degradation with a classification of oxidizability. If there are amines present in the molecule, especially tertiary amines, oxidation is usually rapid if the amine is uncharged (e.g., the free base). A protonated cationic amine is protected and the oxidation rate will be greatly reduced. In general, however, it is the experience of the authors that if a 0.3% solution of hydrogen peroxide induces <5% degradation in 24 hr at room temperature, the compound is not particularly sensitive to peroxides and will likely not require special considerations for development. Alternatively, an ~20% or more degradation in 24 hr may indicate a particularly sensitive compound that could require special efforts in the formulation and/or storage conditions to ensure oxidative stability.

1.5 Analytical considerations of stress testing

Stress testing is likely to be carried out on a single batch of the drug substance. It should include the effect of temperatures [in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing],humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing.

1.5.1 Data gathering

The first task before beginning stress-testing studies is to gather all the relevant information about the compound. Information such as molecular structure, solubility, pK(s), known chemical instability, hygroscopicity, enantiomeric purity, etc. is important. In addition, previously

established analytical methods may provide a starting point for development of more discriminating methods required for the separation of the complex mixtures which may result from stress degradation.

The molecular structure of a compound is very important. For example, one can usually deduce from the structure whether or not the compound will absorb UV radiation and be detectable with a UV detector. The molecular structure also reveals if the compound has ionizable functional groups and will require a mobile phase modifier if HPLC analysis is used. Examination of the molecular structure may also tell something about the chemical reactivity of the molecule. The molecular structure indicates whether the molecule contains any chiral centers. If the molecule is chiral and nonracemic, then an assay to determine chiral stability may be required.

Knowledge of the solubility of the compound, particularly the aqueous solubility, is required in order to design the study. If the aqueous solubility is too low, then an organic co-solvent may be utilized to achieve solutions for stressing.

1.5.2 Preliminary Studies

Unless a significant amount of information about the stability of the molecule is known, it will probably be necessary to conduct some preliminary studies to gain some basic information about the stability of the compound. The samples generated in this preliminary study can also be used to aid in the development of an analytical method, if needed. Generally, the goal of stress testing is to facilitate an approximate 5–20% degradation of the sample under any given condition (if possible after reasonable limits of stressing).

In the preliminary investigation, observations are made regarding sample stability including exposure of solid state samples to heat, humidity, and light and exposure of solutions to pH extremes, oxidative conditions (hydrogen peroxide and a radical initiator such as 2,20-azobisisobutyronitrile (AIBN)), light and heat. Table1.1 lists some typical stress conditions for preliminary studies.

Table 1.1: Typical stress conditions for preliminary studies

Sample condition	Time/exposure
Solid/70°C	1 week
Solid/70°C/75% RH	1 week
Solid/simulated sunlight	2–3 x ICH confirmatory exposure
Aqueous solution/simulated sunlight	2–3 x ICH confirmatory exposure
0.1N HCl solution/ up to 70°C	1–7 days
Aqueous solution/up to 70°C	1–7 days
pH 8 solution/up to 70°C	1–7 days
0.1N NaOH solution/up to 70°C	1–7 days
0.3% H$_2$O$_2$ solution/ambient in the dark	1–7 days
Solution with radical initiator/40°C	1–7 days

1.5.3 Stress test screen

After conducting some preliminary studies and developing analytical methods it is time to design the stress test screen. Unfortunately, it is impossible to devise a universal set of stress conditions since there is significant variability in the stability of drugs. What can be defined, however, are suggested upper limits for the various stress conditions that can be used as starting points for stress-testing studies. If no degradation can be induced at these proposed maximum stress conditions, then it is concluded that the molecule is stable. The proposed upper limits are listed in table 1.2 – 1.3.

Table 1.2: Typical stress conditions for preliminary studies

Sample condition	Time
70°C	4 to 6 weeks
70°C NaCl ~75% RH	28 days
Photostress	Exposure 2–5 times ICH exposure levels defined in Q1B, Photostability Testing of New Drug Substances and Products

Table 1.3: Proposed maximum stress conditions for solutions or suspensions of drug substances

Solution or suspension	Storage condition	Time
0.1N HCl, water, NaOH, and any buffers between pH 1 and 14	70°C	14 days
80/20 ACN/H$_2$O containing a radical initiator such as AIBN	40°C	7 days
Water (consider buffering compounds which have ionizable functional groups both below and above pKa)	Photostress	Exposure 2–3 times ICH exposure levels defined in Q1B, Photostability Testing of New Drug Substances and Products
Dilute hydrogen peroxide (0.3–3%) solutions containing dilute metal salt (e.g., Fe(III), Cu(II))	25°C	7 days
0.1N HCl solution/ up to 70°C	40°C	1 day

1.5.4 Design of study

Taking into account the information derived from the preliminary study, one can devise a more detailed stress test study. Tables 1.4 and 1.5 list the proposed conditions and analytical time points for stress testing of a drug substance that appeared to be reasonably stable at the proposed upper stress conditions. Additional conditions can be added if deemed necessary.

Table 1.4: Proposed conditions and time points for a detailed study (solid-state stress)

Storage condition	Time points
70°C	7, 14, 28 days
70°C NaCl ~75% RH	7, 14, 28 days
Photostress	Exposure 2–3 times ICH guideline

Table 1.5: Proposed conditions and time points for a detailed study (solution stress)

Solution or suspension	Storage condition	Time
0.1N HCl, pH 3, pH 5, pH 7, pH 9, pH 11, 0.1N NaOH, and water	70°C	3, 7, 14 days
80/20 ACN/H$_2$O containing a radical initiator such as AIBN	40°C	3, 7 days
Water (consider buffering compounds which have ionizable functional groups both below and above pKa)	Photostress	Exposure 1 and 3 times the ICH exposure levels defined in Q1B
Dilute hydrogen peroxide (0.3–3%)	25°C	3, 7 days
Solutions containing dilute metal salt (e.g., Fe, Cu)	40°C	1 hr, 1 day

1.5.5 Sample preparation general

Solid-state samples can be prepared by accurately weighing the drug substance into a container that can be stored under the appropriate condition. Suitable containers include volumetric flasks, scintillation vials, etc. The amount of drug substance used is usually dictated by the availability of material, accuracy of balances, and the final concentration desired for analysis. Typical amounts used for solid-state samples would be between 2.0 and 20.0 mg. For example, if the final analytical concentration desired is 0.3 mg/ml, solid-state samples of approximately 3.0 mg in 10.0 ml volumetric flasks could be stressed and then simply diluted to volume at the time of assay. Alternatively, samples could be prepared in scintillation vials, stressed, and then diluted with a known amount of solvent.

Solid-state samples can be pre-weighed into the respective containers before stressing. Pre-weighing the samples simplifies the analysis of stressed samples by eliminating any concerns about changing levels of volatile constituents such as water or organic solvents during thermal stress. The ICH guideline on photostability indicates that samples for photoexposure be less than 3mm in depth.

Occasionally, one will have to deal with hygroscopic drug substances or drug substances containing a significant level of a volatile compound (e.g., solvates). These types of drug substances can pose significant issues when preparing samples for quantitative analysis. Fortunately, most of these issues can be overcome by using a simple approach. A simple method for eliminating volatile content issues is to allow samples to come to equilibrium with the environment and then conducting all of the weighing of samples and standards over as short a time frame as possible. Performing a volatiles analysis (i.e., TGA) both before and after sample weighing will provide assurance that no significant change in volatiles content occurred over the weighing period.

1.5.6 Solution samples

Solution samples can be prepared in a number of different ways. One way is to prepare a single stock solution at a known concentration for each stress condition and then pull aliquots at the desired time points. This method requires that the container used for the solution be tightly closed to prevent evaporation. If evaporation is a problem, it can be overcome by preparing a separate sample for each time point at a concentration higher than the analytical concentration. For example, if the final analytical concentration desired is 0.3 mg/ml, solutions could be prepared at a concentration of approximately 1.0 mg/ml by adding 3.0 ml of the appropriate solvent to samples of approximately 3.0 mg in 10.0 ml volumetric flasks. Prior to assay, the samples can then be diluted to volume to achieve the final analytical concentration.

1.5.7 Suspension or slurry samples

Suspensions or slurries pose a problem since by definition they are not homogeneous. The problem is how to obtain reliable quantitative results from suspensions. One method for dealing with suspensions is to prepare individually weighed samples and stress them at concentrations greater than the final analytical concentration. Prior to analysis the samples are then diluted to the final analytical concentration with a solvent that completely dissolves the sample. For example, if the final analytical concentration desired is 0.3 mg/ml, suspensions could be prepared at a concentration of approximately 1.0 mg/ml by adding 3.0 ml of the appropriate solvent to samples of approximately 3.0 mg in 10.0 ml volumetric flasks. Prior to assay, the samples can then be diluted to volume with a solvent capable of completely dissolving the sample.

1.5.8 Standards

The assay of stressed samples will usually require the use of some type of external standard. The external standard could be an established reference standard, however, the preferred method is to use the same material/lot as is being stressed. This is easily accomplished by weighing additional samples (that will not be stressed) for use as standards at the same time as the stress test samples are weighed. The standards should then be stored under conditions that will assure that no degradation will occur (e.g., freezer). At the time of analysis, the stressed samples are simply assayed vs. the freshly prepared unstressed standards and the results calculated as percent initial.

1.5.9 Solution and buffer preparation

Typically 0.1N HCl and 0.1N NaOH are used for the pH extremes of aqueous solution stressing (i.e., pH 1 and 13). Since neither of these solutions possesses significant buffering capacity, the pH of the solution should be verified following addition of the drug to these solutions. In order to obtain solutions at pH values between 1 and 13, a buffer must be used. It is desirable to use the same buffer for all the pH levels to avoid chemical differences between different buffers, since buffers are not always inert and can sometimes act as catalysts for drug degradation or even react with the drug being studied (Richter et al., 2004). Unfortunately, no single buffer provides buffering capacity across this wide pH range. A common practice is to make the buffer of sufficient ionic strength such that it still offers some pH stability even outside of its normal buffering range (e.g., 50mM phosphate). For example, if pH values of 3, 5, 7, 9, and 11 are desired, a phosphate buffer can be used keeping in mind that the buffering capacity will be low at pH5 and pH 9. If more buffering capacity is required, then other buffers or a combination of buffers can be used. The buffering range for several common buffers is given in table 1.6.

Table 1.6: Proposed maximum stress conditions for solutions or suspensions of drug substances.

