

# 13

## ASSAY AND STABILITY TESTING

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### ABSTRACT

#### I. INTRODUCTION

- A. Scope
- B. Objectives

#### II. STABILITY PROGRAM IN PHARMACEUTICAL DEVELOPMENT

- A. Guidelines for Stability Testing on Drug Substances and Drug Products
- B. Stability Testing at Different Phases of the Drug Development Process
- C. Testing Requirements for Drug Products Filed in the United States and Outside the United States
- D. Design of Stability Program
- E. Specifications Setting in Stability Testing

#### III. STABILITY TESTING BY HPLC

- A. HPLC for Stability Testing
- B. Detector Considerations
- C. Stability-Indicating Methods Development
- D. Case Studies

#### IV. SUMMARY AND CONCLUSION

#### ACKNOWLEDGMENTS

#### REFERENCES

### ABSTRACT

This chapter introduces the general concept of drug stability and the factors affecting drug substance and drug product stability. It discusses the guidelines from the International Conference on Harmonisation (ICH) for stability testing on drug substances and products, and the requirements at different phases of the drug development process in the pharmaceutical industry. The role of high-performance liquid chromatography (HPLC) in stability testing along with the chromatographic techniques and the procedures involved in developing a stability-indicating method are also described. Case studies of challenging HPLC method development on dual drug systems in solid dosage and surfactants with different molecular weights in liquid formulation are presented.

## I. INTRODUCTION

### A. Scope

#### 1. What Is Drug Stability?

The term drug stability refers to the extent to which a drug substance or product retains, within specified limits and throughout its period of storage and use, the same properties and characteristics that it possessed at the time of its manufacture. The type of stability is generally divided into chemical, physical, microbiological, therapeutic, and toxicological.<sup>1</sup> Drug stability can be categorized as pre-market and commercial (marketed product) stability. Pre-market stability, which supports the clinical trial where drug products are stored under different conditions for safety and efficacy evaluation, is usually conducted throughout the clinical trial and during the filing period. Commercial stability is continuous assurance on the post-approval batches for long-term stability monitoring on the drug product. Drug stability assessment generally involves the testing of the drug substance or drug product using a stability-indicating method in order to establish the retest period (for pre-market stability) and shelf life (for commercial stability).

Drug substance (also called the active pharmaceutical ingredient [API] as per USP-NF definition<sup>1</sup>) is the material that is used to manufacture, usually with excipients, the drug product. Drug substances can be derived from chemical synthesis, plant or animal sources, or biological or recombinant technology. In addition to the API, the drug substance can contain product- and process-related substances or impurities. From the earliest stage of drug product development, information on drug substance stability has been an integral part of drug development. Data on the physical and chemical characteristics and other properties of the drug substance are helpful for designing methods that indicate the drug product's stability and are also helpful in designing formal stability studies.

Drug product (also called the dosage form or finished product per USP-NF definition<sup>1</sup>) contains one or more drug substances, usually with excipients, in the final packaging intended for marketing. Stability studies on the drug product serve three purposes: (1) to support the stability of the drug product used in clinical/non-clinical studies, (2) to establish commercial expiry dating, and (3) to determine levels for certain specifications (API, preservatives, etc.) and set the control limits for lot release.

#### 2. What Is a Stability-Indicating Method?

A stability-indicating method is a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product under defined storage condition. A stability-indicating assay method accurately measures the active ingredient(s) without interference from other peaks and is sensitive

enough to detect and quantify the degradation products/impurities.<sup>2</sup> To develop a stability-indicating method, stress testing, in the form of forced degradation and a photostability study, should be carried out at an early stage so that impurities and degradation products can be identified and characterized. Stability-indicating assay analytical methods must be discriminating and validated to ensure the accuracy of the long-term stability study trending.

### 3. Factors Affecting Drug Substance and Product Stability

The stability of drug substances and products can be influenced by environmental factors such as temperature, humidity, light, and oxygen. The major factors that influence drug stability in the solid dosage or liquid form include particle size, pH, solvent system composition, solution ionic strength, cations and anions/excipients compatibility, chemical additives, and primary container and storage conditions.<sup>2</sup> Information obtained from stability studies in different conditions will aid in establishing the retest period of the drug substance and shelf life of the drug product. Strict adherence to the storage requirements specified in the product labeling will help ensure product potency and stability through to the manufacturer's labeled expiration date.

## B. Objectives

### I. General Stability Study

The general purpose of stability testing is to monitor the characteristics and changes of drug substances and drug products under different conditions (temperature, humidity, and light) over time so that the retest period of drug substances or the shelf life of drug products under specified storage conditions can be established. The test conditions should be wide enough to cover the four climatic zones in the world.<sup>2,3</sup> The four climatic zones are defined as (I)—temperate climate (e.g., Northern Europe, Canada, Russia, and the United Kingdom), (II)—subtropical and Mediterranean climate (e.g., United States, Southern Europe, and Japan), (III)—hot, dry climate (e.g., Iran, Iraq, and Sudan), and (IV)—hot, humid climate (e.g., Philippines, Indonesia, and Brazil). With the data obtained from stability studies, optimum packaging that will ensure the quality and integrity of the drug product in different parts of the world can be developed.

The typical attributes evaluated in stability testing include physical, chemical, biological, and microbiological quality characteristics that cover all dosage forms. A stability protocol should be generated for every batch that is evaluated in a long-term stability study. The stability protocol should record the purpose of the stability test, the method used, testing frequency, the storage conditions, the package description, and any additional information that needs to be included.

## 2. Role of HPLC in Stability Testing

The following methods are used to evaluate the stability and purity of drug substances and drug products: thin-layer chromatography (TLC), HPLC, gas chromatography (GC), capillary electrophoresis (CE), and ultraviolet (UV) spectroscopy. However, reversed-phase HPLC analysis is generally considered the most effective method of identifying most drug substance degradation or drug–excipient interactions. Hence, it is the typical choice for stability-indicating and stability-specific methods for small molecules. Reversed-phase HPLC accounts for more than 85% of stability-indicating methodologies for small molecular chemical entities as it is suitable for release testing, assay, and assessing impurities.<sup>4</sup> With its well-established techniques, efficiency, robustness, and ease of use, HPLC plays a pivotal role in drug stability testing.

