CHAPTER 1
INTRODUCTION
1. Introduction

Product quality, efficacy and safety of drugs have always been a major concern for pharmaceutical industries. Stability of drugs is a quality attribute, which is connected with drug substance or product in terms of strength, purity, identity, safety, apparent physical, chemical, microbiological and biological change, and their effect on biological performance of the drug product. Any change with time in any of the quality attributes of drug product is considered as a potential instability, and assessment of this change becomes mandatory as it is directly related to the safety and efficacy of the drug. Stability testing is done primarily to provide evidence that drug substance or the drug product maintains its essential features of quality, identity, purity and strength (within acceptable ranges) throughout the time in which, it is expected to remain safe for further processing or human consumption. It enables to establish the recommended storage conditions, retest period and shelf lives [1].

For the development of a sound scientific protocol for the stability studies, a thorough understanding of the conditions for the drug degradation and some knowledge of the degradation mechanism are required. Series of stress conditions are applied to generate data on drug degradation [2]. As indicated in the International Conference on Harmonization (ICH) guidelines, stress testing or forced decomposition studies are the studies undertaken to elucidate the intrinsic stability of the drug substance and are carried out under more severe conditions of pH, light, oxidative, hydrolytic stress and dry heat than those used for accelerated testing. Stress testing can help in identifying the likely degradation products, which, in turn, can help in establishing the degradation pathways/ intrinsic stability of the molecule, and validating the stability-indicating nature of the analytical procedures used [3].

Stress testing is the major tool that is used to predict stability problems, develop analytical methods, and identify degradation products and pathways. Stress testing has long been recognized as an important part of the drug development process. Recent efforts by the International Conference on Harmonization with regard to impurities and stability have brought an increased regulatory scrutiny of impurities requiring identification and toxicological qualification at very low levels [4]. Efforts to improve and streamline
processes related to early identification of potential impurity problems are important to the goal of providing new and safe medicines, faster [5].

Stress testing or forced degradation studies involve testing of drug substances and drug products under conditions exceeding those used for accelerated testing. Stress testing (or forced degradation studies) is an important part of the drug development process. Stress testing is undertaken to demonstrate specificity when developing stability-indicating assay methods (SIAMs), particularly when little information is available about potential degradation products. Studies also provide information about degradation pathway and degradation product that could form during storage. Stress degradation studies may help facilitate pharmaceutical development as well as in area such as formulation development, manufacturing and packaging, in which knowledge of chemical behaviour can be used to improve a drug product [6].

Stress testing is different from accelerated stability studies as the former is carried out under more severe conditions and includes exposure to extremes of pH, light and oxidative conditions in addition to temperature, whereas accelerated stability studies include testing at high relative humidity and temperature (40 °C/75% RH) only and serves to generate the data which is useful in predicting the changes that might occur in the drug substance or product during normal storage conditions. [3].

Information on degradation products not only helps chemists to synthesize new compounds with improved stability but also facilitate formulators to design alternative dosage forms with better stability. It also facilitate the selection of appropriate storage conditions and the analytical methods to be used to produce improved drug product. In addition, the information on drug degradation products could become crucial to the understanding of potential toxicity or side effects associated with degradation. Therefore, a rapid and accurate elucidation of the drug degradation process has become essential to the much reduced timelines for drug product development [7].

From a regulatory perspective, forced degradation studies provide data to support the following:

- Identification of possible degradation products.
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- Degradation pathways and intrinsic stability of the drug molecule.
- Stability indicating analytical procedures.

Issues addressed in regulatory guidance include:
- Forced degradation studies are typically carried out using one batch of material.
- Forced degradation conditions are more severe than accelerated stability testing such as >50 °C; ≥75% RH; in excess of ICH light conditions; high and low pH, oxidation, etc.
- Photostability should be an integral part of forced degradation study design.
- Degradation products that do not form in accelerated or long-term stability may not have to be isolated or have their structure determined.
- Mass balance should be considered.