Buffer	pKa	Buffer range
Phosphate	2.1	1.1-3.1
	7.2	6.2-8.2
	12.3	11.3-13.3
Citrate	3.1	2.1-4.1
	5.4	4.4-6.4
Formate	3.8	2.8-4.8
Succinate	4.2	3.2-5.2
Acetate	4.8	3.8-5.8
Citrate	3.1	2.1-4.1
	4.7	3.7-5.7
	5.4	4.4-6.4
Tris	8.3	7.3-9.3
Borate	9.2	8.2-10.2

1.6 Acid and base hydrolysis

Acid and base hydrolytic stress testing can be carried out for drug substances and drug products in solution at ambient temperature or at elevated temperatures. The selection of the type and concentrations of an acid or a base depends on the stability of the drug substance. A strategy for generating relevant stressed samples for hydrolysis is stated as subjecting the drug substance solution to various pHs (e.g., 2, 7, 10-12) at room temperature for two weeks or up to a maximum of 15% degradation. Hydrochloric acid or sulfuric acid (0.1M to 1M) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0.1M to 1M) for base hydrolysis are suggested as suitable reagents for hydrolysis. For lipophilic drugs, inert co-solvents may be used to solubilize the drug substance. Attention should be given to the functional groups present in the drug molecule when selecting a co-solvent. Prior knowledge of a compound can be useful in selecting the stress conditions. For instance, if a compound contains ester functionality and is very labile to base hydrolysis, low concentrations of a base can be used. Analysis of samples at various intervals can provide information on the progress of degradation and helps to distinguish primary degradants from secondary degradants.

1.6.1 Procedure of conducting acid-base degradation

It is important to conduct forced degradation studies to obtain degraded samples wherever degradation possible from about 1% to 30%. For acid stress, reflux may be necessary to 0.1N HCl to 2N HCl can be carried out at 60°C, 70°C or 80°C for 30 minutes to 24 hours. For base stress, reflux with 0.1N NaOH to 2N NaOH at 60°C, 70°C or 80°C for 30 minutes to 24 hours. For water stress we should reflux the drug with water at 60°C, 70°C or 80°C for 30 minutes to 24 hours. Stress agent can be changed to achieve degradation if necessary. Co-solvent can be used to dissolve and extract the drug, where necessary.

1.7 Oxidation

Oxidative degradation can be complex. Although hydrogen peroxide is used predominantly because it mimics possible presence of peroxides in excipients, other oxidizing agents such as metal ions, oxygen, and radical initiators (e.g., azobisisobutyronitrile, AIBN) can also be used. Selection of an oxidizing agent, its concentration, and conditions depends on the drug substance. Solutions of drug substances and solid/liquid drug products can be subjected to oxidative degradation. It is reported that subjecting the solutions to 0.1%-3% hydrogen peroxide at neutral pH and room temperature in dark place for seven days or up to a maximum 20% degradation could potentially generate relevant degradation products. Samples can be analyzed at different time intervals to determine the desired level of degradation.

Different stress conditions may generate the same or different degradants. The type and extent of degradation depend on the functional groups of the drug molecule and the stress conditions.

1.7.1 Procedure of conducting oxidative degradation

For this, it is necessary to conduct the following forced degradation studies to obtain degraded samples wherever degradation possible from about 1% to 30%. For oxidation stress, we can treat the drug with 1% H_2O_2 at less than 30°C for 30 min. The oxidative stress testing is initially carried out in 3% H_2O_2 at room temperature for 6 hours and it can be increased/ decreased to achieve sufficient degradation. Stress agent can be changed to achieve degradation if necessary. Co-solvent can be used to dissolve and extract the drug, where necessary.

1.8 Photostability

Photostability testing should be an integral part of stress testing, especially for photo-labile compounds. Some recommended conditions for photostability testing are described in ICH Q1B. Samples of drug substance, and solid/liquid drug product, should be exposed to a minimum of 1.2 million lux hours and 200 watt hours per square meter. The same samples should be exposed to both white and UV light. To minimize the effect of temperature changes during exposure, temperature control may be necessary. The light-exposed samples should be analyzed for any changes in physical properties such as appearance, clarity, color of solution, and for assay and degradants. The decision tree outlined in the ICH Q1B can be used to determine the photo stability testing conditions for drug products. The product labeling should reflect the appropriate storage conditions. It is also important to note that the labeling for generic drug products should be concordant with that of the reference listed drug (RLD).

1.8.1 Procedure of conducting photolytic degradation

Conduct the following forced degradation studies to obtain degraded samples wherever degradation possible from about 1% to 30%. For this we should expose the bulk powder to ultraviolet radiation up to minimum of 200 watts hour/m2 and minimum of 1.2 million lux hour for visible light and photo stability chamber. If photo stability chamber is not available exposure of the powder/content of to intense ultraviolet radiation (both at longer and shorter wavelengths) up to minimum of 7 days in UV cabinet could be an alternative option.

1.9 Heat or thermal degradation

Samples of solid-state drug substances and drug products should be exposed to dry and wet heat, whereas liquid drug products can be exposed to dry heat. It is recommended that the effect of temperature be studied in 10ºC increments above that for routine accelerated testing, and at 75% relative humidity or greater. Studies may be conducted at higher temperatures for a shorter period. Testing at multiple time points could provide information on the rate of degradation and primary and secondary degradation products. In the event that the stress conditions produce little or no degradation due to the stability of a drug molecule, one should ensure that the stress applied is in excess of the energy applied by accelerated conditions (40º for 6 months) before terminating the stress study.

1.9.1 Procedure of conducting heat or thermal degradation

It is necessary to conduct the following forced degradation studies to obtain degraded samples wherever degradation possible from about 1% to 30%. Preferably, the following stress conditions are recommended for specificity study, however stress condition can be decided based on experimental data, or physical properties of the analyte based on literature. If melting point of API is less than 150°C, stress should be at 105°C or 40°C less than melting point whichever is higher. If melting point of API is more than 150°C stress should at the nearest melting point and at 105°C.

1.10 Acceptance criteria of chromatogram generated for stress samples

All requirements of the software are to be met while evaluating peak purity. The purity angle should be less than purity threshold. The peak should not have any flag in purity result table. Mass balance of all stressed samples shall be verified by calculating mass balance (% assay of stressed sample + % impurities) x 100/ % assay of unstressed sample.

1.11 Required criteria of analytical method and calculation to be used to analyze stress samples

The preferred method of analysis for a stability indicating assay is reverse-phase high-performance liquid chromatography (HPLC). Such HPLC is preferred for several reasons, such as its compatibility with aqueous and organic solutions, high precision, sensitivity, and ability to detect polar compounds. Separation of peaks can be carried out by selecting appropriate column type, column temperature, and making adjustment to mobile phase pH. Poorly retained, and highly polar impurities should be resolved from the solvent front. As part of method development, a gradient elution method with varying mobile phase composition (very low organic composition to high organic composition) may be carried out to capture early eluting highly polar compounds and highly retained nonpolar compounds. Stressed samples can also be screened with the gradient method to assess potential elution pattern. Sample solvent and mobile phase should be selected to afford compatibility with the drug substance, potential impurities, and degradants. Stress sample preparation should mimic the sample preparation outlined in the analytical procedure as closely as possible. Neutralization or dilution of samples may be necessary for acid and base hydrolyzed samples.

The analytical method of choice should be sensitive enough to detect impurities at low levels (i.e., 0.05% of the analyte of interest or lower), and the peak responses should fall within the range of detector's linearity. The analytical method should be capable of capturing all the impurities formed during a formal stability study at or below ICH threshold limits. Degradation product identification and characterization are to be performed based on formal stability results in accordance with ICH requirements. Conventional methods (e.g., column chromatography) or hyphenated techniques (e.g., LC-MS, LC-NMR) can be used in the identification and characterization of the degradation products. Use of these techniques can provide better insight into the structure of the impurities that could add to the knowledge space of potential structural alerts for genotoxicity and the control of such impurities with tighter limits. It should be noted that structural characterization of degradation products is necessary for those impurities that are formed during formal shelf-life stability studies and are above the qualification threshold limit. Chromatographic profiles of stressed samples should be compared to those of relevant blanks (containing no active) and unstressed samples to determine the origin of peaks. The blank peaks should be excluded from calculations.

The amount of impurities (known and unknown) obtained under each stress condition should be provided along with the chromatograms (full scale and expanded scale showing all the peaks) of blanks, unstressed, and stressed samples. Additionally, chiral drugs should be analyzed with chiral methods to establish stereochemical purity and stability.

Various detection types can be used to analyze stressed samples such as UV and mass spectroscopy. The detector should contain 3D data capabilities such as diode array detectors or mass spectrometers to be able to detect spectral non-homogeneity. Diode array detection also offers the possibility of checking peak profile for multiple wavelengths. The limitation of diode array arises when the UV profiles are similar for analyte peak and impurity or degradant peak and the noise level of the system is high to mask the co-eluting impurities or degradants. Compounds of similar molecular weights and functional groups such as diastereoisomers may exhibit similar UV profiles. In such cases, attempts must be made to modify the chromatographic parameters to achieve necessary separation. An optimal wavelength should be selected to detect and quantitate all the potential impurities and degradants. Use of more than one wavelength may

be necessary, if there is no overlap in the UV profile of an analyte and impurity or degradant peaks. A valuable tool in method development is the overlay of separation signals at different wavelengths to discover dissimilarities in peak profiles.

Peak purity is used as an aid in stability indicating method development. The spectral uniqueness of a compound is used to establish peak purity when co-eluting compounds are present. Peak purity or peak homogeneity of the peaks of interest of unstressed and stressed samples should be established using spectral information from a diode array detector. When instrument software is used for the determination of spectral purity of a peak, relevant parameters should be set up in accordance with the manufacturer's guidance. Attention should be given to the peak height requirement for establishing spectral purity. UV detection becomes non linear at higher absorbance values. Thresholds should be set such that co-eluting peaks can be detected. Optimum location of reference spectra should also be selected. The ability of the software to automatically correct spectra for continuously changing solvent background in gradient separations should be ascertained.

Establishing peak purity is not an absolute proof that the peak is pure and that there is no co-elution with the peak of interest. Limitations to peak purity arise when co-eluting peaks are spectrally similar, or below the detection limit, or a peak has no chromophore, or when they are not resolved at all.

Mass balance establishment is a regulatory requirement. The mass balance is very closely linked to development of a SIAM as it acts as an approach to establish its validity. The balance would not be achieved unless all degradation products are separated well.

Mass balance establishes adequacy of a stability indicating method though it is not achievable in all circumstances. It is performed by adding the assay value and the amounts of impurities and degradants to evaluate the closeness to 100% of the initial value (unstressed assay value) with due consideration of the margin of analytical error.

Some attempt should be made to establish a mass balance for all stressed samples. Mass imbalance should be explored and an explanation should be provided. Varying responses of analyte and impurity peaks due to differences in UV absorption should also be examined by the use of external standards. Potential loss of volatile impurities, formation of non-UV absorbing compounds, formation of early eluants, and potential retention of compounds in the column should be explored. Alternate detection techniques such as RI LC/MS may be employed to account for non-UV absorbing degradants.

