1.1 Introduction on Development of analytical methods for the determination of related components in pharmaceutical compounds using chromatography technique:

Identification and quantification of impurities is a crucial task in pharmaceutical process development for quality and safety. Related components are the impurities in pharmaceuticals which are unwanted chemicals that remain with the active pharmaceutical ingredients (APIs), or develop during stability testing, or develop during formulation or upon aging of both API and formulated APIs to medicines. The presence of these unwanted chemicals even in small amounts may influence the efficacy and safety of the pharmaceutical products. Various analytical methodologies were employed for the determination of related components in pharmaceuticals. There is a great need for development of new analytical methods for quality evaluation of new emerging drugs.

An impurity as defined by the ICH (*The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use)* guidelines is "Any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product". Analytical methods for impurities estimation should be stability indicating to monitor the stability of pharmaceutical dosage forms during the investigational phase of drug development, and once the drug is marketed, the ongoing stability studies must be conducted/ performed. The purpose of stability testing is to

provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, enables to establish a retest period/shelf lives for a drug substance and a recommended storage condition. Methods can be developed which measure the amount of drug remaining, the amount of drug lost (or the appearance of degradation products), or both. The development of these methods for pharmaceuticals can be approached from several avenues. Related components, related substances, and related impurities terms are synonyms for the term impurities; the use of above terms at different phrases means one and the same (i.e. impurities).

1.2 Sources of impurities in pharmaceutical substances:

The origin of impurities in drugs is from various sources and phases of the synthetic process and preparation of pharmaceutical dosage forms. Majority of the impurities are characteristics of the synthetic route of the manufacturing process. There are several possibilities of synthesizing a drug; it is possible that the same product of different sources may give rise to different impurities. According to the international conference on harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use, impurities are classified as organic impurities, inorganic impurities and residual solvents. Organic impurities may arise from starting materials, by products, synthetic intermediates and degradation products. Inorganic impurities may derived from the

manufacturing process and are normally known and identified as reagents, ligands, inorganic salts, heavy metals, catalysts, filter aids and charcoal etc. Residual solvents are the impurities introduced with solvents [1- 6]. Of the above three types, the number of possible inorganic impurities and residual solvents is limited. These are easily identified and their physiological effects and toxicity are well known. For this reason the limits set by the pharmacopoeias and the ICH guidelines can guarantee that the harmful effects of these impurities do not contribute to the toxicity or the side effects of the drug substances. The situation is different with the organic impurities. Drugs prepared by multi-step synthesis results in various impurities, their number and the variety of their structures are almost unlimited and highly dependent on the route and reaction conditions of the synthesis and several other factors such as the purity of the starting material, method of isolation, purification, conditions of storage etc. In addition, toxicity is unknown or not easily predictable. For this reason the ICH guideline set threshold limit above which the identification of the impurity is obligatory.

1.2.1 Sources of Organic Impurities:

Organic impurities may arise during the manufacturing process and/or storage of the drug substance. These impurities are derived from drug substance synthetic processes and degradation reactions in drug substances and drug products. The process (synthetic process) related impurities can be derived from starting materials, intermediates, reagents,

ligands, and catalysts used in the chemical synthesis, as well as by-products from the side-reactions of the chemical synthesis [7]. Degradation products are derived from the chemical degradation of drug substances and drug products under storage or stress conditions. They may be identified or unidentified, volatile or non-volatile, and include the following [6].

1.2.1.1 Impurities Originating from Drug Substance Synthetic Processes:

Most of the drug substances (low molecular weight) are chemically synthesized. Chemical entities, other than the drug substance, that are involved or produced in the synthetic process can be carried over to the final drug substance as trace level impurities. These chemical entities include raw materials, intermediates, solvents, chemical reagents, catalysts, by-products, impurities present in the starting materials, and chemical entities formed from those starting material impurities (particularly those involved in the last steps of the synthesis). These impurities are usually referred to as process impurities [7]. The goal of process impurity identification is to determine the structures and origins of these impurities. This knowledge is critical for improving the synthetic chemical process, in order to eliminate or minimize process impurities [8].

1.2.1.2 Starting Materials and Intermediates:

Starting materials and intermediates are the chemical building blocks used to construct the final form of a drug substance. Unreacted starting

materials and intermediates, particularly those involved in the last steps of the synthesis, can potentially survive the synthetic and purification process and appear in the final product as impurities [9,10]. For example, in the synthesis of tipranavir drug substance, the “aniline” is the intermediate in the last step of the synthesis. The similarity between the structures of the “aniline” and the final product, it is difficult to totally eliminate it in the subsequent purification step. Consequently, it appears in the drug substance at around 0.1% [11].