## II. STABILITY PROGRAM IN PHARMACEUTICAL DEVELOPMENT

### A. Guidelines for Stability Testing on Drug Substances and Drug Products

#### 1. Introduction

The primary purpose of stability testing is to provide data and supporting evidence on the stability behavior of chemical or biological entities in different forms. As drug stability can be affected by many factors like temperature, humidity, light, pH, oxidation, or combinations of these parameters, it is critical to establish the retest periods of drug substances and shelf lives of drug products under recommended storage conditions. In this section, the general approach of stability testing on drug substances and drug products will be discussed.<sup>5</sup>

#### 2. Stress Testing

In the early stages of product development, a systematic stability study plan is critical, as it can affect the product life cycle and project planning. Before the drug product is available, stress testing (a forced degradation study) of the drug substance under different conditions can help to identify possible degradation products, the degradation pathway, and the stability of the drug substance.<sup>2,5</sup> The design of the stress test will depend mainly on the nature of the drug substance and the excipient composition.

Stress testing should be carried out on at least a single batch of the drug substance at the beginning, and minimally, include the effects of temperatures (a wide range from 20°C to 60°C), humidity (e.g., 60%RH, 75%RH, and dew point), oxidation, hydrolysis (wide pH range), and light (see photostability testing section). The information on the impurities and degradation pathway will help develop a stability-indicating method for formal stability studies of drug substances and drug products. (See chapters 6 and 7.)

### 3. Photostability Testing

Photostability testing is another form of stress testing to ensure that if the drug substances and products are exposed to light, the light will not cause unacceptable changes in the drug's potency or safety profile. To simulate a product left on a window, sitting on a shelf, or left in an open area, light chambers that expose the sample to various types and intensities of light are used. Light or photostability studies can also help determine the optimum packaging required to protect the drug product. Drug substances, in conjunction with excipients and drug products, should be exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200-W h/m<sup>2</sup> to allow direct comparisons. Testing should progress until the results demonstrate that the drug product is adequately protected from exposure to light.<sup>6</sup>

The drug substances and products should be examined for any changes in physical properties and for assay and degradants by a suitably validated method. If protected samples are used as dark controls, the sample analysis should be performed concomitantly. The test method should be capable of resolving and detecting photolytic degradants that might arise in the confirmatory studies. To assure that the product is within proposed specifications during the shelf life, the results of photostability studies should be evaluated to determine whether any changes due to light exposure are acceptable.

For drug substances, photostability testing should consist of two parts: forced degradation testing and confirmatory testing. Forced degradation testing is to evaluate the overall photosensitivity of the material for method development and degradation pathway elucidation. Confirmatory studies should then be undertaken to provide the information necessary for handling, packaging, and labeling. For drug products, one batch is required for confirmatory testing in the development phase, but at least two additional batches should be tested if the results of the confirmatory study are equivocal.<sup>6</sup>

### 4. Selection of Batches and Container Closure System

Stability data on at least three primary batches must be provided.

For drug substances, the batches should be manufactured to pilot scale by the same synthetic route that simulates the final process to be used for production batches. Stability studies should be carried out on the drug substance where it is packaged in a container closure system that imitates the packaging proposed for storage and distribution. For drug products, the primary batches should be of the same formulation and packaged in the same container as proposed for marketing. Two of the three batches should be at least pilot-scale batches; the third batch can be smaller in size. If possible, the drug product batches should be manufactured using different batches of the drug substance.

## 5. Storage Conditions and Testing Frequency

The storage conditions and the length of the studies should be sufficient to cover storage, shipment, and subsequent use. Both drug substances and products should be evaluated under storage conditions that test for thermal stability (temperature effect) and moisture sensitivity (humidity tolerance).

The storage conditions for drug substances and products recommended by the International Conference on Harmonisation (ICH) guidelines are summarized in Table 1. In addition to testing for assay, impurities/degradation products, and general attributes, water content determination is recommended for solid dosage drug products. Alternative storage conditions can also be used with data support and justification.<sup>5</sup>

If drug substances and products are intended for storage in the refrigerator or freezer, real-time data obtained at the long-term storage condition must be provided as supporting evidence for the retest period or shelf life determination. Drug substances and products that require storage below  $-20^{\circ}\text{C}$  should be evaluated on a case-by-case basis.

The stability study conditions recommended by ICH guidelines are as follows:

*Drug substances and products intended for storage in a refrigerator:*

Long term: storage condition at  $5 \pm 3^{\circ}\text{C}$  for 12 months.

Accelerated: storage condition at  $25 \pm 2^{\circ}\text{C}/60 \pm 5\% \text{RH}$  for 6 months.

*Drug substances and products intended for storage in a freezer:*

Long term: storage condition at  $-20 \pm 5^{\circ}\text{C}$  for 12 months.

If a significant change occurs at any time during the 6 months' testing at the accelerated condition, additional testing at the intermediate storage condition should be conducted and evaluated.

**TABLE 1 Drug Substances and Products for Storage in General Cases**

Study	Storage condition	Minimum time period covered by data at submission
Long term <sup>1</sup>	$25 \pm 2^{\circ}\text{C}/60 \pm 5\% \text{RH}$ or $30 \pm 2^{\circ}\text{C}/65 \pm 5\% \text{RH}$	12 months
Intermediate <sup>2</sup>	$30 \pm 2^{\circ}\text{C}/65 \pm 5\% \text{RH}$	6 months
Accelerated	$40 \pm 2^{\circ}\text{C}/75 \pm 5\% \text{RH}$	6 months

<sup>1</sup>Long-term stability studies at  $25 \pm 2^{\circ}\text{C}/60 \pm 5\% \text{RH}$  or  $30 \pm 2^{\circ}\text{C}/65 \pm 5\% \text{RH}$  are subject to applicants' decision.

<sup>2</sup>No intermediate condition is required if  $30 \pm 2^{\circ}\text{C}/65 \pm 5\% \text{RH}$  is the long-term condition.

A significant change for a drug substance or product as per ICH guideline is defined as follows:

- A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures.
- Any degradation product's exceeding its acceptance criterion.
- Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., color, phase separation, resuspendability, caking, hardness, and dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories and melting of creams) may be expected under accelerated conditions.
- Failure to meet the acceptance criterion for pH.
- Failure to meet the acceptance criteria for dissolution for 12 dosage units.

The long-term testing should cover at least 12 months' duration on at least three primary batches at the time of submission and should be continued for a period of time sufficient to cover the proposed retest period of drug substance or shelf life of drug product. For drug substances with a proposed retest period or drug products with a proposed shelf life of at least 12 months, the frequency of testing at the long-term storage condition should normally be every 3 months over the first year, every 6 months over the second year, and annually thereafter through the proposed retest period or shelf life.