Issues not specifically addressed in regulatory guidance:
- Exact experimental conditions for forced degradation studies (temperatures, duration, extent of degradation, etc.) are not specified.
- Experimental design is left to the applicant's discretion [8].

ICH guideline Q1A(R2) clearly mentions that the results from stress testing studies should form an integral part of the information provided to regulatory authorities at the New Drug Application (NDA) stage. It suggests that the degradation products formed under a variety of conditions should be identified and degradation pathways should be established. It is stated that it may however not be necessary to examine specifically for certain degradation products if they have been demonstrated not to form under accelerated or long term storage conditions [3].

European Committee for Proprietary Medicinal Products (CPMP) draft guideline on stability testing of existing active substances and related finished products state that the regulatory authorities for drugs covered under the pharmacopoeial monographs require no forced decomposition studies [9]. However, stress testing is to be done when no data is available in the scientific literature or the official pharmacopoeias. The route of stress testing for determining intrinsic stability of drugs is also mentioned as a requirement in Canadian Therapeutic Products Directorate’s (TPD) draft guideline for stability testing of well established or existing drug substances and products [10].
Association of Southeast Asian Nations (ASEAN) guideline on stability study of drug product states that stress studies are necessary for pharmaceutical formulation, identifying and monitoring potential degradation products during stability testing [11]. Such testing is part of the development strategy and is normally carried out under more severe conditions than those used for accelerated testing.

1.1. Practical aspects

Though various regulatory guidelines (ICH, FDA) provide information about reporting stress studies, they do not describe how to carry out stress testing studies. However, considerable amount of literature can be found on stress testing. Many authors have proposed different approaches to conduct stress testing. These include Singh and Bakshi’s approach [12], Reynold’s approach [13] and S. Klick’s [6] generic approach. Amongst these Singh and Bakshi approach gives the most detailed and a complete method on the conduct of stress testing.

Important practical aspects in conduct of stress testing (Singh and Bakshi approach)

- **Nature of drug**: The new drug is assumed to be labile in specific nature and accordingly, it should be subjected to stress conditions. Initially, drug should be subjected to intermediate conditions such as (0.1N HCl/NaOH 8 h reflux, water 12 h reflux (acid, base and neutral hydrolysis); 3% H$_2$O$_2$ 6 h RT (oxidation); 1.2 x 10$^6$ lux h light exposure (photolytic degradation). If insufficient decomposition occurs harsher conditions mentioned in the approach should be followed; however, if the drug decomposes excessively, milder conditions should be used.

- **Drug concentration**: The studies should be initiated at a concentration of 1 mg/mL. It is suggested that some degradation studies should also be carried out at a concentration at which the drug is expected to be present in the final formulations.

- **Design of the studies**: For every stress study, it is advised to generate four samples and report the results of each. These include: blank solution (stored under normal conditions), blank (subjected to stress in the same manner as the drug solution), zero time sample (containing the drug stored under normal conditions) and fourth the drug sample subjected to stress treatment.
• **Equipment for stress test:** The reactions can simply be carried out in containers like volumetric flasks or stoppered culture tubes and stored in water bath set at the desired temperature. For reactions above 80 °C and reflux conditions, a boiling water bath equipped with a voltage regulator or an oil bath may be used. For oxidative stress, studies should be done in a leak proof stoppered container. A caution is that the headspace left above the solution during the study should be small, for which, either the solution volume can be increased or the size of the container can be selected so that it is sufficient just to accommodate the total volume of reaction solution. For photolytic reaction, if solid drug sample is used, the study should be done in a petri dish, where solid samples are spread evenly as a thin layer. For liquid or dissolved sample, any transparent container can be used which gives maximum exposure.