1.2.1.3 Impurities in the Starting Materials:

Impurities present in the staring materials could follow the same reaction pathways as the starting material itself, and the reaction products could carry over to the final product as process impurities. Knowledge of the impurities in starting materials helps to identify related impurities in the final product, and to understand the formation mechanisms of these related process impurities [7, 10, 11]. One such example is the presence of a 4-trifluoromethyl positional isomer in 3-trifluoromethyl-alpha-ethylbenzhydrol (flumecinol), due to the presence of 4-trifluoromethylbenzene impurity in the starting material, 3-trifluoromethylbenzene. A second example involves a 2-methyl analogue present as a trace impurity in tolperisone, due to the presence of 2-methylpropiophenone in the starting material, 4-methylpropiophenone [2].

1.2.1.4 Reagents, Ligands and Catalysts:

These chemicals are less commonly found in APIs; however, in some cases they may pose a problem as impurities [2, 6]. Chemical reagents, ligands, and catalysts used in the synthesis of a drug substance can be carried over to the final products as trace level impurities. For example, carbonic acid chloromethyl tetrahydro-pyran-4-yl ester (**CCMTHP**), which is used as an alkylating agent in the synthesis of a ß lactam drug substance, was observed in the final product as an impurity. Many chemical reactions are promoted by metal based catalysts. For instance, a Ziegler-Natta catalyst contains titanium, Grubb's catalyst contains ruthenium, and Adam's catalyst contains platinum. In some cases, reagents or catalysts may react with intermediates or final products to form by-products. Pyridine, a catalyst used in the course of synthesis of mazipredone, reacts with an intermediate to form a pyridinium impurity [7].

1.2.1.5 By-Products of the Synthesis:

All chemical reactions are not 100% selective; the side-reactions are common during the synthesis of drug substances. By-products from the side reactions are among the most common process impurities in drugs [6]. By-products can be formed through a variety of side reactions, such as incomplete reaction, overreaction, isomerisation, dimerisation, rearrangement, or unwanted reactions between starting materials or intermediates with chemical reagents or catalysts [7].

1.2.1.6 Products of over-reaction:

In many cases the least or previous steps of the syntheses are not selective enough and the reagents attack the intermediate not only at the desired site. For e.g. in the synthesis of nanodralone decanoate, the last step of the synthesis is the decanoylation of the 17 –OH group. In the course of overreaction the reagents also attacts the 4ene- 3 oxo group leading to an enol ester- type impurity (3, 17β- dihydroxyestra-3, 5- diene disdecanoate) [2, 8].

1.2.1.7 Products of side reactions:

Some of the frequently occurring side reactions (which are unavoidable in drug synthesis) are well- known to the synthetic chemist; other which lead to trace level impurities have to be detected and elucidated during impurity profiling. The formation of diketopiperazine derivative is a typical side reaction in peptide synthesis [2].

1.2.1.8 Impurities Originating from Degradation of the Drug Substance:

Impurities can also be formed by degradation of the end product during manufacturing of bulk drugs. Degradation products resulting from storage or formulation to different dosage forms or aging are common impurities in the medicines [6]. The **definition of degradation product** in the ICH guideline is a molecule resulting from a chemical change in the substance brought about by overtime and/or action of e.g. Light, temperature, pH or water or by reaction with excipient and/or the

intermediate container closure system [2, 12]. For example in the case of aspartame, in the presence of moisture, hydrolysis occurs to form the degradation products i.e. L- aspartyl- L- Phenyalanine and 3-benzyl-6-carboxymethyl 2, 5-diketopierazine. Third degradation product is also known, β-L- aspartyl-L-phenylalanine methyl ester. Aspartame degradation also occurs during prolong heat treatment [2].

1.2.2 Enantiomeric Impurities:

The majority of therapeutic chiral drugs used as pure enantiomers are natural products. The high level of enantio selectivity of their biosynthesis excludes the possibility of the presence of enantiomeric impurities [6]. In the case of synthetic chiral drugs, the racemates which are usually marketed, if the pure enantiomer is administered, the antipode is considered to be an impurity. The reason for its presence can be either the incomplete enantio selectivity of the syntheses or incomplete resolution of the enantiomers of the racemate [3, 13]. Although the ICH guideline excludes enantiomeric impurities, pharmacopoeias consider them as ordinary impurities [2].

A single enantiomeric form of chiral drug is now considered as an improved chemical entity that may offer a better pharmacological profile and an increased therapeutic index with a more favourable adverse reaction profile. However, the pharmacokinetic profile of levofloxacin (S-Isomeric form) and ofloxacin (R- isomeric form) are comparable, suggesting the lack of advantages of single isomer in this regard [2, 6]. The

prominent single isomer drugs, which are being marketed, include levofloxacin (S-ofloxacin), levalbuterol (R-albuterol), esomeprazole (S-omeprazole).

Typical examples of drugs containing enantiomeric impurities:

a) Dexchlorophenarmine maleate (R enantiomer impurity allowed NMT 0.5%)

b) Timolol maleate (R enantiomer impurity allowed NMT 1%)

c) Clopidogrel sulphate (R enantiomer impurity allowed NMT 1%)

In general, an individual API may contain all of the above-mentioned types of organic impurities at levels varying from negligible to significant level [6].