There might be many situations where the mass balance may be difficult to establish. This can be happen due to one or more of the following situations:

  a) Formation of multiple degradation products, involving complex reaction pathways and drug excipient interaction products.
  b) Incomplete detection due to loss of UV chromophore or lack of universal detection.
  c) Loss of drug/degradation products as volatiles.
  d) Diffusive losses into or through containers.
  e) Elution/resolution problems.
  f) Inappropriate or unknown response factors due to lack of standards.
  g) Errors and variability in the drug content assay

1.12 Role of mass balance in pharmaceutical stress testing

The assessment of degradation in pharmaceutical products involves two aspects of analytical measurement. First, a selective analytical method must be available for accurate assay of the parent drug compound, in order to correctly measure any loss. Second, methodology should be in place for quantification of the degradation products formed. Ideally, when degradation occurs, the measured amount of parent drug lost should correlate well with the measured increase in degradation products. This correlation is referred to as mass balance (Kirschbaum , 1988). More recently, the International Conference on Harmonization (ICH) has provided a definition of mass balance or material balance as follows:

The process of adding together the assay value and levels of degradation products to see how closely these add up to 100% of the initial value, with due consideration of the margin of

analytical precision. This concept is a useful scientific guide for evaluating data, but it is not achievable in all circumstances. The focus may instead be on assuring the specificity of the assay, the completeness of the investigation of routes of degradation, and the use, if necessary, of identified degradants as indicators of the extent of degradation via particular mechanisms.

Clearly, from a theoretical standpoint, any true decrease in the mass of parent compound (and other reactants involved) upon degradation is necessarily equivalent to the total mass of all degradation products formed. In a closed system, mass balance would thus be assured if sufficient analytical methods were in place to accurately quantify all species present in the original and degraded material and their environment. However, such is almost never the case. The requirement for a ''closed system'' is rarely met. For example, degradation may produce volatile substances that escape from the sample matrix. Adsorption or other physical losses may also result in inaccurate assessment of amounts of material degraded or produced. That is, it is not typically practical to assay a sample's entire environment (e.g., container, atmosphere, etc.). Finally, one may deliberately choose not to quantify certain degradation products if the given degradation pathway can be monitored by assessing a limited number of key substances. As always, the analyst must balance time and resource demands to provide the information necessary to understand degradation without going to extreme measures to quantify components of little interest.

1.12.1 Why is mass balance important?

Mass balance in pharmaceutical analysis is important for several reasons. By demonstrating that degradative losses of parent drug correlate well with the measured increase in degradation products, an analyst confirms that there are no significant degradation products unaccounted for. Conversely, if one observes, for example, a 20% loss of parent drug but only measures a5% increase in degradation products, it is likely that additional degradation products are formed that are not accurately determined by the given method(s). Because unknown degradation products could potentially be toxic or otherwise compromise the safety of the drug, it is important to have methods that detect all major degradation products. Thus, safety is the primary reason for evaluating mass balance.

Mass balance is also useful in method validation (Kirschbaum, 1988; Riley *et al.*, 1996; Priestner *et al.*, 1993). In order to demonstrate that analytical methods are stability indicating, unstressed and stressed materials are often compared. An increase in degradation products that correlates well with loss of parent drug aids in demonstrating that the methods can be used to accurately assess degradation.

Mass balance is also important in understanding alternative degradation pathways (Conner *et al.*, 1986). For example, let us consider a situation where both acid catalyzed degradation and oxidative degradation produce a substantial loss of parent compound in stress-testing studies. If good mass balance is achieved for the acid-catalyzed degradation, but not for the oxidative degradation, further work to better understand the oxidative degradation pathway(s) is warranted. It may be that the poor mass balance in the latter case results from important oxidative degradation products that are unaccounted for or from structures which need to be more fully elucidated to understand response factor differences.

1.12.2 How can mass balance be measured and expressed?

Mass Balance can be calculated and expressed in a variety of ways. The amount of parent compound lost and of degradation products formed can be expressed in terms proportional either to weight or to number of moles. The term ''mass balance'' is suggestive of a straightforward correlation of mass or weight lost and gained. If all starting materials and degradation products are accounted for, then a correlation in terms of weight is appropriate. However, if degradation involves the formation of products of substantially different molecular weight, or which are not all readily measured, it may be more appropriate to consider molar mass balance.

For the purposes of this discussion, then, the following definitions will be used. Let P be the parent drug and I be the impurity or degradation product. Assume that $M_{P,0}$ and $M_{P,x}$ are the mass of parent compound (and other starting reactants) initially and at time X, respectively; $M_{I,0}$ and $M_{I,x}$ are the total mass of impurities initially and at time X, respectively. Similarly, $N_{P,0}$, $N_{P,x}$, $N_{I,0}$ and $N_{I,x}$ are the analogous number of moles of each.

**Mass balance:** The situation in which the measured mass of parent and other reactant(s) consumed is equivalent to the measured increase in mass of degradation product(s); i.e.,

$M_{P,0}$- $M_{P,x}$ = $M_{I,x}$ - $M_{I,0}$

Molar mass balance: The situation in which the measured increase in moles of degradation product(s) is equivalent to that predicted via a balanced chemical reaction from the number of moles of parent consumed. For a degradation reaction:

$$\alpha P + \cdots \cdots \longrightarrow b I + \cdots \cdots$$
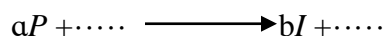
molar mass balance at time x can be expressed as:

$$\frac{N_{P,0} - N_{P,x}}{\alpha} = \frac{N_{I,x} - N_{I,0}}{b}$$

Furthermore, an observed deviation from mass balance can be expressed in either absolute or relative terms, as described below.

*Absolute mass balance deficit (AMBD)***:** The difference between the measured amount of parent compound consumed and of degradation product(s) formed; i.e.,

$$AMBD = (M_{P,0} - M_{P,x}) - (M_{I,x} - M_{I,0}).$$

Although AMBD can be expressed in units of mass, it is commonly expressed in percentage.

*Relative mass balance deficit (RMBD)***:** The absolute mass balance deficit expressed as a percentage of the total amount of parent consumed; i.e.,

$$RMBD = 100\% \times \frac{(M_{P,0} - M_{P,x}) - (M_{I,x} - M_{I,0})}{M_{P,0} - M_{P,x}}$$

The absolute and relative molar mass balance deficit can be analogously calculated using the number of moles in place of mass of parent and degradation product(s).

AMBD and RMBD are both zero in the case of perfect mass balance, positive when the measured increase in degradation products is less than the loss of parent, and negative when the measured increase in degradation products exceeds the loss of parent. The RMBD is particularly

useful in assessing how significant a mass balance issue is, as it is independent of the extent of degradation (in contrast to AMBD). The RMBD, in other words, expresses the relative inaccuracy of the measured increase in degradation products.

Often, stress-testing results from HPLC assays are reported in terms of percentages lost or gained upon degradation, on the basis of peak areas. Consideration should be given to exactly what the reported percentages mean, in order to understand mass balance. Generally, for stress-testing, any counter-ion or other inorganic impurity present is ignored, and percentages are with respect to the total sum of parent compound and related impurities. In addition, for solid-state samples, changes in water or other volatile components should be accounted for. One way to address this is to analyze samples using thermogravimetric analysis (TGA) or Karl–Fischer titration (for water content) to account for loss or gain of volatiles. Another approach is to perform the analyses such that volatiles lost or gained are compensated for in the procedure. This can be accomplished using a method that involves individually weighing samples for each time point to be analyzed (from a lot with a known volatile content) into separate containers to be placed under the stress condition. At the prescribed time point, the individual container (with a known pre weighed amount of sample) is completely dissolved and diluted to a specified volume. Any changes in volatile content are irrelevant to the assay result. Thus, when reporting changes in parent content after stress testing, it is important to clearly state what a percentage change refers to and whether changes in overall mass (e.g., due to water loss or gain) are normalized.

Typically, organic degradation products (related substances) are determined by HPLC. It is important to know whether degradation products are quantified against an external standard or, more commonly, by relative peak area. HPLC peak areas are integrated and the results are, in the simplest case, reported as a percentage of the total of all integrated peaks. Of course, this assumes uniform response factors (e.g., UV absorptivity) for degradation products and parent, and such an assumption may not be valid (Newton *et al.*, 1991; Olsen *et al.*, 1997; Guillemin *et al.*, 1982). Even when valid, or when response factor corrections are used, it is important to remember what the total of the HPLC peak areas excludes. Clearly, any substances that do not respond to the given HPLC detector will not be included in the total. In addition, the assumption is made (and presumably tested) that all degradation products are eluted from the column.

1.12.3 Stress testing and mass balance

The relation of mass balance to stress testing is apparent. As defined by ICH, stress testing is designed to determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify likely degradation products and to validate the stability-indicating power of the analytical procedures. Thus, an assessment of mass balance is an important part in achieving the goals of understanding degradation pathways and evaluating the capability of the analytical procedures to detect all the relevant degradation products.

It is important to remember that the goal of stress testing is not primarily to achieve mass balance in the analytical results, but rather to achieve a full understanding of the degradation chemistry. That is, if the degradation pathways are fully understood, then it is relatively straightforward to determine whether all relevant degradation products are being accurately determined. Typically, this kind of understanding cannot be achieved unless the structures of the main degradation products are known. Correlation of the degradation product structures with scientifically reasonable pathways then enables one to assess whether or not any major products are unaccounted for. This mechanistically driven approach (also referred to as assessment of the completeness of the investigation of the routes of degradation and the use, if necessary, of identified degradants as indicators of the extent of degradation via particular mechanisms.

1.12.4 Causes of and approaches to solving positive mass balance deficit

In these cases, the increase in mass (or number of moles) of degradation products is less than the corresponding decrease in parent. Potential sources and resolution of the problem are described as follows.

(1) Degradation product(s) are not eluted from the HPLC column

There are a number of practical ways to diagnose this problem:

a) the HPLC method can be modified to elute any additional impurities

b) samples can be analysed using UV spectrophotometric analysis

c) samples can be analyzed using the HPLC system without the column present (flow injection analysis) or

d) an alternate/orthogonal separation can be used

(2) Degradation product(s) not detected by the detector used

Ultraviolet absorbance is the most common detection technique for HPLC. Although widely applicable, UV detectors do not detect all compounds. Degradation may produce compounds without chromophores, in which case the observed increase in degradation products will be smaller than the loss in parent compound. The diagnosis (as well as the solution) for the problem may be to use a shorter wavelength or an alternative detector [e.g., evaporative light-scattering detection (ELSD), mass spectrometry (MS), or flame ionization detection (FID)]. It is important to keep in mind that most such detectors, while broadly applicable, are—like UV—not uniform in their response. For example, compounds with significant vapor pressure give poor response by ELSD, and MS response varies greatly with ionizability. However, such detectors can be very useful in confirming the presence of degradation products undetected by the original method (McCrossen *et al.*, 1998; Righezza *et al.*, 1988; Cech *et al.*, 2001; Tang *et al.*, 1993; Cheng *et al.*, 1992).

(3) Degradation product(s) lost from the sample matrix

In some cases, degradation products are inadvertently excluded from the sample tested because of insolubility, volatility, or adsorption losses. Instances of insolubility are usually the most obvious and straightforward to solve. In such cases, visual observation or turbidity measurements may reveal the problem. Use of a different sample solvent or isolation and specific testing of the insoluble material may then be necessary.