• **Preparation of samples for HPLC studies:** Processing is necessary for making proper concentration of drug, acid, alkali or oxidizing agent to make them compatible with HPLC. For this, one approach is diluting the sample enough so that the concentration of reagent falls within the tolerable range. Dilution should preferably be done in the mobile phase. The second approach involves neutralization of acid and alkali solutions to a tolerable pH. However the dilution is a more simple method than neutralization as degradation product may further degrade by changing pH. If stress sample condition is solid particle then those solid particles should be separated and dissolved in appropriate solvent. This sample may help to detect insoluble degradation product, which forms during stress testing.

1.2. **Stability-indicating assay method (SIAM)**

The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. SIAM is an analytical procedure, which is capable of discriminating between the active pharmaceutical ingredient (API) and the degradation product(s) formed under defined storage conditions during the stability evaluation period. In addition, it must be sufficiently sensitive to detect and quantify one or more degradation products [12].
1.3. Regulatory aspects

The ICH guideline Q1A(R2) on Stability Testing of New Drug Substances and Products emphasizes that the testing of those features which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy must be done by validated stability-indicating testing methods [3]. The ICH guideline Q3B(R2) entitled ‘Impurities in New Drug Products’ emphasizes on providing documented evidence that analytical procedures are validated and suitable for the detection and quantitation of degradation products [14]. It is also required that analytical methods should be validated to demonstrate that impurities unique to the new drug substance do not interfere with or are separated from specified and unspecified degradation products in the drug product. The ICH guideline Q6A, which provides note for guidance on specifications, also mentions the requirement of stability-indicating assays under Universal Tests/Criteria for both drug substances and drug products [15]. The same is also a requirement in the guideline Q5C on Stability Testing of Biotechnological/Biological Products [16]. Since there is no single assay or parameter that profiles the stability characteristics of such products, the onus has been put on the manufacturer to propose a stability-indicating profile that provides assurance on detection of changes in identity, purity and potency of the product. Unfortunately, none of the ICH guidelines provides an exact definition of a stability-indicating method. Elaborate definitions of stability-indicating methodology are, however, provided in the US-FDA stability guideline of 1998. Stability-indicating methods according to this guideline were defined as the ‘validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference’ [17]. Even the United States Pharmacopeia (USP) has a requirement listed under ‘stability studies in manufacturing’, which says that samples of the products should be assayed for potency by the use of a stability indicating assay [18]. Current ICH guideline on Good Manufacturing Practices for Active Pharmaceutical Ingredients (Q7A), also clearly mentions that the test procedures used in stability testing should be validated and be stability indicating [19].
Regulatory authorities does not explicitly require stress testing be performed or reported at the Phase 1 and 2 of Investigational New Drug (IND) stages although it is encouraged to facilitate selection of stability indicating methods [20]. 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 [21]. 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 [3] in degraded samples from formal stability trials, an assessment of the drug’s stereochemical stability [16], 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 [15]. 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 [16]. 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 [21]. 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 [15].

1.4. Techniques employed

In last decade titrimetric, spectrophotometric and chromatographic techniques have been commonly employed in analysis of stability samples. But nowadays hyphenated techniques are also used. Titrimetric and spectrophotometric methods are simple and inexpensive but they are not sensitive as that of chromatographic method and have limited specificity. In contrast to these methods, chromatographic methods are sensitive, accurate and can detect trace amounts of degradation product/impurities as well as are capable of separating multiple components. In chromatographic methods, HPLC has been very widely employed. It has gained popularity in stability studies due to its high-resolution capacity, sensitivity and specificity. Non-volatile, thermally unstable or polar/ionic compounds can also be analyzed.
by this technique [22]. In addition to the spread of HPLC/MS, another new development in this field has been the introduction of column packings with ultrafine particles (<2 µm) enabling short columns (5 cm or less) to be used and rapid analyses (e.g., 5 min or even less than 1 min) to be carried out by UPLC (ultra performance liquid chromatography). UPLC fulfils the promise of increased resolution, speed, and sensitivity predicted for liquid chromatography [23]. Analytical methods, which are reported till date in literature, are enlisted in Table 1.1.