For accelerated studies, a minimum of three time points (including the initial and final time points, 0 and 6 months) of at least 6 months is recommended. If a significant change is observed at the accelerated storage condition, testing at the intermediate storage condition for a minimum of four time points (including the initial and final time points) from a 12-month study is recommended.

## **B. Stability Testing at Different Phases of the Drug Development Process**

Stability testing is required throughout all stages of drug development, from early feasibility through clinical trials (pre-filing) to NDA (filing) and product post approval (commercial stability). The requirements and the type of study will change during the drug product development life cycle as the impact is different at various stages.

During the early discovery phase, the primary function of stability studies is to determine the stability characteristics of the drug. Knowing these characteristics helps researchers select and design the most satisfactory chemical or molecular entity for the desired pharmaceutical profile and indication. The pharmaceutical profile focus on obtaining

optimum drug stability (chemical and physical stability where physical forms can exist in polymorphs, free acid, salt, base, hydrates, or solvates), suitable pre-formulation, and achievable manufacturing process. Pre-formulation testing is the study of physicochemical properties; preliminary information regarding particle size, distribution, crystal shape, mechanical properties, surface area, and other physical information also plays an important role in estimating drug stability, manufacturability, and the performance of the chemical or molecular entity.<sup>2</sup> Informal stability studies can be carried out in-house with proper documentation that contains all the relevant early-stage information. At the beginning of product development, the information on drug substance stability and its interaction with the excipients in the pre-formulation (excipient compatibility) is crucial where excipient compatibility is considered as an early form of stability study. The effect of temperature and hydrolysis across a wide range of pH, moisture, oxidation, and photolysis under long-term and accelerated conditions should be performed so that the rate and mode of degradation pathways (or mechanism, if possible) can be established. The primary aim of the initial stability evaluation is to ensure that a safe and stable formulation can be developed.

Formulations change as the drug goes through its development stages; therefore, stability studies should be conducted on the promising formulations under both accelerated storage conditions: 40°C/75% RH for short-term and normal storage conditions, and 25°C/60% RH or 30°C/65% RH for intermediate to long-term storage conditions. These studies must be initiated before clinical studies begin to ensure that the clinical formulations are likely to remain stable during clinical studies. During the later stages of product development, the goal is to develop a formulation that has a shelf life of 2–3 years for consumer use and optimum packaging that adequately protects the drug product. Formal stability studies with specified attributes under designated storage conditions and packages usually begin in Phase I or Phase II and continue after market approval (Phase IV). Stability testing must be performed to ensure that the drug products are not only chemically stable but also physically stable. All the chemical testing should be performed by stability-indicating methods where the same method is used throughout the stability-testing period for a particular attribute. A new method can be used if equivalency is demonstrated. If changes are involved in formulation, packaging or testing procedures, the methods must be revalidated according to ICH and FDA guidelines.

### **C. Testing Requirements for Drug Products Filed in the United States and Outside the United States**

Regulatory agencies in various geographical regions enforce guidelines to ensure the quality and uniformity of pharmaceutical substances.

The documents, established separately in the United States, Great Britain, Europe, and Japan, provide standards and specifications for all facets of pharmaceutical materials including their testing, packaging, and storage. With the globalization of the pharmaceutical industry, a need arose to harmonize these separate standards, and the ICH was formed. This committee is responsible for harmonizing the requirements of pharmaceutical products among the participating members: The United States, Europe, and Japan. *The European Pharmacopeia (EP)*, *British Pharmacopeia (BP)*, and *Japanese Pharmacopeia (JP)* are the three largest and most influential official compendia outside the United States. The *United States Pharmacopeia* and *National Formulary (USP/NF)* is the largest and most comprehensive of the national compendia. A detailed discussion of all the pharmacopeia is beyond the scope of this chapter.<sup>7</sup> Overall, methods and acceptance criteria must be acceptable to regulatory authorities in each region. The standards and specifications established by the four major pharmaceutical compendia and harmonized by the ICH guidelines assure uniform product quality throughout the world.

## D. Design of Stability Program

### 1. Matrixing and Bracketing

In an ideal full stability study design, samples for every combination of all design factors are tested at all time points. In a reduced design, samples for every factor combination are not all tested at all time points. Due to the many combinations of formulations, dosages, and container types and sizes in the drug products, a full stability study testing program of all samples for every combination at all time points is time-consuming and costly. Hence, a reduced design can serve as an alternative to save time and cost in the drug development stage if the shelf life of the drug product can be adequately predicted. The concept of bracketing (samples are tested only at extreme conditions for any time point) and matrixing (a selected subset of the total number of stability samples is tested at specified time points) can be applied to the reduced design; assumptions and the statistical analysis should be stated clearly in the study plan.<sup>8</sup>

Bracketing is a design of a stability schedule in which only samples on the extreme of design factors are tested at all time points as in a full design. The design assumes that the stability of any intermediate levels is represented by the stability of the extremes tested. Design factors (strength, container size, and fill size) are variables to be evaluated in a study design for their effect on product stability. Bracketing can be applied to studies with multiple strengths of identical or closely related formulations.<sup>2</sup> With justification, bracketing can be applied to studies with multiple strengths where relative amounts of drug substance and excipients change in a formulation. In cases where different excipients

are used with different drug strengths, bracketing generally should not be applied. If the stability of the extremes in the bracketed design is shown to be different, the intermediates should be considered no more stable than the least stable extreme.

Matrixing is the design of a stability schedule where only a fraction of the samples are tested at specified time points. The design assumes that the stability of each of the subsets of samples represents the stability of all samples in the study. Matrixing with reduced testing is an alternative for monitoring stability where multiple factors are involved for the same product. The variables such as different batches, different dosage strengths, and different fills in a container of the same formulation can be matrixed, and the stability of the product studied. The different storage conditions and test attributes may not be matrixed, but should be studied in their own matrix design. If matrixing is applied to time points, all the factors in the design should be tested at the initial and final time points, and at two additional time points through the first 12 months. Matrixing design is usually "one-half reduction" and "one-third reduction" referring to the reduction applied to the full study design. A protocol should be written clearly and followed for all testing. The key aspect of matrixing is that it should be a well-balanced design.