TGA with associated vapor spectral analysis (e.g., IR, MS) may be a useful approach. Finally, if the degradation can be generated at low temperatures, it may be possible to minimize volatility simply by keeping the sample cold.

Degradation products may also adsorb to the sample container or to insoluble excipients. In the latter case, the diagnosis and resolution of the problem is the same as if the degradation product were insoluble. If adsorption to the container is a potential issue, then the most straightforward approach is to compare results using different container materials (e.g., glass, polypropylene). In some cases, changing the sample solvent (e.g., pH, solvent strength) may minimize the

adsorption. Occasionally, it may be necessary to use particularly strong solvents to remove the adsorbed material.

(4) Parent compound lost from the sample matrix

In rare cases, the parent compound may itself be lost from the sample matrix due to volatility or adsorption. Often, even if such losses occur, they will be insignificant in proportion to the quantity of parent present. However, if significant, the decrease in assay results would not be due to degradation and so would not correspond to any increases in degradation products. Generally, information obtained prior to stress testing (e.g., melting and/or boiling point, vapor pressure, tendency to adsorb to various materials) should provide indications of this potential problem.

(5) Degradation product(s) co-eluting with the parent compound in the related substance method

If a degradation product co-elutes with the parent compound in both assay and related substance methods, then the resulting mass balance depends on the response factor of the impurity relative to that of the parent compound under the given conditions. If the impurity formed has a lower response factor, then there will be a ''positive'' mass balance deficit. If the response factor of the impurity is greater than that of the parent, then there will be a ''negative'' mass balance deficit. Examination of the parent compound peak by photodiode array (PDA)–UV detection (and using UV-homogeneity algorithms) or by MS detection may reveal the co-eluting impurity as peak heterogeneity. Of course, PDA detection is effective only for impurities that have distinct UV spectra and that do not perfectly co-elute with the parent. In addition, the sensitivity of PDA to detecting peak heterogeneity is dependent on how different from the parent the spectral properties of the impurity are. For some related substances, PDA detection may be unable to detect impurities at levels below a few percent. LC–MS may provide greater sensitivity to a wider range of potential co-eluting impurities; however, LC–MS is more expensive and more restrictive in the types of mobile phase that can be used. Alternatively, an orthogonal separation technique (e.g., CE) can be used to check for co-eluting impurities. If a co-elution problem is discovered, the related substance method should be modified to separate the co-eluting impurity.

(6) Degradation products are not integrated due to poor chromatography

Some degradation products may not chromatograph well (e.g., due to adverse interactions with residual silanols, on-column conversion or interconversion from one product to another, etc.) and, although they may elute from the HPLC column, the resulting broad peaks can easily be missed and remain unintegrated, especially when the broad peak area is present at low levels. Alternatively, if the parent drug degrades to a large number of products that are poorly resolved, the chromatogram observed upon analysis may not reveal discrete peaks but rather an elevated baseline. At low levels, such an elevated baseline can easily escape detection. Situations such as this are not uncommon, especially when isocratic HPLC methods are used. Running a blank (and overlaying the chromatogram) can be very helpful in determining if a baseline elevation is from the sample or simply an artifact of the chromatography. Experiments to determine if low mass balance results are from poor chromatography are the same as those that would be performed to determine if degradation products are not eluting from the column.

1.12.5 Causes of and approaches to solving negative mass balance deficit

In these cases, the increase in mass (or number of moles) of degradation products is greater than the corresponding decrease in parent. Potential sources of the problem are described below.

(a) Inaccurate quantification of degradation product(s) due to differences in response factors.

(b) Unaccounted-for reactants involved in addition reaction(s) to the parent compound. Consider reactants from matrix (oxygen, excipients, container ingredients). Compare molar mass balance to weight mass balance; i.e., molar mass balance may be more appropriate in this case.

(c) Impurities arising from source(s) other than degradation of the parent compound. Such impurities could be present in the mobile phase, sample solvent, or column or could be from the sample matrix. Run a blank sample matrix, or one with varying concentrations of parent under the same conditions, to determine which—if any—impurities are unrelated to amount of parent present.

(d) Degradation product(s) co-eluting with the parent compound in the parent compound assay. Check for peak homogeneity via PDA–UV, LC–MS, and/or use an orthogonal separation technique. Modify conditions to separate if necessary.

1.12.6 Practical approaches to solving response factor problems

Poor mass balance is inevitable if response factors (e.g., absorptivity at the given wavelength) differ significantly between impurities and the parent compound when uncorrected peak areas are assumed to represent actual relative amounts (Newton *et al.*, 1991). A positive mass balance deficit results if the response factors of the degradation products are less than that of the parent. A negative mass balance deficit results when the degradation products have larger response factors. Thus, relative response factors (RRFs) of impurities are an important consideration in assessing mass balance.

Occasionally, the UV response factors can be reasonably assumed to be quite similar; for example, the parent and degradation product(s) share the same chromophoric backbone, with structural differences only in regions unassociated with the chromophore. Favorable comparison of UV spectra (e.g., from a PDA array detector) can help confirm such a situation (Olsen *et al.*, 1993).

More often, structures are unknown or differ significantly in the chromophoric region of the molecule such that the assumption of similar response factors is questionable at best. Traditionally, the process for establishing response factors involves the use of isolated samples of individual impurities. For accurate determinations, the purity of each such sample must be known. For synthetic samples, the purity is generally estimated using a combination of HPLC (with UV, light-scattering, or other appropriate detection), NMR, and some method to determine volatile impurities (e.g., TGA, Karl–Fischer). This process involves a significant amount of time and effort (i.e., expense). The effort required is typically even greater for impurities that are not readily synthesized (e.g., low–level process impurities and degradation products). Such impurities need to be isolated and purified using standard techniques such as preparative TLC or HPLC. The risk of having contaminants in the isolated impurities is magnified when compared with synthetic samples because of the large amounts of solvents used, the possibility of non-chromophoric components (e.g., solvents or column bleed), the presence of counter ions (e.g., trifluoroacetic acid, acetate, etc.), and the impracticality of using crystallization (with the low levels isolated) to enhance the purity. Moreover, in some cases, the degradation products are unstable and thus very difficult to purify. In order to assess sample purity, amounts of ≥50 mg

are often needed. Isolation of these amounts of impurities can be very time consuming and costly. Once material of known purity is available, the determination of the response factor(s) is straightforward. A measured concentration is prepared and processed as per the analytical method, to determine the detector response (under the given conditions) per unit weight or molar concentration.

A potential alternative for determining UV response factors is to use two HPLC detectors: a standard UV absorbance detector and a second detector that has a response uniformly proportional to weight or concentration. For example, if a detector could provide accurate information on the relative amounts of the impurities and parent compound, then this information, combined with the UV peak areas, would supply the desired RRF information without the need for a purified impurity sample. One could reasonably question the need for a UV detector and RRF values at all if such an alternative detector was available, as it would directly provide information on relative amounts of impurities/parent. However, UV detectors are inexpensive, rugged, and readily available; therefore, RRF values, once determined, are widely applicable to situations in which no other detector is available.

1.13 Forced degradation in QbD paradigm

A systematic process of manufacturing quality drug products that meet the predefined targets for the critical quality attributes (CQA) necessitates the use of knowledge obtained in forced degradation studies.

A well-designed, forced degradation study is indispensable for analytical method development in a QbD paradigm. It helps to establish the specificity of a stability indicating method and to predict potential degradation products that could form during formal stability studies. Incorporating all potential impurities in the analytical method and establishing the peak purity of the peaks of interest helps to avoid unnecessary method re-development and revalidation.

Knowledge of chemical behavior of drug substances under various stress conditions can also provide useful information regarding the selection of excipients for formulation development. Excipient compatibility is an integral part of understanding potential formulation interactions

during product development and is a key part of product understanding. Degradation products due to drug-excipient interaction or drug-drug interaction in combination products can be examined by stressing samples of drug substance, drug product, and placebo separately and comparing the impurity profiles. Information obtained regarding drug-related peaks and non-drug related peaks can be used in the selection and development of more stable formulations. For instance, if a drug substance is labile to oxidation, addition of an antioxidant may be considered for the formulation. For drug substances that are labile to acid or undergo stereochemical conversion in acidic medium, delayed release formulations may be necessary. Acid/base hydrolysis testing can also provide useful insight in the formulation of drug products that are liquids or suspensions.

Knowledge gained in forced degradation studies can facilitate improvements in the manufacturing process. If a photo-stability study shows a drug substance to be photolabile, caution should be taken during the manufacturing process of the drug product. Useful information regarding process development (e.g., wet versus dry processing, temperature selection) can be obtained from thermal stress testing of drug substance and drug product.

Additionally, increased scientific understanding of degradation products and mechanisms may help to determine the factors that could contribute to stability failures such as ambient temperature, humidity, and light. Appropriate selection of packaging materials can be made to protect against such factors.

Stability- related issues can affect many areas, including the following:
   a) Analytical methods development.
   b) Formulation and packaging development.
   c) Appropriate storage conditions and shelf-life determination.
   d) Safety/toxicological concerns.
   e) Salt selection/polymorph screening.
   f) Manufacturing/processing parameters.
   g) Absorption, distribution, metabolism, and excretion (ADME) studies.
   h) Environmental assessment.

1.13.1 Analytical methods development

In order to assess the stability of a compound, one needs an appropriate method. The development of stability–indicating analytical method, particularly an impurity method, is a "Chicken and egg" type of problem. That is, how does one develop an impurity method to detect degradation products when one does not know what the degradation products are? Stress testing study can help to address this dilemma. Stress the parent compound under particular stress conditions can generate samples containing degradation products. These samples can then be used to develop suitable analytical procedures. It is important to note that the degradation products generated in the stressed samples can be classified as "potential" degradation products that may or may not be formed under relevant storage conditions. It is also important to note that not all relevant degradation products may form under the stress conditions. Both accelerated and long-term testing studies of the drug substance and formulated drug product are used to determine which of the potential degradation products actually form under normal storage conditions and are, therefore, relevant degradation products.

1.13.2 Formulation and packaging development

The knowledge gained from stress testing is useful for formulation and packaging development. Well-designed stress-testing studies can determine the susceptibility of a compound to hydrolysis, oxidation, photochemical degradation, and thermal degradation (Singh *et al.*, 2013; Effat *et al.*, 2011; Zarana *et al.*, 2014; Srinivas *et al.*, 2014; Effat *et al.*, 2014; Ali *et al.*, 2012; Riddhiben *et al.*, 2011; Smela, 2005). This information is then taken into consideration when developing the formulation and determining the appropriate packaging. For example, if stress-testing studies indicate that a compound is rapidly degraded in acid, and then consideration might be given to developing an enteric coated formulation that protects the compound from rapid degradation in the stomach. If a compound is sensitive to hydrolysis, then packaging that protects from water vapor transmission from the outside may be helpful to ensure long-term storage stability. Alternatively, if the compound is sensitive to base-catalyzed degradation, then a formulation with a slightly acidic microenvironment might be needed. Other degradation mechanisms {e.g., oxidative degradation or photo degradation can also be prevented or minimized by the use of appropriate packaging and / or formulation. Knowledge of potential drug-excipient interactions is also critical to developing the best formulation, and therefore it is

also important to conduct drug-excipient compatibility studies and formulated product stress-testing studies.