Table 1.1 Techniques employed in stability-indicating method.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Principle Technique</th>
<th>Technique</th>
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<tbody>
<tr>
<td>1.</td>
<td>Electrometric</td>
<td>Titrimetric, Polarographic</td>
</tr>
<tr>
<td>2.</td>
<td>Spectrophotometric</td>
<td>Ultra violet (UV)/Visible (VIS), Fluorescence, Nuclear magnetic resonance (NMR)</td>
</tr>
<tr>
<td>3.</td>
<td>Chromatographic</td>
<td>Thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), Gas chromatography (GC), High performance liquid chromatography (HPLC), Capillary electrophoresis (CE)</td>
</tr>
<tr>
<td>4.</td>
<td>Hyphenated techniques</td>
<td>Gas chromatography–Mass spectrometry (GC-MS), Liquid chromatography–Mass spectrometry (LC-MS), LC-MS-MS, Liquid chromatography–Nuclear magnetic resonance (LC-NMR), Capillary electrophoresis–Mass spectrometry (CE-MS)</td>
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</table>

1.5. Identification and characterization of degradation products

The isolation of degradation products has been used as a general approach for their structure elucidation. However, considerable time and the use of several techniques, such as a preparative scale chromatography and multiple extractions, are required to isolate sufficient quantities of degradation products for spectroscopic identification. Moreover, special care is required during the isolation processes in cases where the degradation products are labile, since further degradation of the degradants will occur [24].
HPLC hyphenated analytical techniques such as LC-DAD-UV, LC-MS and LC-NMR, in which a chromatographic separation is coupled online with one or more information-rich detectors, have quickly become indispensable tools for low level impurity and degradation products identification. All of these techniques have complementary selectivity, often requiring analysis by all of them to completely define an unknown molecular structure [25].

LC-MS has been one of the powerful techniques for the identification of small quantities of drug degradation products [26-29]. Over the last decade, atmospheric pressure ionization (API) LC-MS has largely replaced direct probe electron impact and chemical ionization mass spectrometry for structural elucidation. This on-line approach also provides an easy means of volatilizing relatively nonvolatile analytes to obtain molecular weight information (MS) and structural information based on collision-induced-dissociation (MS/MS and MS\(^n\)).

However, sometimes the structures assigned with the help of LC-MS alone remain tentative, especially when structural, conformational, and optical isomers need to be identified. In such cases, many a times NMR experiments are required to obtain detailed structural information on each component. During these experiments, each component has to be separated and isolated from an HPLC run for NMR analysis. With the advent of LC-NMR, this additional isolation step is eliminated and the structural characterization work is thus accelerated [30]. Recently, LC-NMR has been increasingly applied to obtain detailed structural information on degradation products.

Stability testing function is an evolutionary concept covering the life cycle of pharmaceutical product development. The objective of stability testing varies during various stages of product development. For example, during the early discovery phase, the primary focus is to generate stability characteristics of a chemical ingredient or biological entity, which will be helpful in the design and development of drug or biological products. During later stages of product development, the goal of stability testing is to establish shelf life for formulations packaged in a final package intended for commercial introduction. Stability testing principles can be subdivided into various stages of development. The objective of stability testing differs in each stage of development. The following sequence describes several stages of drug development during which stability characterization should be carried out.
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- Stability testing of new chemical or new molecular entities (discovery phase).
- Stability testing of formulations for preclinical safety testing or toxicology testing (preclinical stage).
- Pre-formulation stability testing of new chemical entities (pre-IND stage).
- Accelerated and normal storage testing of clinical formulations (IND stage).
- Stability testing of early formulations, formulation mixtures, and packaging materials evaluation (product development stage).
- Long-term primary stability testing of final marketable formulations in proposed packages for marketing for product registration and approval (NDA stage).
- Stability testing of production batches post regulatory approvals (approved product stage).
- Stability testing of revised products (revised product stage).