The applicability of matrixing is affected by various factors, especially for the first few batches. The knowledge about the drug substance stability and the information obtained from the pre-formulation work will greatly assist in the design. If the drug substance is well characterized with sufficient stability information, and stability is established for the current formulation with less variability, the applicability of matrix design will be better. However, matrixing is not recommended for formulations with poor drug substance stability and highly variable data. Matrixing design in site qualification lots (SQLs) and post approval lots can be determined based on the historical data. Due to the reduced amount of data collected, shorter retest periods or shelf life should be considered. With appropriate justification, a change to full testing or to a less reduced design can be considered during the course of a reduced design study. The use of any reduced design should be justified. Reduced design of a stability study must be carefully planned and well balanced. The decision to use bracketing or matrixing or both must take into full consideration the information needed and the potential risks involved.

## **2. Statistical Analysis of Stability Data**

As the stability data can be affected by many factors like formulation, manufacturing, storage conditions, in-process and GMP controls, analytical methods, and process validation, the biggest challenge is to figure out the source of the variability in the stability results. At least three batches of the drug substance or product are required to establish the acceptance criteria for future production batches as a measurement

standard. The degree of variability of individual batches affects the confidence that a future production batch will remain within the acceptance criteria throughout its retest period or shelf life.

Assuming the drug substance does not show any compatibility problem with the excipient in the stress or forced degradation studies at early stage, long-term and accelerated formal stability supporting data should be evaluated to determine the critical factors that could affect the quality and performance of the drug product. For example, the temperature or humidity conditions during the scale-up process and exposure to excessive oxygen during the drying process could change the assay profile.

Statistical analyses like linear regression, poolability tests, and statistical modeling are examples of mathematical tools that can be used as quantitative ways to evaluate the stability data.<sup>9</sup> Although normal manufacturing and analytical variations are to be expected, it is important that the drug product be formulated with the intent to provide 100% of the labeled amount of the drug substance at the time of batch release. If the assay value of the batches used to support the registration application is higher than 100% of the label claim at the time of batch release, after taking into account manufacturing and analytical variations, the shelf life proposed in the application can be overestimated. On the other hand, if the assay value of a batch is lower than 100% of the label claim at the time of batch release, it might fall below the lower acceptance criterion before the end of the proposed shelf life. Hence a careful and systematic approach should be adopted in the presentation and evaluation of the stability information.

An approach for analyzing data of a quantitative attribute that is expected to change with time is to determine the time at which the 95% one-sided confidence limit for the mean curve intersects the acceptance criterion.<sup>2</sup> If analysis shows that the batch-to-batch variability is small, it is advantageous to combine the data into one overall estimate by applying appropriate statistical tests (e.g., *p*-values for level of significance of rejection of more than 0.25) to the slopes of the regression lines and zero-time intercepts for individual batches. If it is inappropriate to combine data from several batches, the overall shelf life should be based on the minimum time a batch can be expected to remain within the acceptance criteria.

## E. Specifications Setting in Stability Testing

“Specifications” refers to a list of tests regarding analytical procedures and appropriate acceptance criteria which are numerical limits, ranges or other criteria provided in an approved application that confirm the quality of the drug substances, drug products, intermediates, raw materials, reagents, and other components.<sup>10</sup> Typically, the specification is a set of numerical values/ranges or characteristics derived from formal/informal

stability results and any experimental data to ensure the consistent, high-quality production of drug substances and drug products. Stability testing of drug products should include general attributes like appearance, average assay, organic/inorganic impurities, release assay, and water content so that sufficient data at different time points can be collected to propose acceptance criteria. Often, only a limited amount of data is available at the time of filing, and this can impose many challenges in setting acceptance criteria. Hence, acceptance criteria may need to be revised as more experience is gained throughout the development of the product. The basis of the specification is to focus on the safety and efficacy of the final drug products. An adequate rationale and data for specifications must be provided.

Specifications set for each test attribute should be evaluated at each test point. Specifications can be set for “release” of the product and for “shelf life” as discussed in ICH guidelines Q6A and Q6B. The shelf life of the product is determined by all available stability information. Justifiable differences between shelf life and release acceptance criteria are appropriate.

Specifications remain a binding standard of quality between the regulatory agency and the applicant. In general, a drug product or drug substance conforms to a specification when (1) the article is tested according to the listed analytical procedure, and (2) values obtained are within the listed acceptance criteria.<sup>7</sup> In other words, methods and specifications are related. If the method is modified or optimized for better performance, a change in the specification can be justified and the regulatory agency needs to be informed. An example of a drug product specification is shown in Table 2.

### III. STABILITY TESTING BY HPLC

#### A. HPLC for Stability Testing

Among all the different analytical techniques used in the pharmaceutical industry, chromatography is the most commonly used technique for drug analysis. HPLC is the most popular technique due to its high accuracy, precision, and ease of usage. Planar chromatography, which consists of TLC and paper chromatography (PC), is less commonly used because it is considered to be a less precise technique, but is a feasible approach for quick pre-screening in searching for impurities and determining the purity of a drug substance.<sup>7</sup> Recent technology includes chiral chromatography, which is used to separate racemic mixtures into individual enantiomers that may have different therapeutic effects.<sup>2</sup> Another separation technique that is growing faster and becoming more popular is CE. CE separates compounds by driving the mobile phase with a voltage differential where the voltage differential results in electroosmotic flow.

**TABLE 2** Specification Table of a Drug Product (A Typical Example)

Product name: Analgesic-ABC

Product ID: Lot # 5678abc

Dosage strength: 10 mg

Stage: IND Phase I

Test attribute	Method no.	Specification	Result	Pass/fail
Appearance	#5-001	Round, white tablet with "abc" imprint on one side	Conforms to specification	Pass
Average Assay	#1-001	95–105% label claim	94%	Fail
Impurities	#1-002	Individual known impurities <0.2%	0.7%	Fail
		Total impurities <2.0%	2.5%	
Dissolution testing	USP <711>	10 min >25% LC 30 min >75% LC 45 min >90% LC	10 min = 30% LC 30 min = 80% LC 45 min = 99% LC	Pass
Water content	USP <921>	Report value	1.01%	Pass
Residual solvent	#2-001	<2000 ppm	500 ppm	Pass

Average assay and impurities test did not conform to specification. Upon investigation and confirmation of the results obtained, the product failed the specification. The product is at the Phase I stage where the formulation is not optimized, the manufacturing process is not robust enough, and the analytical methods are not rugged, hence a higher percentage of lots could have failed. In addition, the specification was not set appropriately to reflect the stage of the drug development.