1.3 Requirement for control of impurities:

Impurities often posses unwanted pharmacological or toxicological effects by which any benefits from their administration may be outweighed [2]. Impurities will have different disastrous efficacy, different bioavailability, adverse effects and toxic effects. In case of chiral impurities one isomer may produce the desired therapeutic activities, while the other may be inactive or in worst cases, produce unwanted effects, for example consider the tragic case of the racemic drug of n-phthalyl-glumatic acid imide that was marketed in the 1960's as the sedative Thalidomide. Its thereapeutic activity resided exclusively in the R-(+)-enantiomer. It was discovered only after several hundred births of malformed infants that the S-(+)-enantiomer was teratogenic. It is not only that one enantiomer reacts

and the other does not but also in some instances different enantiomers can have different effects as shown in Table 1.1.

Table: 1.1 Examples of pharmaceutical products, and its effect of chirality

Compound	Isomer	Effect
Thalidomide	S-isomer	Teratogenic
	R-isomer	Sleep inducing, anti-nausea
Barbiturates	S-isomer	Depressant
	R-isomer	Convulsant
Opirates	R,S-isomer	Narcotics
	S,R-isomer	Non-addictive cough mixture
Labetalol	S,R-isomer	Alpha-blocker
	R,R-isomer	Beta-blocker
Pencillamine	D-isomer	Anti-arthritic
	L-isomer	Toxic

1.3.1 Pharmacopoeial Status:

The quality of a chemical active substance with respect to organic impurities is controlled by a set of tests within a pharmacopoeial monograph. Individual monographs are periodically updated to keep pace with scientific progress and regulatory developments. Following the revised ICH Q3A (R2) impurity testing guideline major pharmacopoeias will continue publishing new or revised relevant monographs and general chapters. Active substances found to contain an organic impurity not detected by the relevant pharmacopoeial tests prescribed below are not of

pharmacopoeial quality, unless the amount and the nature of this impurity are compatible with GMP [14].

Two general chapters (<466> & <1086>) of the US Pharmacopoeia (USP) deal with **organic impurity testing**. Concepts and definitions are clearly described although different terminology from that of ICH is used. Until now, one of three types of tests in bulk pharmaceutical chemicals is ordered:

1. A chromatographic purity test coupled with a non- specific assay
2. A chromatographic purity- indicating method that also serves as an assay
3. A specific limit test for known impurities, a procedure that requires reference standards for these impurities [15].

In the future, new and revised USP individual monographs will include tests that actually control specified and unspecified organic impurities. Where different routes of synthesis yield different impurity profile, different analytical procedures will be proposed. All specified impurities will be separately limited, with a further limit of 0.10% for any unspecified (unknown) impurity. Total impurities above the disregard limit should be less than 1.0%. USP also proposes that a suitable test for detecting impurities that may have been introduced from extraneous sources should be employed in addition to tests provided in a specific monograph [13, 14, 15].

The European Commission decided that the principles and terminology of the revised ICH Q3A should be implemented in the European Pharmacopoeia (EP) monographs of the active substances; both new and already published [16]. A new general chapter concerning the control of impurities in pharmaceutical substances was introduced in the fifth edition of the EP, while a revision of the monograph entitled Substances for Pharmaceutical Use has also been done. According to the policy of EP control of the relevant organic impurities in synthetic drug substances is often accomplished by the test of related substances. Currently, it is a limit test (comparison of the peak areas), but will progressively be changed to utilize a quantitative acceptance criterion [3, 17].

Some individual monographs already satisfy this demand. More tests are ordered, if the general test does not control a given impurity or there are other special reasons [16]. Potential impurities with a defined structure that are known to be detected by the tests in a monograph, but are not known to be present in medicinal substances above the identification threshold, are referred to as detectable impurities. They are limited by a general acceptance criterion [17]. EP individual monographs published in the new format include a separate section in which all impurities (specified and detected) are listed. Unidentified specified impurities are not listed in this section, but their specific acceptance criteria along with appropriate analytical characteristics (e.g., retention time) are reported in the text, wherever it is applicable [13].

However, previous EP monographs were not having a related substances test in the new explicit style are to be read and interpreted according to the recent amendments. During the coming years, EP individual monographs now published in the old format will be revised to contain related substances tests and lists on specified and other detectable impurities. Monographs containing tests for related substances based on TLC will also be revised [3, 13, 16, 17].

1.3.2 Pharmacopoeial norms for the enantiomeric impurities:

B.P 2001 has recommended following norms for the enantiomerically pure drug substance. It describes the way in which the stereochemistry of a substance is identified and/or controlled.

1- Many medicinal substances that contain one or more chiral centers and that are already on the market have been made available for pharmaceutical use as racemic mixtures with little known about the biological activities of the separate isomers. This has been reflected in the monograph in the pharmacopoeia and a test to show that the substance is a racemic mixture has not usually been included unless it was known that at least one of the separate enantiomers was also available commercially. Nevertheless, with increasing concern by regulatory authorities for substances to be made available as single isomers, tests for enantiomeric composition will become more common [9].