During the discovery phase, stability testing of a new chemical entity or a new molecular entity is required to help select the most satisfactory chemical or molecular entity possessing the right pharmacological, toxicological, and pharmaceutical profiles. The pharmaceutical profile is mostly focused towards the optimum chemical and physical stability characteristics, a good preformulation profile, and satisfactory manufacturing potential. The emphasis initially is not only towards selecting the right chemical entity but also towards selecting the appropriate physical form (for example a satisfactory polymorphic form if applicable, or a physical form with the most desirable handling behavior), and, where applicable, the base, salt, ester, hydrates, solvates or other forms with optimum stability. It is not uncommon to find vast differences in stability characteristics of different salts of the same chemical entity. In addition, preliminary information regarding particle size, distribution, crystal shape, crystal habits, mechanical properties, and specific surface area can play key roles in affecting the stability, manufacturability, and biological performance of the chemical entity. These studies help to establish the boundaries within which one must operate to design formulations for toxicology and for initial clinical testing. In the current competitive accelerated drug development environment, early physicochemical investigation has to be completed not only in the shortest time possible but also with limited quantities of drug substance [31].
1.6. Decision trees for respective stress conditions

As per S. Singh et.al, [13] an approach was suggested to carry out stress degradation studies. This approach was based upon the labile behavior of the drug, for which they proposed a systematic classification of drugs based on the susceptibility to stress conditions and decision trees to stop the stress condition at a particular time interval. From the decision trees a decision can be taken on whether to increase or decrease the strength of the reaction conditions. The increase or decrease is done step-wise and those stress conditions are accepted wherever a sufficient degradation is obtained. Figure 1.1, suggests that in order to study hydrolytic degradation (under acidic and alkaline conditions) of a new drug, whose stability behavior is not known. In such case one can start by refluxing the drug in 0.1 N HCl/NaOH for 8 h, considering that the drug is labile. If a reasonable degradation is observed on subjecting the drug to this treatment, no further studies need to be carried out. In case no degradation is seen, drug should be subjected to refluxing in 1 N acid/alkali for 12 h. For a drug which can withstand even these conditions, more extreme conditions of acidity or alkalinity such as refluxing in 2 N HCl/NaOH for 24 h may be tried. The reaction should be monitored, and if still satisfactory change is not obtained, the drug should be refluxed in 5 N HCl/NaOH for up to 24 h. The drug may be declared to be “practically stable” if no hydrolytic products are formed on subjecting the drug to this harsh condition. Going to the other side of starting condition, if a total degradation is seen after refluxing in 0.1 N HCl/NaOH for 8 h, the strength of acid/alkali can be decreased to 0.01 N along with decrease of temperature to 40 °C while keeping the time as same 8 h. A drug showing complete degradation even in these mild conditions, then it should be treated with 0.01 N HCl/NaOH for 2 h at 25 °C and if still complete degradation is taking place, drug is extremely labile and has to be tested under very mild conditions of temperature and pH.
Fig. 1.1. Decision tree for performing stress studies for hydrolytic degradation under acid and alkali conditions.

Stress testing under neutral conditions can be started by refluxing the drug in water for 12 h (Fig. 1.2). Refluxing time should be increased to 1 day in case no degradation is seen. It should be increased further to 2 days if no change is observed. In case of negligible degradation, the drug may be refluxed for a period of 5 days. If still found stable, the drug may be declared non-degrading in neutral conditions. For this study, it may be advisable that a sufficient volume of solution should be taken for the reaction initially, so that the time period can be continually increased, as required, and there is no need to restart the reaction afresh. For a drug undergoing complete degradation on refluxing in water for 12 h, both time and temperature of exposure may be decreased to 8 h and 40 °C, respectively. More mild
conditions, like keeping the drug in water up to 2 h at 25 °C, should be tried if no intact drug is left after exposure to above mentioned conditions.