1.13.3 Appropriate storage conditions and shelf-life determination

Determining appropriate storage conditions for a drug substance or product requires knowledge of conditions that induce degradation and the degradation mechanisms. Most of this information can be obtained from stress-testing studies combined with accelerated stability testing.

Accurate shelf-life predictions, however, are best made with data from formal long-term stability studies, although recent studies utilizing an "accelerated stability assessment protocol" have demonstrated a high degree of kinetics predictability.

1.13.4 Safety/toxicological concerns

Stress-testing studies are useful for assessing whether known toxic compounds or potential genotoxic compounds are formed by degradation of the parent drug. If the formation of (a) toxic compound(s) is possible, steps can be taken early on to inhibit the formation of the toxic compound(s) and to develop sensitive analytical methods to accurately detect and quantify the formation. Stress-testing studies can also facilitate preparation/isolation of a degradation product for toxicological evaluation when synthetic preparation is not feasible.

1.13.5 Salt selection/polymorph screening

Stress-testing studies can help the salt and polymorph selection process by providing rapid information related to chemical and physical stability. The chemical and physical stability of different salt and polymorphic forms can be dramatically different, highlighting the importance of using stability as part of the rationale for selection. The importance of such considerations is illustrated by the estimation that 50% of all drug molecules are administered as salts and this percentage may be growing due to the increasing need to improve solubility by salt formation.

1.13.6 Manufacturing/processing parameters

Degradation can also occur during manufacturing or processing steps. Knowledge of conditions that lead to degradation of the parent compound can help in designing appropriate controls/conditions during manufacturing/processing. For example, if a compound is susceptible

to degradation at low pH, then either the manufacturing steps under low pH conditions can be avoided or the time and /or temperature can be more carefully controlled to minimize the degradation. It is not uncommon to observe degradation during formulation processing, for example, wet granulation, milling, etc. An understanding of the degradation that may occur during the formulation processing steps can help in choosing conditions to ensure maximum stability of the drug substance (e.g., oxidative susceptibility may lead to the use of processing in an inert-gas atmosphere; hydrolytic instability may lead to the elimination of wet granulation processes in favor of drug processing conditions such as direct compression or roller compression).

1.13.7 Absorption, distribution, metabolism, and excretion (ADME) studies

ADME characteristics of a drug are extensively studied prior to marketing. These studies typically involve identification of the major metabolites, a process that can be difficult owing to the complex matrix (living organism) and often very low levels. Occasionally, degradation products detected in stress-testing studies are also metabolites. In these cases, it is usually easier to generate larger quantities of the metabolite for characterization using the stress condition rather than isolation it from the living organism. It is also possible that nonenzymatic degradation can occur in vivo, and therefore an understanding of what degradation pathways might be relevant under physiological conditions can be important to understanding the ADME of a drug.

1.13.8 Environmental assessment

The environmental assessment deals with the fate of the drug in the environment. The information gained from the stress testing can be useful designing and interpreting environmental studies, as the degradation of the drug in the environment will often be similar to degradation observed during stress-testing studies (e.g., hydrolytic, photolytic and oxidative degradation). Knowledge of the degradation chemistry of pharmaceuticals is also useful in designing effective wastewater treatment for destroying the drug compound in cost effective and environmentally friend ways.

1.14 Degradation kinetics

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to a establish a retest period for the drug substance of a shelf life for the drug product and recommended storage conditions (Steven *et al.*, 2011; John, 2002; Keith *et al.*, 2002; Patrick *et al.*, 2010; Luis *et al.*, 2010). The rate and mechanisms of reactions with particular emphasis on decomposition and stabilization of drug products are essential for pharmacists and pharmaceutical scientists to study, understand, and interpret conditions of instability of pharmaceuticals products as well as to be able to offer solutions for the stabilization of these products (Eyring, 1935; Effat *et al.*, 2014). Knowing the rate at which a drug deteriorates as various hydrogen ion concentrations allows one to choose a vehicle that will retard or prevent the degradation. Patients expect that products will have a reasonable shelf life. Application of degradation kinetics in pharmacy results in the production of more stable drug preparations, the dosage and rationale of which may be established on sound scientific principles. Thus, as a result of current research involving the kinetics of drug systems, the pharmacist is able to assist the physician and patient regarding the proper storage and use of medicinal agent.

Shelf life (also referred to as expiration dating period) is the time period during which a drug product is expected to remain within the approved specification for use, provided that it is stored under the conditions defined on the container label.

Expiration date is the date placed on the container label of a drug product designating the time prior to which a batch of the product is expected to remain within the approved shelf-life specification if stored under defined conditions and after which it must not be used.

The half-life is the time required for one-half of the material to disappear. The shelf-life is the time required for 10% of the material to disappear.

1.14.1 Drug impurities, degradants and the importance of understanding drug degradation
A drug impurity is anything that is not the drug substance (or active pharmaceutical ingredient, API) or an excipient according to the definition by the US Food and Drug Administration (FDA).

Impurities can be categorized into process impurities, drug degradation products (degradants or degradates), and excipient and packaging-related impurities. Process impurities are produced during the manufacture of the drug substance and drug product, while degradants are formed by chemical degradation during the storage of the drug substances or drug products. The storage conditions are typically represented by the International Conference on Harmonisation (ICH)- and World Health Organization (WHO)-recommended stability conditions which simulate different climatic zones of the world. Certain process impurities can also be degradants, if they continue to form in storage under stability conditions. Packaging-related impurities, also called leachables, are typically various plasticizers, antioxidants, UV curators, and residual monomers that leach out of the plastic or rubber components and labels of the package/container of a drug product over time.

Those process impurities that are not degradants may be controlled or eliminated by modifying or changing the process chemistry. On the other hand, control or minimization of drug degradants requires a clear understanding of the drug degradation chemistry, which is not only critically important for developing a drug candidate but also for maintaining the quality, safety, and efficacy of an approved drug product. Specifically, knowledge of drug degradation is not only vital for developing adequate dosage forms that display favorable stability behavior over the registered product shelf-life, but is also critical in assessing which impurities would be most likely to be significant or meaningful degradants so that they can be included in the specificity mixture when developing and validating stability-indicating analytical methodologies. A common problem in the development of stability-indicating HPLC methods using stress studies (or forced degradation) is a lack of proper evaluation if the stress-generated degradants would be real degradants or not. From a practical point of view, the real degradants are those that can form under long term storage conditions such as the International Conference on Harmonisation (ICH) stability conditions. On the other hand, various artificial degradants can be generated during stress studies, in particular when excessive degradation is rendered or the stress conditions are not consistent with the degradation pathways of the drug molecule under the usual stability conditions. For example, forced degradation of a ketone-containing drug, pentoxifylline, using 30% hydrogen peroxide at room temperature for eight days produced a germinal

dihydroperoxide degradation product. This compound is highly unlikely to be a real degradant of the drug product.

Most drugs undergo at least certain level of metabolism, that is, chemical transformation catalyzed by various enzymes. Except in the case of pro-drugs, drug metabolites can be considered as drug degradants formed in vivo. Chemical degradation and drug metabolism can produce the same degradants, even though they may go through different reaction intermediates or mechanisms. In vitro chemical reactions have been used to mimic enzyme-catalyzed drug metabolism processes, in order to help elucidate the enzymatic mechanisms for the catalysis. On the other hand, understanding the mechanisms of drug metabolism may also facilitate the elucidation of drug degradation pathways in vitro.

Regardless of their origins, certain drug degradants can be toxic, which is one of the main contributors to undesirable side effects or adverse drug reactions (ADR) of drugs. In the early stage of drug development, the degradants (including metabolites) and degradation pathways (or bioactivation pathways in the case of reactive metabolites) of a drug candidate need to be elucidated, followed by toxicological evaluation of these degradants. Dependent upon the outcome of the evaluation, the structure of the drug candidate may have to be modified to avoid the formation of a particular toxicophore based on the understanding of the degradation chemistry (or bioactivation pathways) elucidated. Failure to uncover toxic degradants, usually the low level ones, in the early development stage can lead to hugely costly failure in later stage clinical studies or even withdrawal of an approved drug product from the market.

1.14.2 Characteristics of drug degradation

The vast majority of therapeutic drugs are either organic compounds or biological entities. The latter drugs include protein and nucleic acid (RNA and DNA)-based drugs which are biopolymers comprising small molecule building blocks. Stress studies or forced degradation can help elucidate the structures of real degradants and the degradation pathways of drugs. Nevertheless, caution needs to be taken in differentiating the real and artificial degradants.

Drug degradation chemistry differs from typical organic chemistry in several ways. First, the yield of a drug degradation reaction is usually very low, from approximately 0.05% to a few percentage points at the most. Dependent upon the potencies and maximum daily dosages of the drugs, ICH guidelines require that the impurities and/or degradants of a drug be structurally elucidated, once they exceed certain thresholds, which are typically between 0.05% and 0.5%, relative to the drug substances. For potential genotoxic impurities, they need to be characterized and controlled at a daily maximum amount of 1.5 mg for drugs intended for long term usage. Such low yields would be meaningless from the perspective of the regular organic chemistry. Second, due to the low yields and limited availability of samples, particularly stability samples of formulated drugs, the quantity of a drug degradant is usually extremely low, posing a serious challenge for its isolation and/or characterization. Despite the advent of sensitive and powerful analytical methodologies such as high resolution tandem liquid chromatography-mass spectrometry (LC-MS/MS), liquid chromatography-nuclear magnetic resonance (LC-NMR), and cryogenic micro NMR probes, the identification of drug degradants remains one of the most challenging activities in pharmaceutical development. Third, the typical conditions and reagents of drug degradation reactions are limited in scope. For example, the ICH long term stability conditions for different climatic zones specify the requirements for heat and moisture (relative humidity, RH), for example, 25°C/60% RH and 30 °C/65% RH, while the ICH accelerated stability condition requires heating at 40°C under 75% RH. In addition to moisture, the other most important reagent in drug degradation reactions is molecular oxygen. Since molecular oxygen is ubiquitous and difficult to remove from drug products, oxidative degradation of drugs is one of the most common degradation pathways. Often, the impact of molecular oxygen can be indirect. For example, a number of polymeric drug excipients such as polyethylene glycol (PEG), polysorbate, and povidone, are readily susceptible to autooxidation, resulting in the formation of various peroxides including hydrogen peroxide. These peroxides can cause significant drug degradation once formulated with drug substances containing oxidizable moieties. In contrast, reductive degradation is rarely seen in drug degradation reactions owing to the lack of a reducing agent in common drug excipient that is strong enough to cause meaningful reductive degradation. Other possible reagents in drug degradation reactions are usually limited to drug excipients and their impurities. For example, excipients consisting of oligosaccharides and polysaccharides with reducing ends, such as lactose and starch, are frequently used in drug

formulation. The aldehyde functionality of these excipients can react with the primary and secondary amine groups of drugs to undergo degradation via the Maillard reaction.