Chemical definition (monographs other than those of the European pharmacopoeia)

1-In the case of substances containing a single chiral centre, the descriptor '(RS)'-is included at the appropriate position in the chemical definition of the substances to indicate a racemic mixture.

2- For substances containing multiple chiral centres and comprising mixture of all possible stereomers the term 'all-rec' has been used, for example Isoaminile. In those few substances existing as diastereomeric mixtures, that is where in one or more centers the stereochemistry is explicit but in other centers it is not, each centre is defined either as the specific (R)- or (S) – configuration , or as racemic (RS)-, respectively.

Tests:

3- In future, when a monograph describes an enantiomer, it will include both a test for specific optical rotation under identification and a test using methods such as chiral chromatography, to control enantiomeric purity.

4- When both the racemic mixture and the enantiomer are available, the monograph for the racemic mixture will specify a test for angle of rotation together with a cross reference under identification. The test for angle of rotation will normally specify limits of +0.10° to -0.10°in order to limit the presence of optically active impurities and demonstrate equal proportions of the enantiomers.

5- When only the racemic mixture is available, the monograph for the racemic mixture will simply specify a test for angle of rotation [9, 18].

1.3.3 ICH Guideline:

According to ICH Guideline, each impurity must be investigated with respect to both chemistry and safety aspects. The former include identification (structural characterization), reporting and quantitation using suitable analytical procedures, while the latter include a process of acquiring and evaluating data concerning the biological safety of an impurity (qualification). Individually listed impurities, limited with specific acceptance criteria, are referred to as specified and they can be either identified or unidentified.

Unspecified impurities are limited by a general acceptance criterion. A decision tree for the identification and qualification along with the corresponding thresholds, which are dependent on the maximum permitted daily dose (MDD), is given by ICH. Summing up, the following list of organic impurities must be presented in the specification of a synthetic drug substance:

- Each specified identified or unidentified impurity
- Any unspecified impurity
- Total impurity

Specified unidentified impurities are referred to by an appropriate qualitative analytical description (e.g. relative retention time) [3, 4, 12, 13, 19]. Below are the ICH topics, codes of quality guidelines.

Table: 1.2 List of topics, codes and corresponding quality guidelines developed by ICH

Topics / Code	Quality guidelines
Q1A(R2)	Stability Testing of New Drug Substances and Products
Q1B	Stability Testing: Photo stability Testing of New Drug Substances and Products
Q1C	Stability Testing for New Dosage Forms Annex to the ICH Harmonised Tripartite Guideline on Stability Testing for New Drugs and Products
Q1D	Bracketing and Matrixing Designs for Stability Testing of Drug Substances and Drug Products
Q1E	Evaluation of Stability Data
Q1F	Stability Data Package for Registration in Climatic Zones III and IV
Q2(R1)	Validation of Analytical Procedures: Text and Methodology
Q3A(R2)	Impurities in New Drug Substances
Q3B(R2)	Impurities in New Drug Products
Q3C(R4)	Impurities: Guideline for Residual Solvents
Q4B	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions
Q4B Annex 1	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Residue on Ignition/Sulphated Ash General Chapter
Q4B Annex 2	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Test for Extractable Volume of Parenteral Preparations General Chapter
Q4B Annex 3	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Test for Particulate Contamination: Sub-Visible Particles General Chapter
Q4B Annex 4A	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbiological Examination of Non-Sterile Products: Microbial Enumerations Tests General Chapter
Q4B Annex 4B	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbiological Examination of Non-Sterile Products: Test for Specified Micro-Organisms General Chapter
Q4B Annex 4C	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbiological Examination of Non-Sterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use General Chapter
Q4B Annex 6	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Uniformity of Dosage Units General Chapter
Q4B Annex 7	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the

	ICH Region on Dissolution Test General Chapter
Q4B Annex 8	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Sterility Test General Chapter
Q4B Annex 9	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Tablet Friability General Chapter
Q4B Annex 10	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Polyacrylamide Gel Electrophoresis General Chapter
Q5A(R1)	Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin
Q5B	Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of R-DNA Derived Protein Products
Q5C	Quality of Biotechnological Products: Stability Testing of Biotechnological/ Biological Products
Q5D	Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products
Q5E	Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process
Q6A	Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances
Q6B	Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products
Q7	Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients
Q8(R2)	Pharmaceutical Development
Q9	Quality Risk Management
Q10	Pharmaceutical Quality system
Q8/Q9/Q10 Q&As	**Quality Implementation Working Group on Q8, Q9 and Q10** Questions & Answers

1.4 Control of Organic Impurities:

Control of the organic impurities in new drug substances is based on the Maximum Daily Dose and total daily intake (TDI) of the impurities. Table 1.3 provides the ICH threshold for control of the organic impurities in new drug substances. Depending on whether the Maximum Daily Dose is higher or lower than 2g, organic impurities in a new drug substance at (or greater than) 0.05% or 0.1% requires identification. Control of organic

impurities in new drug products are outlined in Table 1.4. Based on the Maximum Daily Dose, the identification thresholds for organic impurities in new drug products are divided into 4 groups to give more consideration to low dose drug products. For most new drug products, the Maximum Daily Dose is between 10 mg–2 g/day, therefore, any impurities at 0.2% or greater would have to be identified [5, 7 and 12].