**Fig. 1.2.** Decision tree for performing stress studies for hydrolytic degradation under neutral conditions.

For determining the susceptibility of the drug to oxidative decomposition, testing may be started by keeping the drug in 3% hydrogen peroxide (H₂O₂) for 6 h at room temperature (Fig. 1.3). The period of reaction should be increased to 24 h in case there is no sufficient degradation. Still if there is no change, the reaction should be conducted in 10% H₂O₂ for 24 h. For a drug which does not oxidize even under these conditions, more extreme conditions of 30% H₂O₂ for 24 h may be tried. The drug may be declared to be “practically stable” if no degradation products are formed on subjecting the drug to this condition. In an event of
decomposition of whole drug under the starting conditions, the strength of H$_2$O$_2$ should be
decreased from 3% to 1% and the reaction may be monitored for a period sufficient to yield
the desired percent of decomposition. The drugs undergoing complete degradation even
under these conditions are highly prone and should be tested in very dilute oxidising agent
with an exposure for very short duration.

Fig. 1.3. Decision tree for performing stress studies under oxidative conditions.

In order to get an idea about photostability (Fig. 1.4), the drug substance should be initially
subjected to an illumination up to 1.2 x 10$^6$ lux hours which is the ICH recommended
exposure and the reaction should be monitored periodically. The exposure may be increased
by 5 folds in case there is negligible degradation. The drug may be declared photostable if
the increase in exposure to 6.0 x10$^6$ lux hours has no effect on the stability of the drug.
Fig. 1.4. Decision tree for performing stress studies for photolytic degradation.

Over the last century mass spectrometry (MS) has emerged as an indispensable analytical technique. Historically, the issues of concern in MS research have mainly been the development and refinement of the hardware employed, whereas now a lot of emphasis is put on data evaluation and interpretation. The possibility to discover new biomarkers using MS is a rapidly growing field of research. Biomarkers are compounds that potentially can be used for early diagnostic or disease/treatment surveillance purposes. The measurement should then convey information of the biological condition being tested. However, stress is given towards the difficulties of validating the analytical procedures involved. Even though MS has become a mature technique, there are still limitations to the instrumentation available today. Especially in quantitative analysis, many researchers report unwanted increase or decrease of the MS signal due to other constituents in the sample, which potentially will impair the analysis. Therefore, the quest for more robust technical solutions and analytical methods will continue.

The potential of MS, and also LC-MS, is recognized in the huge amount of data produced. However, in order to fully appreciate the data gathered, proper techniques for data treatment and evaluation have to be incorporated. The application of different mathematical tools is
therefore a prerequisite for the realization of the high potential of MS. Also, in order to ascertain optimized conditions for analyses, it is essential to identify and properly adjust the parameters most likely to affect the obtained results. The field of chemometrics offers the means for successful planning and evaluation of the experiments.

The uses for mass spectrometry continue to grow at an unprecedented rate. Everyday, new applications are developed as the instrument continues to advance as fast as the need. Some specific applications include analyses of small (drugs), medium (siderophores) and moderately large (peptides) compounds. Several technical developments have been converging into a generic new approach to proteomics. The specific advances include high throughput protein identification by multidimensional chromatography, automated tandem mass spectrometry and sequence database searching, accurate quantification by the application of stable isotope dilution theory to protein analysis and the targeted isolation of selected analytes by the use of highly selective chemistries. Recent advances in instrumentation and methods have improved the sensitivity and throughput of mass spectrometry-based approaches so that truly proteome-wide analyses are now becoming feasible. The 2004 Nobel Prize for chemistry was recently awarded to John B Fenn and Koichi Tanaka “for their development of soft ionization methods for mass spectrometric analyses of biological macromolecules”.