In applying HPLC for separation purposes, the solubility of the compound is the primary consideration. As a rule of thumb, HPLC requires solubility of the analytes in the mobile phase. The choice of separation is based on the size, polarity, and ionic nature of the solute. The preliminary information required is the molecular size and solubility of the compound in water to select the appropriate column prior to optimizing other HPLC parameters. A few chromatographic column types are commonly used for separation. Normal phase (NP) is defined as a polar stationary phase with a nonpolar mobile phase typically used for nonpolar analytes. Reversed phase (RP) is defined as a nonpolar stationary phase with a polar mobile phase, which is the reverse of the NP. In the pharmaceutical industry, RP chromatography is the most popular mode for small molecules <1000 Da. Analytes that are soluble between weakly and relatively polar solvents separated by a nonpolar stationary phase (C18, C8, phenyl or cyano columns) would be the best way to separate small molecules. Ion-exchange chromatography (IEC) and ion chromatography (IC) are all separation techniques for charged analytes

(inorganic and organic ions). Size exclusion chromatography (SEC) is a technique that separates analytes based on their sizes in solution and can handle high-molecular weight samples in a short time with simple method development.<sup>7</sup>

In developing a stability-indicating assay method, the basic criterion is to separate the API from the major related substances so that the potency data will not be affected. For a drug product with different dosages, the all-strength method is the best option for efficiency purposes. By changing the sample preparation through varying the volumetric flask size or dilution scheme, the assay target concentrations for different dosages can fall in a narrow range where the analysis can be combined using the same calibration curve or a single point standard. For reporting assay, the typical unit is % label claim (%LC) or mg/system; the final choice usually depends on the specification setting.

During early drug development, combining assay and impurity in one single test method is recommended as more information can be extracted in a shorter time. As the drug product enters a later stage, it is better to separate the active assay by isocratic elution and the impurities/degradants by gradient elution for higher capacity and better analysis for dimers. As a general guideline, gradient methods are more suitable for multi-drug formulations.

In stability testing, HPLC is the most frequently used chromatographic technique for assay. HPLC has illustrated the benefits of speed, resolution, accuracy, and sensitivity, and HPLC can interface with many detectors that other analytical techniques are unable to offer.

## B. Detector Considerations

To select a good detector, the basic criteria include sensitivity at low concentrations, linearity over a wide range, and tolerance to temperature or solvent composition changes.<sup>11</sup> A stability-indicating assay method must be specific, selective, and must separate the impurities from the active drug well to avoid interference. In the pharmaceutical industry, the majority of small molecules contain chromophores that display good absorbance in the UV region. Hence, HPLC interfaced with a UV detector becomes the natural choice for RP chromatography. The API peak should have fairly high absorbance where the absorbance value of the highest concentration should be between 1 and 1.5; a wide linearity range should also be developed. A mass spectrometry compatible method can be developed for confirmation or to serve as an alternate method.

However, samples that have no UV absorbance, exist in ionic form, or require structural information have to couple with other types of detectors. Refractive index (RI) detector is almost a universal detector in that it responds to almost any solute (UV-absorbing molecules, sugars,

polymers, and pharmaceutical excipients). The drawbacks are inadequate sensitivity, low tolerance to temperature and pressure, baseline instability, and incompatibility with gradient elution. Another type of universal detector is the evaporative light-scattering (ELS) detector. The advantage of ELS is that it can be used for gradient elution, especially for impurity analysis. However, ELS is amendable to nonvolatile and semi-volatile analytes. Electrochemical detectors are mainly designed for ions and can be performed in either oxidative or reductive modes. The main disadvantage is a poor signal-to-noise ratio as a result of dissolved oxygen in the solution. Fluorescence (FL) and direct-current amperometry (DCA) detectors both have high sensitivity and selectivity, but the former detection mode always requires sample derivatization, which may cause problems like incomplete reaction or multiple derivatives formation, and hence is less preferred. The combination of LC and MS (mass spectrometer) is a powerful tool as molecular weight and structural information can be obtained from the mass spectra. LC-MS and GC-MS have become increasingly popular in the pharmaceutical industry. The main applications are to monitor the purity of drug substances and identify the structure of new chemical entities or intermediates.<sup>7,12</sup>

Other than selecting the appropriate detector with the correct mode of chromatography, optimizing the LC parameters is crucial in developing a stability-indicating assay method. The lower the injection volume the sharper the peak will be, but the area count for the low concentrations in the calibration curve should give an acceptable percentage of recoveries and reproducibility. Flow rate must be well-adjusted to avoid backpressure exceeding 3000 psi. The mobile phase selected should avoid interference and absorbance from the matrix and excipient. The wavelength selected for the UV detector should provide maximum detection sensitivity and signal-to-noise ratio on top of a wide linearity range. Compounds that do not have UV absorbance are the ultimate challenge in method development. The detector must be sensitive enough to detect low concentrations, which often require alternative ways like increasing injection volume, serial detectors, or sample derivatization to become a UV-absorbing molecule. For example, an isocratic RI detector can detect a non-UV absorbing molecule, but the detection mode needs to switch to electrochemical (conductivity or amperometry) if the impurities contain inorganic compounds. As gradient elution is not possible with an RI detector, a separate HPLC method must be developed for organic impurities that are different in nature from the API.

In combining the optimized chromatography parameters and the appropriate detector, the accuracy, precision, and robustness (including standard and sample stability) of the method must be tested to ensure that it can be defined as a stability-indicating method. (See section V and VI of chapter 3.)

## C. Stability-Indicating Methods Development

### 1. Different Routes of Administration

In recent years, drug delivery technologies have grown extensively that go beyond conventional oral dosage forms. For example, intravenous, transdermal, and implant technologies are widely used for sustained drug delivery over increased periods of time. The type of drug, its characteristics, and the intended indication determine the mode of delivery.

In developing HPLC stability-indicating methods, sample preparation is the most important part of drug method development, regardless of the route of administration. Sample preparation of the planned dosage forms should be optimized based on the drug's characteristics. Solid and liquid dosage forms can readily be extracted or dissolved in appropriate solvents or media, whereas the sample preparation of transdermal patches needs special attention. For example, it is important to consider (1) the effect of the viscosity of polymers involved in controlled-release tablets, and (2) the effect of external excipient glue-like adhesives that come into contact with the drug and/or the semi-solid dosage form preparation. The stability of the product is dictated by the combined effect of the excipient (solid or liquid) mixture and API. Hence, a stability-indicating method must be developed for the excipient, the drug substance, and the drug product. The HPLC conditions can be generalized regardless of the type of dosage form—solid dosage, liquid dosage, or transdermal patch.