Owing to the variety of dosage forms of formulated drugs, degradation of drugs can occur in various states including solid (tablets, capsules, and powders), semi-solid (creams, ointments, patches, and suppositories), solution (oral, ophthalmic, and optic solutions, nasal sprays, lotions, injectables), suspension (suspension injectables), and gas phase (aerosols). Obviously, a drug molecule can exhibit different degradation pathways and kinetics in different dosage forms.

1.14.3 Thermodynamics and kinetics of chemical reactions

A change in Gibbs free energy, ΔG, of a chemical reaction governs the propensity of the reaction to proceed. ΔG is defined as follows:

$$\Delta G = \Delta H - T\Delta S \text{-----------------Eq. 1.1}$$

where ΔH is the change in the reaction enthalpy, T is the reaction temperature (in Kelvin), and ΔS is the change in the reaction entropy.

For a thermodynamically favored reaction, that is, a reaction that occurs spontaneously, if allowed by the reaction kinetics, the ΔG of the reaction is negative. In other words, the free energy of the products is lower than that of the reactants in such a case. A schematic diagram of a thermodynamically favored reaction is presented in figure 1.1. In contrast, a thermodynamically unfavorable reaction has a positive ΔG.
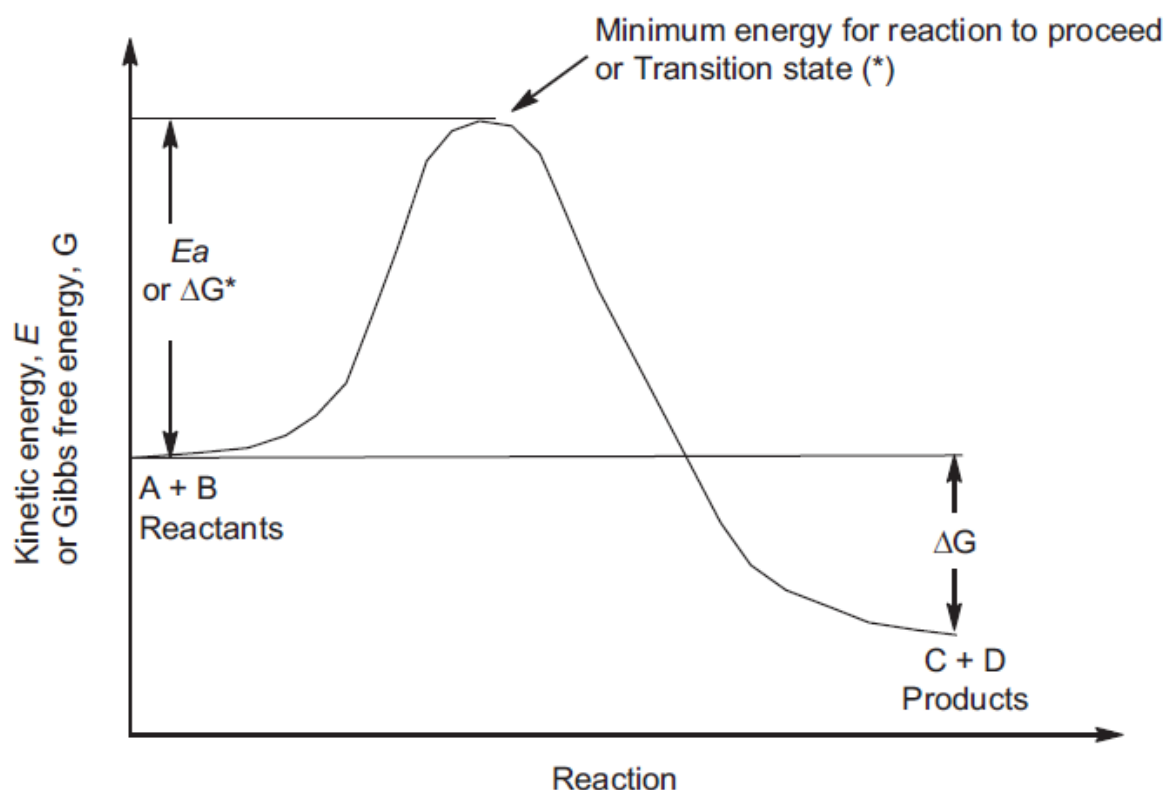
Figure 1.1: Schematic diagram of a thermodynamically favored reaction, where the Gibbs free energy of the reaction, ΔG, is negative. Ea is the activation energy per the collision theory, while ΔG* is Gibbs free energy of activation according to transition theory.

ΔG determines if the reaction of A+B $\longrightarrow$ C+D is favored or not, but it does not determine how fast the reaction, whether thermodynamically favored or not, would take place. The rate of the reaction or its kinetics is governed by the energy that is necessary to activate the reactants to a certain state so that they can convert to their products. There are two theories describing this process: collision theory and transition state theory. Collision theory is embodied in the well-known Arrhenius equation (Eq.1.2), which was first proposed by van't Hoff in 1884 and later justified and interpreted by Arrhenius in 1889.

$$k = Ae^{-Ea}/RT \text{-----------------Eq.1.2}$$

where k is the reaction rate constant, A is the pre-exponential (or frequency) factor which can generally be approximated as a temperature-independent constant, Ea is the activation energy which is defined as the minimum energy the reactants must acquire through collision in order for the reaction to occur, R is the gas constant, and T is the reaction temperature (in Kelvin).

According to the Arrhenius equation, the rate constant of a reaction is temperature dependent and by taking the natural logarithm of Eq. 1.2 , the Arrhenius equation takes the following format (Eq 1.3).

$$\text{In } k = \frac{-E_a}{R} \cdot \frac{1}{T} + \text{In } A \text{----------------------Eq. 1.3}$$

This expression shows that the higher the temperature, the faster the reaction rate. Additionally, if one measures the reaction rate constants (k) at different temperatures (T), one should get a linear relationship by plotting ln$k$ versus1/T. Hence, the activation energy, Ea, can be obtained from the slope ($-Ea/R$) of the linear plot and ln$A$ from the y-intercept.

$$k = \frac{k_B T}{h} e^{-\Delta G^*/RT} \text{----------Eq. 1.4}$$

Despite its widespread use, the Arrhenius equation and its underlying collision theory have been challenged over time. The major competing theory appears to be transition state theory which was developed independently by Eyring, and Evans and Polanyi in 1935. The equation derived according to transition state theory is the Eyring equation, also called the Eyring–Polanyi Eq. 1.4:

where ΔG^* is Gibbs free energy of activation, k_B is the Boltzmann constant, and h is Planck's constant.

This equation bears some resemblance to the Arrhenius equation in that the $k_B T/h$ item corresponds to the pre-exponential factor, A, and ΔG^* correspondsto the activation energy, Ea. Nevertheless, in the Eyring equation, ΔG^*, in addition to $k_B T/h$, is temperature dependent, as $\Delta G^* = \Delta H^* - T\Delta S^*$. Hence, the Erying equation can be written as Eq. 1.5 after taking natural logarithm and rearrangement:

$$\ln k/T = (-\Delta H^*/R)(1/T) + \ln k_B/h + \Delta S^*/R \text{------Eq. 1.5}$$

where ΔH^* is enthalpy of activation and ΔS^* is entropy of activation.

Hence, ΔH^* can be obtained from the slope $(-\Delta H^*/R)$ of a linear plot of $\ln k/T$ versus $1/T$, while ΔS^* can be obtained from the y-intercept $(\ln k_B/h + \Delta S^*/R)$. Therefore, one can obtain both Ea (from Eq 1.3) and ΔH^* and ΔS^* (from Eq. 1.5) from a single dataset of reaction rate constant, k, versus reaction temperature, T. Although application of the Eyring equation enables one to obtain both ΔH^* and ΔS^* values and the ΔS^* value should help elucidate the reaction mechanism, it appears that the use of Arrhenius equation exceeds the use of Eyring equation, at least in the hydrolytic stability studies of drugs. With respect to the numeric difference between the values of Ea and ΔH^*, we can again rearrange the Eyring Eq. 1.5 into the following format (Eq. 1.6):

$$\ln k = (-\Delta H^*/R)(1/T) + \Delta S^*/R + \ln k_B/h + \ln T \text{--Eq. 1.6}$$

Among the last three items of the equation, only $\ln T$ is a variable of reaction temperature, while the other two are constants. However, for reactions that are studied within a relatively narrow window of temperature, say no greater than 100 K above room temperature (298 K), a temperature change of 100 K with regard to $\ln T$ does not appear to have too much impact on the overall value of the summation for the last three items. Hence, the Arrhenius equation may be considered a simplified version of the Eyring equation when reactions are studied within a relatively narrow range of temperature; the vast majority of the degradation reactions of drugs fall into this category. Therefore, numerically the value of Ea would not be too much different from that of ΔH^*. Indeed, in a hydrolysis study of a group of sulfamides, the difference between the two values is no more than 1 kcal mol^{-1}.

1.14.4 Reaction orders, half-lives and prediction of drug product shelf-lives

If a reaction only involves a single reactant, A, and the rate of this reaction is proportional to its concentration, the reaction order of this unimolecular reaction is said to be 1 with regard to A and the reaction is a first order reaction. This relationship can be expressed by Eq. 1.7:

$$K = k\,[A]\text{---------------------Eq.1.7}$$

where K is the reaction rate, k is the reaction rate constant, and [A] is the concentration of A.

For a first order reaction as illustrated above, K can be expressed as:

$$K = -d\,[A]/dt\text{------------------Eq 1.8}$$

Where t is the reaction time. As a result, the first order reaction equation can be rewritten as:

$$-d[A]/dt = k[A] \quad \text{or} \quad d[A]/[A] = -kdt \text{ ------------------Eq. 1.9}$$

Integration of the equation results in the following:

$$[A] = -[A]_0 \, e^{kt} \quad \text{or} \quad [A]/[A]_0 = -e^{kt} \text{ -------Eq. 1.10}$$

where $[A]_0$ is the initial concentration of A. The reaction time when half of A is consumed, that is, $[A]/[A]_0 = -1/2$, is the half-life of A, $t_{1/2}$. The equation now becomes:

$$e^{kt_{1/2}} = -1/2 \text{ -------Eq. 1.11}$$

By taking the natural logarithm and rearranging the resulting equation, $t_{1/2}$ can be calculated by Eq. 1.12:

$$t_{1/2} = \ln 2/k = 0.693/k \text{----------------------Eq. 1.12}$$

Thus, for a first order reaction, the half-life of the reactant can be readily calculated from the reaction rate constant, k. While a true unimolecular reaction is not very common, a great number of reactions are bimolecular reactions. The rate of the latter reaction can be expressed by Eq. 1.13, if the reaction order for either A or B is 1:

$$K = k[A][B] \text{------------------Eq. 1.13}$$

where K is the reaction rate, k is the reaction rate constant, and $[A]$ and $[B]$ are the concentrations of reactants A and B, respectively.