Over the past decade, we have witnessed how proteomics has dealt with growing pains and delivered elegant and effective solutions to the numerous challenges that it faced. Even so, comprehensive, reproducible, quantitative, accurate and precise information on proteins is yet to be achieved with a throughput high enough to realize the great potential of mass spectral techniques in all areas of biosciences. Then again, the promise of proteomics for advancing our understanding of diseases (e.g., cancer) is immense and it is our responsibility to see these challenges to fruition.

Of the numerous types of analytical techniques used in drug discovery and development, mass spectrometry (MS) has become one of the most powerful tools for the analyses of a wide range of chemical and biological entities. A large portion of ADME assays are now being incorporated into drug discovery programs in a high throughput mode because they have enhanced sensitivity, selectivity, and ease of automation that is available with LC-MS
and LC-MS/MS in comparison with traditional analytical methods (LC-UV, florescence, NMR, etc.). Additionally, MS is useful for chemists as well as researchers from neighboring disciplines such as physics, medicine, or biology as a powerful analytical tool. Tandem MS/MS is a powerful technology has opened an avenue for newer and broader applications. Traditionally, separation of an analyte from coeluting matrix components and/or metabolites was achieved by modifying the chromatographic conditions. However, HPLC method modifications come at the expense of time and resource consumption and most often end up being methods with longer run times, which are usually not suitable for high throughput quantitative assays. There are several examples where utilization of higher mass resolution in quantitative assays is advantageous over modifying chromatographic conditions, changing ionization methods, selecting different multiple reaction monitoring (MRM) or selective reaction monitoring (SRM) ion pairs, changing different mass spectrometers, or changing the sample processing/clean-up methods. Indeed the demands of the pharmaceutical and biotechnology industries have driven to some extraordinary recent advances in mass spectrometry technology.

In the past decade, with the commercialization of mass spectrometers using soft ionization techniques such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), the coupling of high-performance liquid chromatography (HPLC) and mass spectrometry (MS) has become one of the most powerful techniques for characterization of impurities or degradation products. The mass fingerprinting techniques such as LC-MS/TOF, LC-MS^n and the recently introduced LC-MS^n-TOF have proven to be very useful tools for on-line characterization and structure elucidation of degradation products in trace concentrations. The strategy involves understanding of fragmentation pattern of the drug and the degradation products using ion-trap MS^n analyses, while measurement of accurate molecular mass/empirical formula can be done employing LC-MS/TOF. In addition, complementary tools like isotopic abundance and H/D exchange mass spectrometry are employed, wherever applicable [32, 33].

In fact mass spectrometry (MS) has emerged as a front-runner technique in pharmaceutical analysis, covering both qualitative and quantitative aspects. The area of use of MS is increasing at such an unprecedented rate that new applications are getting developed almost...
on daily basis. Coupled to it is advancement in the instrumentation, which is trying to keep pace with the ever increasing demands on sensitivity and throughput, guided at times by the increasing stringency of regulatory requirements.

1.7. Pharmaceutical applications

1.7.1. Drug candidate synthesis and screening

Modern drug discovery practice is heavily dependent on the synthesis of combinatorial libraries. As the preparation of large libraries for lead discovery became routine, emphasis is going to be placed on analytical techniques that focus mainly on throughput and quality. Analytical and preparative RPLC in conjunction with MS offers such a potential [34, 35]. As emphasis increases on speed, modern trap and time of flight (TOF) instruments can be employed to measure many mass spectra per second. On the other hand, attraction towards fourier transform-ion cyclone resonance (FT-ICR) is increasing in the pharmaceutical industry owing to its capability to deliver more information per measurement. The instrument has undergone rapid development and is now applied in many fields. FT-ICR has contributed to the dereplication and chemical fingerprinting of natural products, such as in bioactive marine macroorganisms and elemental composition determination of the natural products, such as antibiotics [36].