### 2. Single Drug Vs. Dual Drug System

Developing and validating a stability-indicating assay method becomes more challenging when multiple drugs are present in a drug product. Since developing and marketing new chemical entities (NCEs) for multiple indications is a difficult task, pharmaceutical companies are looking into creating products by combining two or more known, compatible APIs to treat multiple diseases and achieve better patient compliance. Method development for two or more compounds and their related impurities becomes very complex if the solubility and the  $pK_a$  values vary greatly and the UV profiles are not similar. It generally poses more issues in developing sample preparation to fully extract the drugs and in optimal HPLC methods for analysis. If the solubility and the  $pK_a$  values of the APIs involved are similar, where all the active components are totally soluble in water, the method development is much easier.

In addition, the API's dosage strength also plays an important role in developing a stability-indicating method. The value of forced degradation studies conducted in the early stage of development is critical in identifying the related impurities and degradants and their origins. In the case of two APIs with disparate, new impurities seen in stability samples, the samples must be evaluated carefully and the impurities reported to their corresponding origins. Incorrect identification of the origin source

and quantitation against the inappropriate API can cause stability issues (e.g., impurities calculated against the wrong dosage API could have over- or underestimated the actual level, and thus could pose regulatory issues as a result of wrong trending and tracking). The confirmed presence of a new, unknown peak in the sample should initiate further investigation as to the source and identification of the structure. The unknown peak should be trended at specified time points and the structure identification and characterization should be performed and quantitated against the correct API.

### 3. Active Drug Assay and Impurities Method

For routine analysis in a stability program, a stability-indicating method is required for analyzing both the API and impurities. Stability-indicating analysis for an API is crucial since it measures the potency of the drug at an initial time point and the loss of potency during storage. The evaluation of impurities with a good stability-indicating method is also important in measuring the impurities and degradants, which could have toxicological effects when administered to humans. Forced degradation studies in which the product is artificially exposed to high stress conditions can be supportive in developing a stability-indicating assay method.

A well-defined, precise, and validated method will help to determine the drug content accurately, whereas an assay method capable of detecting at low levels can help calculate drug losses during the manufacturing process. Different vendors can supply common APIs. The API characterization accompanied by information from the manufacturer on the synthetic route determines the impurities profile and the method used for the active assay.

Isocratic elution uses the same mobile phase composition throughout the chromatographic analysis and is the preferred choice for API assay analysis. Isocratic separation is relatively simple and rugged, and produces a stable baseline. However, it may not be appropriate for impurities analysis if the API produces impurities/degradants that have widely divergent affinities for the selected column. In this case, gradient elution is required, where the composition of the mobile phase changes from a weak to a strong one throughout the run. Although gradient separation does not produce as good a baseline as isocratic elution, and is not compatible with all detectors, it shortens analysis time and accommodates a greater variety of compounds in a mixture. Therefore, gradient elution during initial method development significantly saves time and provides an informative background against which to ultimately develop an isocratic method, if possible.<sup>7,11</sup>

Other than selecting the column and mobile phase for the correct mode of separation, optimizing different HPLC parameters (injection volume, run time, wavelength, and detector) is equally important for achieving acceptable capacity factor ( $k'$ ), resolution ( $R$ ), and tailing factor ( $T$ ).

In developing a commercially viable method, the stability of samples, standards, and reagents used for the HPLC method must be considered. For the stability of standard solutions and reagents, long-term stability of up to weeks is desirable. For the stability of sample solutions, a minimum of 3 days is ideal. Generally, the reagents for standard and sample preparation should be the same or very similar to the mobile phase composition.

#### 4. Dissolution Method

Any drug that enters the body must first be disintegrated into small aggregates and eventually dissolved. The dissolution measurement can then be correlated with the biological performance of the drug. Dissolution testing is a measurement of drug solubility over time. The purpose of the dissolution test is an *in vitro* evaluation to compare the *in vivo* performance of the solid dosage formulation. The commonly used apparatus of dissolution tests are USP Type I (basket method) and Type II (paddle method) in vessel, which is more suitable for immediate release tablets, where sampling typically occurs at 5–15-min-intervals for a total of 1–2 h. USP Type VII allows samples to be released in calibrated tubes of 25–75mL arranged in designated rows (where total row numbers = total time intervals) containing selected medium. The tablets are typically released every 2–3 h for 24 or more hours based on the design of the delivery system; Type VII is primarily designed for controlled-release tablets.<sup>1</sup>

The medium selected for the dissolution test must consider the drug solubility. Aqueous media with a typical pH range between 1 and 7 to mimic the human gastrointestinal tract are preferred over organic solvents. The operating parameters of the dissolution setting should be optimized to ensure complete dissolution.<sup>7</sup>

To develop an HPLC stability-indicating method for Type I or II dissolution, the linearity must be wide enough, in combination with good sensitivity and minimal interference, to accommodate concentrations from low (possibly LOQ) to very high end, as the samples drawn represent the cumulative drug amount dissolved over time. As for an HPLC method that is designed for Type VII dissolution, the linearity should accommodate the lower concentrations since it is a drug measurement of a controlled-release system.

#### 5. Testing of Preservatives in Pharmaceutical Products

A preservative is a substance that prevents or inhibits microbial growth and extends the shelf life of the drug products. In most pharmaceutical drug products, only a few compounds are typically selected as preservatives. For efficiency, a generic method should be developed for the types of preservatives that are more commonly used. For example, butylated hydroxytoluene (BHT) is an antioxidant commonly used in many solid dosage formulations to retard oxidative degradation of the excipients.