For a dimerization reaction of reactant A, $K = k[A]^2$, and the reaction order for A is 2. Frequently during studies of the kinetics of bimolecular reactions, the concentration of one reactant, for example $[B]$, can either be kept constant experimentally or at a large excess with respect to the other reactant. The latter category includes the hydrolysis of drug molecules in aqueous solutions, where water is reagent B in large excess. Consequently, $[B]$ becomes or can be approximated as a constant and the bimolecular rate expression can be written as $K = k'[A]$, where $k' = k[B]$. In such a case, the bimolecular reaction becomes a pseudo first order reaction and the half-life of A can be calculated using the formula for first order reaction shown in Eq. 1.12 above.

In order to calculate the shelf-lives of drug products, more meaningful reaction times would usually be when 5% or 10% of the drug substances are degraded. Frequently, it is desirable to

perform an accelerated stability study at a higher temperature, T_2, from which the degradation rate constant of the accelerated stability study, k_1, is obtained, to 'predict the shelf-life of the drug product at a regular stability temperature (e.g. 298 K), T_2. In principle, this can be readily done for drug products that follow first or pseudo first order degradation kinetics, since the degradation rate constant at the regular stability temperature, k_2, can be calculated according to the following formula, Eq. 1.14, based on the Arrhenius equation (Eq. 1.2):

$$\frac{k_2}{k_1} = \frac{e^{-E_a/RT_1}}{e^{-E_a/RT_2}} \quad \text{or} \quad k_2 = k_1 e^{\frac{E_a}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)} \text{-----Eq. 1.14}$$

Hence, the shelf-life, t, can be calculated or predicted based on a calculation using Eq. 1.14. Nevertheless, in many cases, such predication produces tremendous errors, rendering this approach of no or little practical value. This is due to a number of factors. For example, the degradation mechanism may not be the same at different temperatures. Thus, the dependence of k on T would deviate from the Arrhenius equation. In addition, the exponential relationship between k and T means that a small error in the k_1 value at T_1, could propagate into a huge variation in the k_2 value at T_2.

Because of the limitation of the above approach, various non-linear statistical models for predicting drug product shelf-lives have been developed with varying degrees of success. Some of these models take into consideration the degradation types of reaction orders at or greater than two using a polynomial degradation model. Last, it needs to be pointed out that in the past decade or so, shelf-lives of drug products have been increasingly constrained by the occurrence of degradants rather than by the loss of active ingredient potency due to the advancement of analytical methodologies and tightening of regulatory requirements.

1.15 Impurity profiling

The stability of a drug substance or a drug product is a critical parameter which may affect purity, potency and safety (Alsante *et al.*, 2005; Reynolds, 2002).Changes in drug stability can risk patient safety by formation of a toxic degradation product(s) or deliver a lower dose than

expected (Volans *et al.*, 1990). Therefore, it is essential to know the purity profile and behavior of a drug substance under various environmental conditions.

Two issues of fundamental importance in drug therapy are efficacy and safety. The safety of drug therapy is determined by two main factors:

a) The pharmacological-toxicological profile of the drug substance, i.e. the relation of the beneficial and adverse effects of the drug materials to the human organism.

b) Adverse effects caused by the impurities in the bulk drug material and its dosage forms

An impurity is defined by the ICH Guidelines is "Any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product" (International Conference on Harmonisation, 2006), while the definition of impurity profile in the same is "A description of the identified and unidentified impurities presents in a medicinal product". Although no definition is given for the term "impurity profiling", it is generally considered to be the common name of a group of analytical activities the aim of which is the detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities as well as residual solvents in bulk drugs.

As per the IUPAC "A sample is sufficiently pure when the amount of each of the impurities which may interfere with the specific purpose for which the sample is required is so low that their combined effect is negligible within the desired limits of accuracy".

The best way to characterize the quality of a bulk drug sample is to determine its purity. There are two possible approaches to reach these goals: the determination of the active ingredient content with a highly accurate and precise specific method or the determination of its impurities.

1.16 Organic impurities

Organic impurities, often called related, ordinary or synthesis-related impurities can originate from various sources and from various phases of the synthesis of bulk and the preparation of pharmaceutical dosage forms.

Degradation products can be formed during the synthesis and the isolation of the end product and also upon storage of the bulk drug and especially during formulation and storage of the dosage form.

1.17 Sources of organic impurities

1.17.1 Last intermediate of the synthesis

Impurities falling into this category are often called "probable" or "expected" impurities. For example, the last step in the synthesis of paracetamol is the acetylation of 4-aminophenol: the latter (4-aminophenol) is a probable impurity in the bulk drug material.

1.17.2 Products of incomplete reaction during the synthesis

If the last intermediate has two functional groups and the final step involves the same reaction on both, there is always a possibility that only one of them reacts and a partially reacted impurity appears. Impurities this kind also falls into the category of probable impurities. For example, in one of the systhesis of ethynodioldiacetate; the final step is the diacetylation of ethanodiol (17α-ethinylestra-4-ene-3β,17-diol). Since the reactivity of the secondary 3-hydroxy group is much higher than that of the tertiary 17-hydroxy, a probable (and real) impurity is ethynodiol-3 acetate.

1.17.3 Products of overreaction

In many cases the reaction of the final step is not selective enough and the reagent attacks the intermediate in addition to the desired site. For example in the course of the decanoylation of nandrolone (19-nortestosterone, 17β-hydroxy-ester-4-ene-3-one) to form nandrolonedecanoate the 4-ene-3one can also be decanoylated to form an enol ester type impurity estra-3,5-diene-3,17β-diol-bis-decanoate.

1.17.4 Impurities originating from impurities in the starting material of the synthesis

Impurities present in the starting materials of the drug synthesis can also be source of impurities in the drug material. In these cases the impurity undergoes the same reactions as the main component leading to mainly isomeric impurities. As an example of this the appearance of the isomeric 4-trifluoromethyl impurity in 3-trifluoromethyl-α-ethylbenzhydrol(flumecinol) is a

consequence of the presence of 4-trifluoromethylbromobenzene impurity in 3-trifluoromethylbromobenzene which is the starting material of the synthesis.

1.17.5 Impurities origination from the solvent of the reaction

In some cases the solvent of a reaction or an impurity in the solvent is also transformed during the synthesis leading to an impurity. For example, one of the first steps in the systhesis of pipecuronium bromide is the catalytic elimination of methanesulphonic acid from 3β-hydroxy-5α-androstane-17-one methylsulphonate to form 5α-androst-2-ene-17-one. In the course of the optimization of the reaction conditions of this reaction an impurity was identified as 3β- phenyl-5α-androstane-17-one. In this experiment the solvent mixture contained benzene and the catalysts were silica and aluminium chloride. The obvious reason for the formation of the 3-phenyl derivative was a Friedel-Crafts type reaction between the active ester and benzene.

1.17.6 Impurities originating from the catalyst

The use of homogeneous catalysts may lead to the formation of rarely occurring impurities in which the catalyst molecule is incorporated. An example of this is the tosylation of prednisolone at 21 position catalysed by pyridine in the course of a synthesis of mazipredone. An impurity in the intermediate prednisolone-21-tosylate was found to be the quaternary 21-pyridinium derivative of prednisolone.

1.17.7 Products of side-reactions

In the majority of cases side reactions are unavoidable beside the main reactions in organic syntheses even if pure starting materials and reagents are used and the reaction conditions are carefully optimized.

1.17.8 Degradation products as impurities

Transformation/degradation of the final product of the drug synthesis can take place in the reaction mixture of the final step or during isolation, drying, etc. For this reason degradation products form a group of impurities in drugs. Papvarine can be oxidized under the conditions of the final step of the synthesis to papaverinol and papaveraldine. The quantity of these products

increases under storage conditions; for this reason they can be impurities and also degradation products.

1.17.9 Enantiomeric impurities

In the case of chiral drugs administered as the pure enantiomer the antipode is considered to be impurity.

1.18 Residual solvents

Residues of solvents are usually present at least at trace level in bulk drug materials and pharmaceutical formulations. The origin of these solvent residues in bulk materials are as follows:

a) The solvent of the crystallization
b) The binding of some solvents to the drug material prior to the crystallization
c) Chromatographic solvent
d) Volatile components by adsorption from the air

Solid dosage forms may contain volatile components originating from various sources:

a) Solvent residue from the bulk drug materials
b) Solvent residue from the excipients
c) Water, alcohol, 2-propanol, chloroform, dichloromethane and other solvents are used in wet granulation technologies and in the application of the active ingredients to the powder mixture of excipients by spraying
d) The same solvents are used to dissolve various polymeric materials used for the preparation of film coated tablets and various sustained release formulations

1.19 Inorganic impurities

There are various possible sources for inorganic impurities in drug:

a) The starting materials, reagents and solvents of the synthetic manufacturing process can be sources for salts of inorganic acids
b) Heavy metals from reaction vessels
c) Filter, filter aids and adsorbents
d) Heterogeneous catalyst

e) The degradation of the drug materials

1.20 Toxicological aspects of impurity profile

The licensing of medicinal products for sale is based upon three premises:

a) The product will be efficacious in the disease to be treated

b) It will be sufficiently safe (taking into account other possible therapy)

c) It will be of as good a quality as can reasonably be achieved

The limitation to1 mg daily oral intake of an uncharacterized or poorly characterized impurity will probably satisfy a safety assessment for regulatory purpose.