Table: 1.3 Organic impurity Threshold in new drug substances based on ICHQ3A [4, 5 and 19]

Maximum daily dose[1]	Reporting Threshold[2,3]	Identification Threshold[2,3]	Qualification Threshold[2,3]
≤2g/day	0.05%	0.1 or 1.0mg/day intake (whichever is lower)	0.15% or 1.0mg/day (whichever is lower)
> 2g/day	0.03%	0.05%	0.05%

Note: [1-] The amount of drug substance administered per day

[2-] Higher reporting thresholds should be scientifically justified

[3-] Lower thresholds can be appropriate if the impurity is unusually toxic

Table: 1.4 Organic impurity Threshold in new drug products based on ICH Q3B [4, 12 and 19]

Reporting Thresholds	
Maximum Daily Dose [1]	Threshold[2,3]
≤1 g	0.1%
> 1 g	0.05%

Identification Thresholds

Maximum Daily Dose[1]	Threshold[2,3]
< 1 mg	1.0% or 5 µg TDI, whichever is lower
1mg – 10mg	0.5% or 20 µg TDI, whichever is lower
>10 mg - 2 g	0.2% or 2 mg TDI, whichever is lower
> 2 g	0.10%

Qualification Thresholds	
Maximum Daily Dose [1]	Threshold[2,3]
< 10 mg	1.0% or 50 µg TDI, whichever is lower
10 mg - 100 mg	0.5% or 200 µg TDI, whichever is lower
>100 mg - 2 g	0.2% or 3 mg TDI, whichever is lower
> 2 g	0.15%

Note: TDI – total daily intake

[1-]The amount of drug substance administered per day

[2-]Thresholds for degradation products are expressed either as a percentage of the drug substance or as total daily intake (TDI) of the degradation product. Lower thresholds can be appropriate if the degradation product is unusually toxic.

[3-] Higher thresholds should be scientifically justified.

1.5 Stability testing of New Drug substances and Drug Products:

Analytical methods for impurities estimation should be stability indicating to monitor the stability of pharmaceutical dosage forms during the investigational phase of drug development, and once the drug is marketed, for the ongoing stability studies which must be conducted / performed. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature,

humidity and light, enables to establish a retest period/shelf lives for a drug substance and a recommended storage condition. Methods can be developed which measure the amount of drug remaining, the amount of drug lost (or the appearance of degradation products), or both. The expert Working Group of the *International Conference on Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human Use* developed a guideline on stability testing for registration application within the European Union, Japan and the United States. The goal of the ICH stability guideline was to exemplify the core stability data package required for new drug substances and products in the European Union, Japan and the United States such that the data generated in any of the regions is mutually acceptable in the other two. The guideline applies to the information required for the registration applications of new molecular entities and drug products, but not to abbreviated or abridged applications, clinical trail applications, and so on. The test conditions were selected based on the climatic conditions in three areas so that test data provides evidence on the variation in quality with time under the influence of a variety of representative environmental factors. These data in turn allow recommended storage conditions and shelf lives to be established.

1.5.1 Drug substance:

The primary stability studies for the drug substance show that it will remain within specification during the retest period. Long-term (12-

month) and accelerated (6-month) testing are performed on at least three batches. Batches can be manufactured at a minimum of pilot scale, but should use the same synthetic route and a method of manufacture that simulates the final process to be used at manufacturing scale. In addition, supporting stability on laboratory-scale batches may be submitted. The quality of the batches placed on stability should be representative of the quality of –

(a) Material used in preclinical and clinical studies

(b) Material to be made at a manufacturing scale.

The first three batches made post approval should also be placed on long-term stability using the product registration protocol. Testing should cover physical, chemical, and microbiological properties susceptible to change during storage and likely to affect product quality, safety, and/or efficacy. Validated stability-indicating methods should be used. The number of replicates to be run depends on the results of validation studies, and limits should be derived from material used in preclinical and clinical studies, including both individual and total upper limits for impurities and degradation products. The length of the studies and the storage conditions should cover storage, shipment, and subsequent use, although use of the same conditions as for the drug product will facilitate comparative review and assessment. Other conditions should be included as scientifically justified. Temperature-sensitive drugs should be stored at the labeled long-term storage temperature, and accelerated testing should

be conducted at 15°C above the designated long-term storage temperature with appropriate relative humidity conditions.