1.7.2. Metabolites identification and bioanalysis

Fast and accurate methods in drug metabolism and pharmacokinetic (DMPK) studies are highly demanding in drug discovery programs. The tandem LC-MS systems allow a chemically diverse range of drug candidates to be analyzed quickly [37]. The LC-MS based in-vivo pharmacokinetic screening approaches allow analysis of large number of samples, thereby reducing the time in which results can be obtained. In a conventional set-up, single auto-sampler/HPLC column, and MS systems are used and samples are injected one at a time. The strategies used to amplify throughput include fast chromatography, automated data processing, and pooling strategies (cassette dosing, pooling after individual dosing, simple sample screens, etc). Two auto samplers and columns, in parallel with a single triple quadruple mass spectrometer, reduce HPLC column equilibration time and common holdups associated with sample loading. The LC-MS and LC-MS/MS techniques are also very useful.
in metabolite identification, as they have the ability to predict as well as detect the metabolites in complex samples of urine, bile, and plasma [38]. The metabolite prediction is based on the fact that metabolites are produced from select in-vivo reactions. The software then imposes the predicted metabolites upon the resolved ones and carry out detection based on the fragmentation overlap. Also, in general, metabolites preserve most of the core structure of the parent drug, and hence show similar fragmentation pattern, generating mass spectra that designates major substructures.

1.7.3. Impurity identification and profiling

Impurities in drug substances are synthetic by-products, starting materials, intermediates, etc., while in case of drug products these constitute degradation products formed on storage. Toxic impurities, regardless of source, are of particular concern during safety evaluation, process research activities and during dosage form development, because of the rigorous regulatory requirements outlined by various international agencies, including ICH [15]. The mass fingerprinting techniques, like LC-MS/TOF, LC-MS\(^0\) and the recently introduced LC-MS\(^n\)/TOF have proven to be very useful tools for on-line characterization and structure elucidation of impurities in trace concentrations. Alternately, mass based preparative instruments are employed for specific isolation [39]. When a new impurity is encountered, retention time, molecular weight information and sub-structural fragmentation pattern obtained using MS\(^n\) and/or TOF helps in rapid identification [40]. CE-MS has also been implemented in the impurity profiling of pharmaceutical products [41].

1.7.4. Stability studies of drug substances and drug products

During the development phase, drug substances as well as drug formulations are subjected to stress testing under a variety of stress conditions, such as temperature, humidity, acidity, basicity, oxidation, light, etc. The same facilitates validated analytical method development and provides extrapolative information for upcoming formulation and packaging studies. For these studies, practical approaches to attain structural elucidation of degradation products using modern LC-MS or LC-MS/MS techniques and information obtained from them, such as retention time, molecular weight and fragmentation pattern, has gained paramount importance. The strategy for identification of degradation products during early development requires fast and sensitive LC-MS analytical methods [42]. Consequently, a structure
database can be built from outcomes, which is employed to distinguish unstable structures within the drug structure as well as for the rapid identification of degradation products generated during these studies [43]. These techniques are also extended later during the analyses of long term as well as accelerated study stability samples to obtain useful and relevant information.

The inherent analytical advantages of mass spectrometry, including speed and sensitivity, combined with recent advances promise to make the technique a mainstay with respect to variety of pharmaceutical applications. The recently developed ionization techniques, such as Direct Analysis in Real Time (DART) and Desorption Electrospray Ionization (DESI), and analyzers, such as orbitrap, allow any type of component to be studied by MS. In addition, MS/MS, MS^n and MS/TOF add the capability for structural analysis of compounds that are present at low level and/or are present in complex mixtures.

The sensitivity and high throughput capability of MS should make it a powerful tool for the early stages of drug discovery, including combinatorial chemistry and DMPK studies. The same is the case even with routine analysis, including impurity profiling, stability testing and quality control activity. The gentle nature of relatively new ionization techniques and the structural analysis capability of MS have the promise to extend mass spectrometry towards more new areas [44].