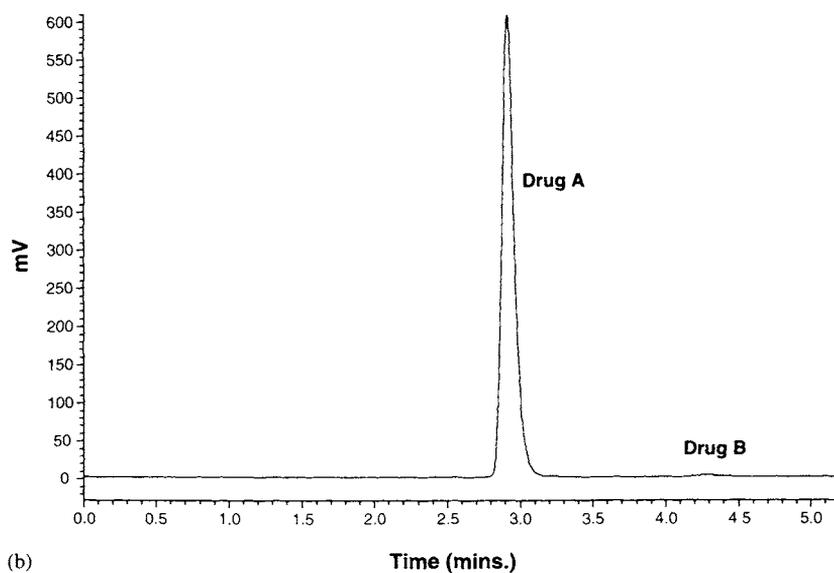
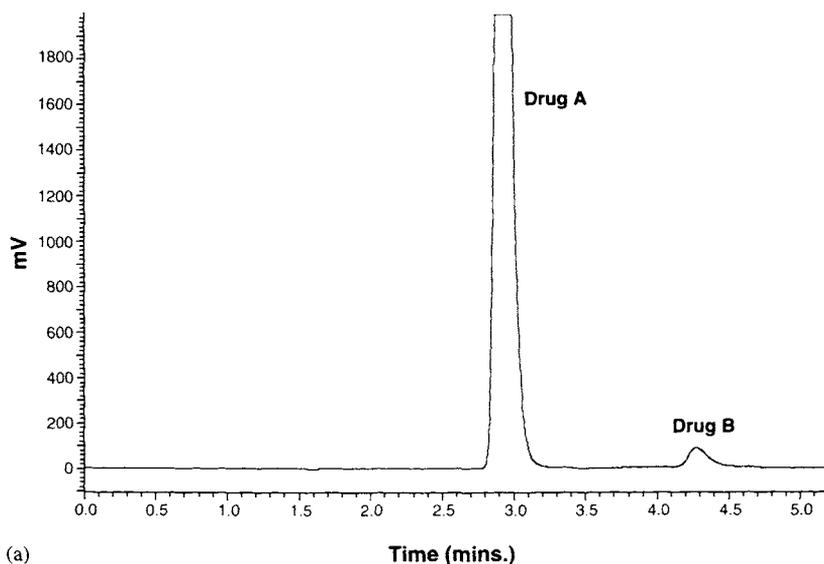
Hence, a generic or universal HPLC method interfaced with a UV detector for BHT can be used on any drug as long as the acceptance criteria on accuracy, precision, robustness, and other necessary requirements have been met. Similar to appearance, drug release, assay, and impurities, preservative testing is also required if a certain degree of preservative has to be included in the drug product to ensure an adequate shelf life.

For a liquid or semi-solid pharmaceutical dosage form, it is crucial to include a preservative in the formulation. Commonly used preservatives in these systems include sodium benzoate, EDTA, sorbic acid, and parabens.<sup>13</sup> A generic HPLC method is also recommended for the preservatives used in liquid formulations for routine monitoring to ensure the stability of the preservative itself and it must be validated specific to its use with the dosage form. (See chapters on Sample Preparation and Method Development.)

#### D. Case Studies

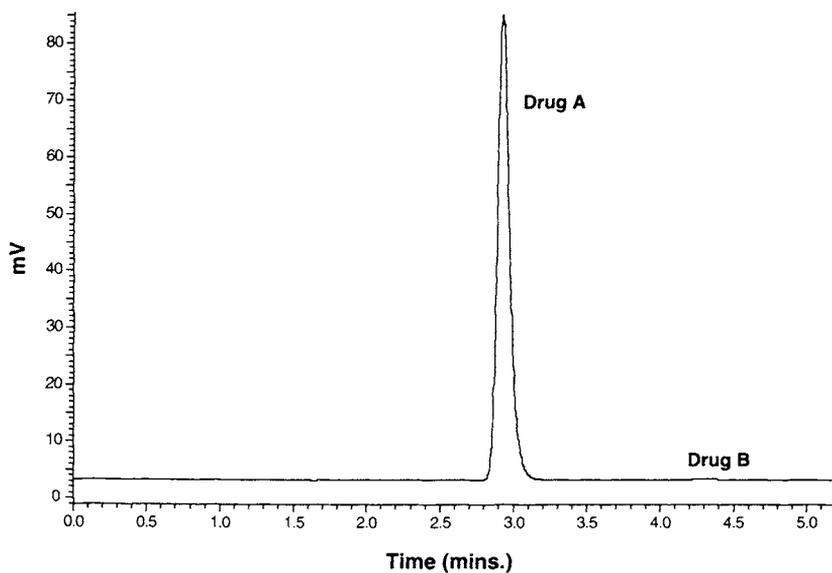
##### *Case study 1: Stability-indicating method development for dual drug system*

An oral dosage form was developed that consisted of two APIs, Drug A and Drug B, in a respective dosage ratio of  $\sim 20$  to 1 in the system. Drug A is relatively hydrophobic and Drug B is hydrophilic. The  $pK_a$  value of Drug A is known, but the  $pK_a$  value of Drug B is unknown. The two drugs had similar UV profiles in the low UV region, and Drug A had a slightly different profile at the mid-UV region. Combined standards were prepared in a mixture of organic and aqueous media to enhance Drug B's solubility. An HPLC column was selected and the mobile phase was optimized to separate both compounds with a run time of approximately 5 min for optimal routine dissolution samples analysis. The separation was acceptable and resolution was good for both drugs, but sensitivity was an issue. Drug A with high dosage along with very high absorptive nature presented a peak with high area count and the peak height was off scale at the selected wavelength, while Drug B with low concentration and low absorption exhibited poor signals (Figures 1a and b). To accommodate the wide range required to encompass the dissolution sample concentration, various approaches were attempted. One approach was to collect data from the detector in two channels (two different wavelengths) where a slightly weaker signal was chosen for Drug A and a maximum absorptive wavelength was selected for Drug B. The problem was solved only if a photodiode array (PDA) detector was used. If a regular dual wavelength detector was chosen, the baseline became wavy and the peak shapes were distorted due to a different detection mechanism (Figure 2). Since the detectors available with a dual channel are programmable, a wavelength-switching technique was explored using a single channel at a given time and this technique produced promising results. The wavelength