At the time of regulatory submission, a minimum of 12 months at 25°C ± 2°C/60% RH ± 5 (long term) and 6 months at 40°C ± 2°C and /75% RH ± 5% (accelerated) is required. If significant changes are noted at the elevated temperature, additional testing at an intermediate condition, such as 30°C ± 2°C/65% RH ± 5% should be conducted. The registration application should include a minimum of 6 months of data from a 12-month study at the intermediate condition. Significant change at 40°C and 75% RH is defined as failure to meet specification. Long-term-testing should be continued to cover all retest periods. Normally, testing under long-term conditions is performed every 3 months for the first year, every 6 months for the second year, and then annually. Containers employed in the long-term stability study should be the same or simulate actual packaging used for storage and distribution. As the application is pending review, accumulated stability data should be submitted. Accelerated or intermediate temperature data may be used to support shipping conditions and evaluate the effect of short-term excursions outside the label storage conditions.

Because long-term stability is used to establish appropriate retest periods, it should be noted that the degree of inter batch variability affects the confidence that a future batch will remain within specifications for the entire retest period. As a rule, determination of the time at which the 95%

one-sided confidence limit for the mean degradation curve intersects the acceptable lower specification limit is acceptable, combining data into one overall estimate to account for variability. Before combining the data, apply appropriate statistical tests (e.g., p test) to be sure it is allowable. If inappropriate to combine data, the retest period may depend on the minimum time a batch is actually measured to remain in specification. The nature of the degradation relationship determines the need for transformation of the data for linear regression analysis. This relationship can generally be fitted to a linear, quadratic, or cubic function on an arithmetic or logarithmic scale.

Statistical methods can be used to test the goodness of fit of the data on all batches and combined batches, where appropriate, to the assumed degradation curve. If the data show little degradation or variability, a retest period can be justified without statistical analysis and a limited extrapolation of real-time data may be undertaken when supported by the accelerated data. Any extrapolation must be justified, because it assumes that the same mechanism of degradation will continue beyond the observed data; this evaluation should include assay, degradation products, and any other appropriate attributes.

The storage temperature range should be based on the stability data and used in accordance with the national or regional requirements. Specific labeling requirements should be stated, particularly for drugs that

cannot freeze; terms such as ambient and room temperature should be avoided.

1.5.2 Drug Product:

The stability program for the drug product should be based on knowledge of the drug substance and experience from experimental and clinical formulations. Unless specifically noted in this section, the requirements for drug substances also apply to drug products. Accelerated and long-term data should be provided on three batches of the same formulation and dosage form in the containers and closure proposed for marketing. This revision of the ICH guideline specifies only solid oral dosage forms, and it states that two of the three batches placed on stability should be at least pilot scale, but that a third may be smaller—for example, 25,000-50,000 tablets or capsules. As with drug substance, at least 12 months of long-term stability data should be submitted at the time of regulatory filing. When possible, manufacture stability batches of the finished product using identifiably different batches of drug substance. Data on laboratory-scale batches of drug product is not acceptable as primary stability data, but may be submitted as supportive information, as may data on associated formulations or packaging. If required, preservative efficacy testing and assays on stored samples should be performed to determine content and efficacy of antimicrobial preservatives. Differences between release and shelf-life specifications for antimicrobial preservatives should be supported by preservative efficacy

testing. Limits for tests such as dissolution and particle size require reference to results of bioavailability and clinical batches.

Storage at high relative humidity is important for solid oral dosage forms, but is not necessary for products such as solutions, suspension, and so on, stored in containers designed to provide a permanent water barrier. Low relative humidity (10-20%) is appropriate for products of high water content stored in semi permeable containers. Testing of unprotected drug product can be a useful part of stress testing and package evaluation, as can studies in related packaging materials. If a product needs to be reconstituted or diluted, stability in the final form should also be addressed.

1.6 Stress testing route to the development of stability-indicating analytical methods (SIAMs):

Studies were under taken to elucidate the intrinsic stability of the drug substance. Such testing is part of the development strategy and is normally carried out under more severe conditions than those used for accelerated testing. A more detailed description of stress testing is provided near the beginning of the ICH stability guideline, under the "Drug substance" heading: "Stress testing of the drug substance can help identify the likely degradation products, which in turn can help establish the degradation pathways and the intrinsic stability of the molecules and validate the stability indicating power of the analytical procedures used".

The nature of the stress testing will depend on the individual drug substance and drug product involved.

Stress testing is likely to be carried out on single batch of drug substance. It should include the effect of temperatures (in 10º C increments (e.g., 50ºC, 60ºC etc.) above that for accelerated testing), humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across wide range of pH values when in solution or suspension. Photo stability testing should be an integral part of stress testing. The standard conditions for photo stability testing are described in ICH Q1B.

Examining the degradation products under stress conditions is useful in establishing the degradation pathways, developing and validating suitable analytical procedures. However, it may not be necessary to examine specifically for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions.