1.21 Chemistry of the selected 5-HT$_3$ antagonists

The IUPAC name of Ramosetron HCl is (*R*)-(1-Methyl-1H-indol-3-yl)(4,5,6,7-tetrahydro-1H-benzo[d]imidazol-6-yl)methanone hydrochloride. The drug substance is slightly yellowish white, odorless crystalline powder. It is soluble in water. The melting range is 215-230°C. The structure of Ramosetron HCl is shown below.
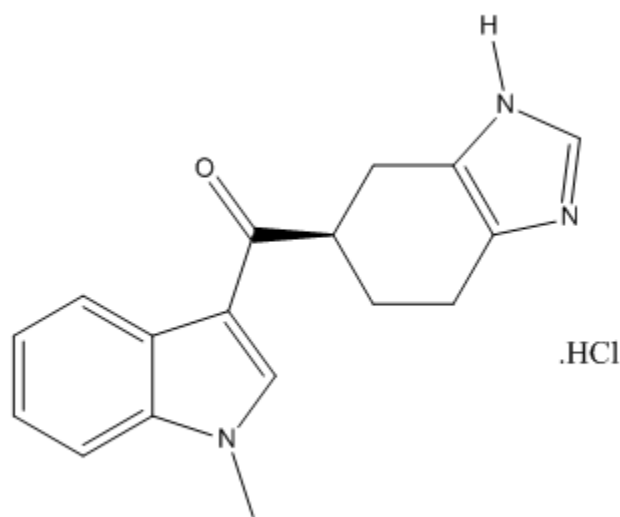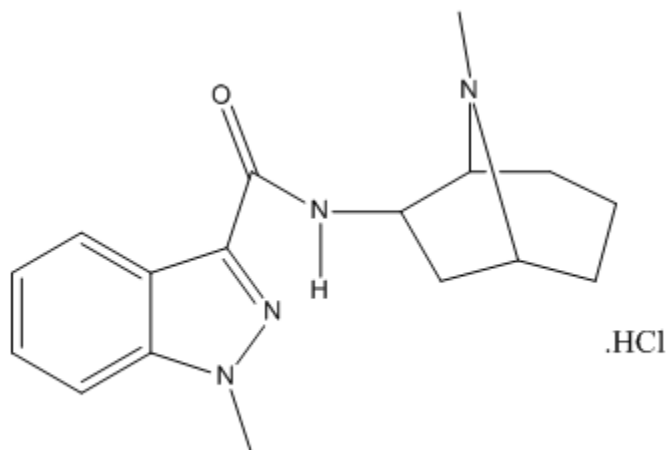

Ramosetron hydrochloride

Ondansetron hydrochloride is the soluble form of ondansetron, a tetrahydrocarbazolone derivative with an imidazolylmethyyl group. It is a white or almost white powder. It is sparingly soluble in water, soluble in methanol, sparingly soluble in ethanol (96 per cent), and slightly soluble in methylene chloride. Its structure is demonstrated below.
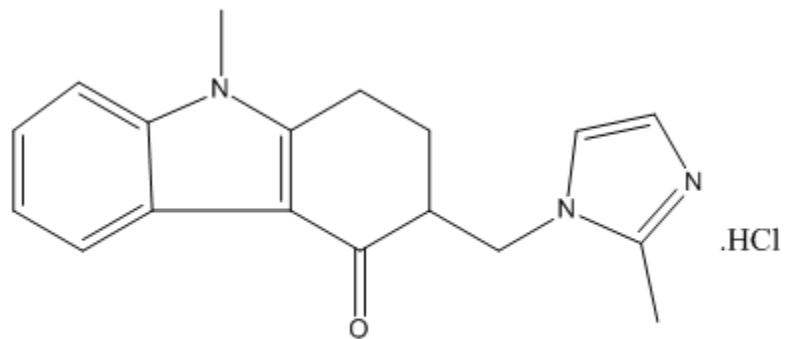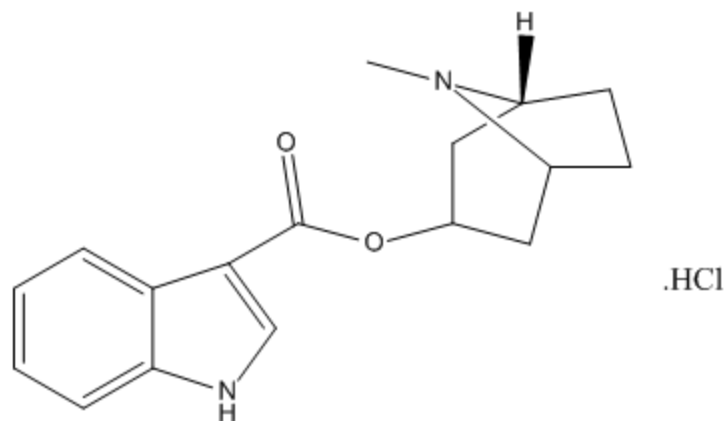
Ondansetron hydrochloride

The IUPAC name of Granisetron is 1-methyl-*N*-[(1*R*, 3r, 5*S*)-9-methyl-9-azabicyclo [3.3.1]non-3-yl]-1*H*-indazole-3-carboxamide hydrochloride. It is white or almost white powder. It is freely soluble in water, sparingly soluble in methylene chloride, slightly soluble in methanol. The structure of Granisetron HCl is shown below.
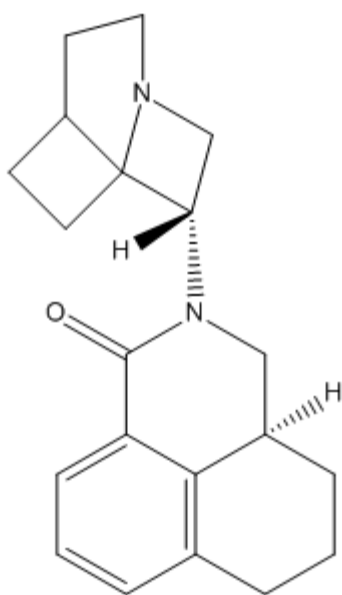

Granisetron hydrochloride

The IUPAC name of Tropisetron is (1*R*,3r,5*S*)-8-methyl-8-azabicyclo [3.2.1]oct-3-yl 1*H*-indole-3-carboxylate. The drug substance is white or almost white powder. It is freely soluble or soluble in water, sparingly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride. The structure of Tropisetron HCl is presented below.

Tropisetron hydrochloride

Palonosetron HCl is a solid substance at room temperature and melts at 87 to 88 °C (189 to 190 °F). The infusion and capsules dosage forms contain Palonosetron hydrochloride. The hydrochloride salt of Palonosetron is easily soluble in water, soluble in propylene glycol, and slightly soluble in ethanol and isopropyl alcohol. The molecule has two asymmetric carbon atoms. It is used in form of the pure (S, S)-stereoisomer. Its structure is shown below.

Palonosetron hydrochloride

1.22 Therapeutic activity of the selected 5-HT$_3$ antagonists

Ramosetron hydrochloride is the hydrochloride salt of Ramosetron, a selective serotonin (5-HT) receptor antagonist with potential antiemetic activity. Upon administration, Ramosetron hydrochloride selectively binds to and blocks the activity of 5-HT sub-type 3(5-HT$_3$) receptors located in the vagus nerve terminal and in the vomiting center of central nervous system (CNS), suppressing chemotherapy-induced nausea and vomiting.

Ondansetron hydrochloride is a potent antagonist of 5-hyroxytryptamine (serotonin) type 3(5-HT$_3$) receptors. Ondansetron is used effectively for the treatment of nausea and vomiting after surgery, radiotherapy or cancer chemotherapy.

Granisetron is a serotonin 5-HT$_3$ receptor antagonist used as an antiemetic to treat nausea and vomiting following chemotherapy. Its main effect is to reduce the activity of the vagus nerve, which is a nerve that activates the vomiting center in the medulla oblongata. It does not have much effect on vomiting due to motion sickness. This drug does not have any effect on dopamine receptors or muscarinic receptors.

Tropisetron is a serotonin 5-HT$_3$ receptor antagonist used mainly as an antiemetic to treat nausea and vomiting following chemotherapy, although it has been used experimentally as an analgesic in cases of fibromyalgia.

Palonosetron is a 5-HT$_3$ antagonist used in the prevention and treatment of chemotherapy-induced nausea and vomiting (CINV). It is used for the control of delayed CINV-nausea and vomiting and there are tentative data to suggest that it may be more effective than Granisetron.

1.23 Rationale of the work

Stress degradation studies to elucidate the intrinsic stability of the drug substance is part of the development strategy and is normally carried out under more severe conditions than those used for accelerated testing (Steven *et al.*, 2011; John, 2002). Stress testing of drug substance can help identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule, and validate the stability indicating power of

the analytical procedures used (Keith *et al.*, 2002; Patrick *et al.*, 2010). The nature of the stress testing will depend on individual drug substance and the type of the drug product involved.

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental stress factors, such as temperature, humidity, and light. Stability testing is also important to establish a retest period for the drug substance during the shelf life for the drug product and recommended storage conditions (Luis *et al.*, 2007; Richard *et al.*, 2007). The rate and mechanisms of reactions with particular emphasis on decomposition and stabilization of drug products are essential for formulation scientists to study, understand, and interpret conditions of instability of pharmaceutical products as well as to be able to offer solutions for the stabilization of these products (Harry, 2002). Knowing the rate at which a drug deteriorates at various hydrogen ion concentrations allows one to choose a vehicle that will retard or prevent the degradation. Application of degradation kinetics in formulation results in the production of more stable drug preparations, the dosage form and rationale of which may be established on sound scientific principles (Birajdar *et al.*, 2015). Degradation and kinetics studies of some members of 5-HT$_3$ receptor antagonists, such as, Dalosetron mesylate (Birajdar *et al.*, 2015; Gillespie *et al.*, 1993; Hu *et al.*, 2012; Huebert *et al.* 1996; Johnson *et al.*, 2003; Lerman *et al.*, 1996; Mcelvain *et al.*, 1997; McElvain *et al.*, 1997; Miller *et al.*,1993), Ondansetron HCl (Patel *et al.*, 2015; Singh *et al.*, 2013), Granisetron HCl (Effat *et al.*, 2011; Bhalerao *et al.*, 2013; Mokhtar *et al.*, 2013), Palonosetron HCl (Vishnu *et al.* 2013; Leon *et al.*, 2006) and Ramosetron HCl (Fujii *et al., 2000;* Zarana *et al.*, 2014) influence the research intuition to take under kinetics studies.

Many research works have been conducted with 5-HT$_3$ antagonists covering pharmacological evaluation, comparative efficacy and safety, design, synthesis, structural and biological study, applications in CNS-related disorders, progress in reducing nausea and emesis and Method development and validation. However, few studies have been conducted with 5-HT$_3$ antagonists in the areas of enantio-separation by computer simulation and validation, compatibility study with glass and plastics, degradation in acidic, basic and oxidative conditions and stability indicating HPLC method development. Very limited data are available for 5-HT$_3$ antagonists on stress degradation studies, degradation kinetics and Impurity profiling.

1.24 Objectives of the work

The key objective of this research is to reveal the factors producing degradants and growing impurities of some selected 5-HT$_3$ antagonists available in Bangladesh. The reported data will help to assist stability indicating method development and validation. This research works will help to know the degradation kinetics of the selected 5-HT$_3$antagonists. The finding of this thesis must be the key information to investigate genotoxic impurities. To set specifications based upon impurity profiling, this thesis may be a vital source of scientific data.

The information which will be evident from this research work will be helpful for a formulation scientist to develop dosage forms of the selected 5-HT$_3$ antagonists following Quality by Design (QbD) approach. This thesis will provide necessary information for the selection of appropriate salt form of the selected 5-HT$_3$ antagonists APIs, the packaging mode selection, appropriate storage conditions and shelf-life determination.

Pharmacologist may be able to extract information regarding absorption, distribution, metabolism, and excretion (ADME) studies. Environmental scientist will get necessary information for the destruction and waste management of the selected 5-HT$_3$ antagonists.

The main objectives of the studies are:

a) To conduct stress degradation/forced degradation and kinetics studies of the selected 5-HT$_3$ antagonists.

b) To develop and validate stability indicating analytical method.

c) To investigate potential degradants and growing impurities.

d) To set product specification.

e) To determine shelf-life of the product.

f) To select appropriate salt form of the selected5-HT$_3$ antagonists.

g) To help formulation scientist to develop robust formulation followed by quality-by-design (QbD).

h) To generate waste management information of the selected 5-HT$_3$ antagonists.