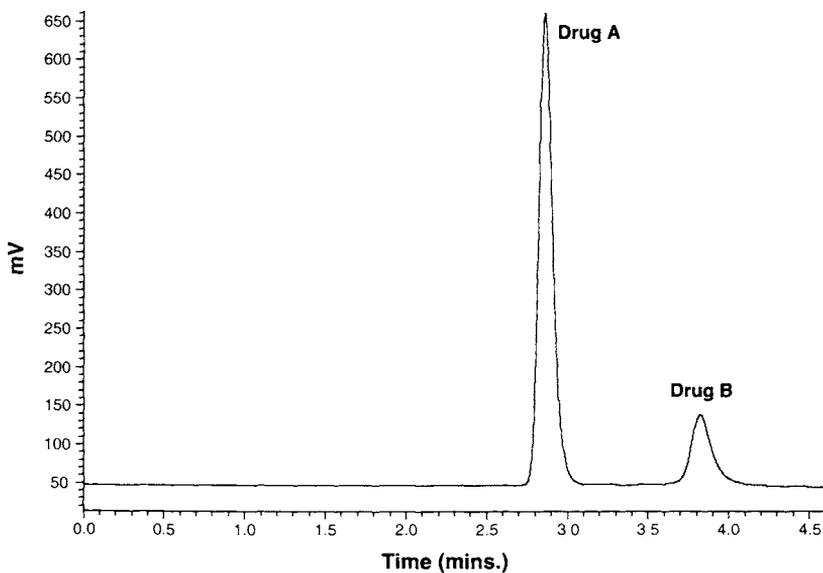


**FIGURE 1** (a) HPLC chromatogram of Drug A and Drug B at single channel (single wavelength:  $\lambda_1$ ). (b) HPLC chromatogram of Drug A and Drug B at single channel (single wavelength:  $\lambda_2$ ).

was programmed for the first eluted peak and it was switched immediately afterwards to a second wavelength that was compatible with the second peak (Figure 3). Resolution, capacity factor ( $k'$ ), tailing factor ( $T$ ),



**FIGURE 2** HPLC chromatogram of Drug A and Drug B at dual channel (dual wavelength:  $\lambda_2$  only).



**FIGURE 3** HPLC chromatogram of Drug A and Drug B at single channel (dual wavelength with wavelength switch:  $\lambda_2 \rightarrow \lambda_1$ ).

and other suitability parameters were optimized with this new approach. Linearity was developed for the range required for both drugs, and accuracy and repeatability were established. The concern of method development for the dual drug was solved for dissolution and drug content method. Hence, the method was applied to stability studies and to routine formulation development.

The real challenge appeared when a method was to be developed for impurities in the drug product. To add to the complexity, both APIs under study listed at least four individual impurities for each compound. Given the nature of both APIs, detecting all the impurities by an isocratic method was not feasible. A two-step gradient was developed to successfully separate all known impurities with good resolution using dual channel detection (no wavelength switching). The method was successfully validated to Phase-I requirement. Once the stability samples were under testing, new unknown peaks were found in the sample chromatograms. The evaluation as to which API these unknowns belonged to was to be done. As described earlier, as the UV profiles for both drugs are similar in the low UV region and the region was chosen to accommodate the low dosage due to its low sensitivity, all the unknown peaks were also detected at this wavelength. Since the newly discovered impurities were present at this wavelength, they were quantitated against the low dosage form, and some values became alarmingly higher than the ICH threshold. After confirmation of the presence of these peaks at the next stability time point, structural identification and characterization were initiated. The results confirmed that a few unknown peaks seen in the Drug B channel were actually related to Drug A. When quantitated against Drug A, the levels of these impurities were negligible. As the occurrence of new unknown peaks will continue further at future time points, the process of identification and characterization should be an ongoing process for impurities method development and throughout the stability study.

*Case study 2: Stability-indicating method development for surfactants*

This is a methodology adopted for separation and quantification of two surfactants with a slight difference in molecular weights. A liquid formulation (cleaning solution) was developed incorporating two forms of surfactant with different molecular weights. Size exclusion chromatographic technique did not provide successful results. A RP chromatography was developed for the separation. One molecular form of the surfactant was at a higher concentration than the other. The second surfactant did not provide enough sensitivity with the method. Hence, on-column concentration was attempted. For the test method, two mobile phases with the same composition, but one with higher organic composition and the other with lower organic composition, were prepared. A low-pressure, six-valve, column-switching unit was incorporated into the HPLC system after the

autosampler and before the detector. A shorter column (5-cm) C18 column was attached to one of the ports in the column-switching valve. This column was used for concentrating sample in the column. A 15-cm C18 column was attached at another port of the valve, which was the outlet to the detector in the second position (position B) of the valve system. Initially the weaker mobile phase was allowed to run through the system until the HPLC system equilibrated. During this time the switching valve was in the first position (position A), and the mobile phase flowed through the 5-cm column, while the effluent went to waste as set by the valve position. The sample was injected repeatedly and concentrated in the 5-cm column as the weaker mobile phase could not partition the compound from the column. After the accumulation of a set sample size, the valve position was switched to position B, and the pump was programmed to run the stronger mobile phase through the 5-cm column and onto to the 15-cm column for good separation and detection of both compounds. The method was reproducible; accuracy and precision were developed and used for monitoring of the indicated stability studies. The column-switching technique is not a commonly used approach since the reproducibility in a commercial laboratory is difficult. This approach is used as a last resort for difficult applications as described above.

#### IV. SUMMARY AND CONCLUSION

Stability testing is an integral part of pharmaceutical product development and is an ongoing activity throughout the entire drug development process. Product integrity and shelf life are based on stability testing. The pharmaceutical industry is challenged by the frequently changing regulatory requirements. The need for improvement in analytical techniques poses challenges and opportunities in pharmaceutical stability testing. A wealth of specific information is available in the FDA stability guidance and in ICH stability guidelines. HPLC and other analytical techniques play important roles in stability testing. The precision, ease of use, and ruggedness of HPLC methods are by far preferred over other separation and quantitation techniques. Making continuous efforts to learn updated technology and to remain informed of constantly changing regulations is the way to succeed in the pharmaceutical industry.

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