Results from these studies will form an integral part of the information provided by the regulatory authorities. The description of stress testing was slightly modified in the revised stability guideline from the original description in ICH Q1A. The original Q1A description contains this additional paragraph:

Stress testing is conducted to provide data on forced decomposition products and decomposition mechanisms for the drug substance. The severe conditions that may be encountered during distribution can be covered by stress testing of definitive batches of drug substance.

The ICH definition of stress testing for drug product shown below:

Studies undertaken to asses the effect of severe conditions on the drug product. Such studies include photo stability testing (ICH Q1B) and specific testing on certain products (e.g., metered dose inhalers, creams, emulsions, refrigerated aqueous liquid products).

From the ICH definition, it is now clear that there is now a (regulatory) differentiation between “accelerated testing” and “stress testing”. Stress testing is distinguished by both severity of the conditions and the focus or intent of the results. Stress testing, which is also often referred as “forced degradation,” is an investigation of the “intrinsic stability” characteristics of the molecule, providing the foundation for ***developing and validating analytical methods*** and for developing stable formulations. Stress testing studies are intended to *discover* stability issues, and are therefore *predictive* in nature. Stress testing studies are not part of the “validated” formal stability program. Rather, pharmaceutical stress testing is a research investigation requiring a scientific expertise and judgment.

It is interesting to consider some of the conditions that have historically been employed to consider in the stress testing of pharmaceuticals, documented both in the “Analytical Profiles of Drug

Substances" [20] and by Singh and Bakshi [21]. Acidic stress conditions can be found to vary from 0.1 N HCl at 40°C for 1 week (with "negligible degradation") [22], to 0.1 N HCl at 65°C for 21 days (71.6% degradation) [21], to 0.1 N HCl at 105° C for 2 months (with "considerable degradation"), to 4 N HCl under refluxing conditions for 2 days (66% degradation) [23], to 6.5 N HCl at 108° C for 24 hr (50% degradation), to concentrated HCl at room temperature (56.5% degradation) [24]. Similar elevated temperatures, times, and base strength have been employed for basic stress conditions. For example, conditions can be found to vary from 0.1 N NaOH at 40°C for 1 week (with negligible degradation) [22], to 0.1 N NaOH at 65°C for 21 days (68% degradation) [21], to 0.1 N NaOH under refluxing conditions for 2 days (68% degradation) [23], to 1 N NaOH under boiling conditions for 3 days (7.2% degradation) [25], to 5 N NaOH under refluxing conditions for 4 hr (100% degradation) [26]. In terms of oxidative degradation studies, hydrogen peroxide has been employed at strengths from 0.3% to 30% [27]. Studies were often conducted at elevated temperatures, e.g., 37°C for 6 hr [3% hydrogen peroxide, 60% degradation [28], 50°C for 72 hr (3% hydrogen peroxide, 6.6% degradation), and even refluxing conditions for 30 min (3% hydrogen peroxide, extensive degradation) [26] or 6 hr (10% hydrogen peroxide, no significant degradation) [29].

As these examples illustrate that historically there has been tremendous variation in the conditions employed in acid/base and

oxidative stress testing studies. There has also been tremendous variation in defining the appropriate “endpoint” of the stress testing studies, i.e., length of time (and temperature) or amount of degradation that is sufficient to end the stress exposure. Perhaps the most dramatic variability in stress testing conditions is observed in the photo- stressing of drugs [30], where the lamps and exposures range from short wavelength Hg arc lamps (254 nm, UVC range), fluorescent light, artificial light, halogen lamps to xenon lamps. The variability of exposure to type of light during pharmaceutical photo-stability studies has also been documented by surveys of practices in the pharmaceutical industry [31-34].

From the information provided above, it is apparent that stress-testing conditions have varied greatly from compound to compound and from investigator to investigator. Extremely harsh conditions have been commonly used in the past to ensure degradation, even if the conditions far exceeded plausible exposures.

More recently, several articles relevant to stress testing have appeared in the pharmaceutical literature. A paper by Singh and Bakshi [21] in 2000 provides the most thorough collection of references to various degradation studies of drug products, documenting the diversity of conditions and approaches to stress testing. This paper attempts to provide a classification system (Extremely labile, Very labile, Labile, Stable) based on a defined systematic approach. It is not clear from the

article on what basis (scientific or otherwise) the classification system was devised; however, the paper does define “endpoints” to stressing (albeit, fairly harsh endpoints), allowing for the conclusion that a particular compound may be regarded as “stable” under a certain set of conditions.

In 1992 (and again in 1994), Boccardi provided some needed guidance on oxidative stress testing by asserting that most pharmaceutical oxidative degradation was the result of autoxidation and that hydrogen peroxide was not a very good reagent to mimic autoxidation processes [35,36]. Boccardi was the first to describe the use of radical initiators such as azobisisobutyronitrile (AIBN) for oxidative pharmaceutical stress testing, and he provided a simple procedure with mild conditions, which he termed as “The AIBN Test.” In 1996, Baertschi [37] presented and discussed an approach to stress testing that had defined limits of harshness and exposure time. In 1998, Weiser [38], while discussing the role of stress testing in analytical method development, suggested a set of conditions for performing stress testing that was arguably milder than many of the historical studies cited above. In 2001, Alsante et al. [39] provided a guide to stress testing studies that suggested defined limits to the stress conditions of 1 N HCl and 1 N NaOH for a maximum of 1 week at room temperature. In 2002, the views of the Pharmaceutical Research and Manufacturer’s Association (PhRMA) were summarized in an article on forced degradation studies published in Pharmaceutical Technology [40]. The PhRMA article did not discuss specifics of conditions of stress,

but rather focused more on what kinds of stress testing should be performed for drug substances and products and on the regulatory requirements.

Recent publications on the topic of stress testing/forced degradation studies reveal that there is still a tremendous variability in the conditions employed. A few examples will be discussed here, although this discussion is not intended to be an exhaustive review of the literature.

A degradation study of haloperidol utilized 1 M HCl and 1 M NaOH (refluxed for 5 hr), and 30% hydrogen peroxide (70°C for 5 hr) for the most stressful conditions of the study [41]. These conditions appear to have been chosen to enable production of known degradation products (six degradation products shown) to facilitate HPLC method validation efforts. A degradation study of ibuprofen produced 13 degradation products, several of which had never been detected before [42]. In this study, oxidative studies were carried out utilizing potassium permanganate (0.05 M) at room temperature up to 16 hr in 0.5 M NaOH; up to 33% hydrogen peroxide at room temperature for 22 hr; and potassium dichromate (0.1 N) at room temperature up to 14 days in 0.5 M HCl. Solid-state studies utilized 50°C up to 8 months and 100°C up to 16 hr to detect volatile degradation products. An NMR study of the aqueous degradation of isophosphoramide mustard was conducted in buffered aqueous solutions in the pH range of 1-13 [43]. The degradation of sumatriptan in 0.1 N HCl, 0.1 N NaOH, and in 3% hydrogen peroxide was studied using LC/MS and

LC/MS/MS [44]. The solutions were heated at 90ºC for 30 min to 9 hr. Photostability was assessed by exposure to UV irradiation at 254 min for 24 hr (no indication of irradiation intensity). A study of the major oxidative degradation products of SCH 56592 was conducted by exposure of the drug substance in the solid state to 150ºC for 12 days with identification of the major products using LC-MS and LC-NMR [45]. Singh et al. describe stress degradation studies of ornidazole [46] and prazosin, terazosin, and doxazosin [47] under conditions designed to be in "alignment" with the ICH Stability guideline (Q1AR). In the case of ornidazole, significant degradation was seen under acidic conditions of 0.1 M HCl to 5 M HCl at 80ºC for 12-72 hr, although no degradation products were detected (presumably because of degradation to non-chromophoric products). Studies under basic conditions of 0.1 M NaOH at both 80ºC and 40ºC revealed complete degradation at time zero. Milder studies were then conducted at pH 8 and 40ºC. Oxidative studies involved 3% and 30% hydrogen peroxide at room temperature for 24 and 48 hr, with losses of 8% and 53% of the parent, respectively. Photo-degradation studies utilized Option 2 of the ICH photostability guideline with exposures up to 30 days at 7000 lux (over 5 million lux-hr exposure). Similar conditions were employed for prazosin, terazosin, and doxazosin. In these recent examples of stress testing studies, it is apparent that there is still a great diversity of conditions employed to induce degradation, although the diversity is arguably less than was observed prior to publication of the ICH guidance.

This continued diversity of approach could be interpreted in a couple of ways. One interpretation is that stress-testing studies are inherently a research undertaking, and, therefore, flexibility and scientific judgment are required, leading to diverse conditions and approaches. Another interpretation is that there is (appropriately or inappropriately) very little guidance (either regulatory or in the scientific literature) on the specification of the conditions or appropriate endpoints of pharmaceutical stress testing.

1.7 Techniques employed in literature reports for the development of SIAMs: If one critically evaluates the literature reports, titrimetric, spectrophotometric and chromatographic techniques have been commonly employed in the analysis of stability samples.

1.7.1 Titrimetric and spectrophotometric

In these methods, usually the objective is the analysis of drug alone in the matrix of impurities, degradation products, impurities, etc., and also other drugs in case of the combination products. Their advantage is low cost and simplicity, though some times they are not sensitive. Due to limitation of specificity there are hardly any reports these days on the use for the assay of stability samples. However, a few reports involving derivative spectroscopy have been published lately.

1.7.2 Chromatographic

Because of very nature of requirement of separation of multiple components during analysis, chromatographic methods have taken

precedence over conventional methods of analysis. Other than separation of multiple components, the advantage of chromatographic methods is that these possess greater accuracy and sensitivity for even small quantities of degradation products performed. Various chromatographic methods that have been used are thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), HPLC (High Performance Liquid Chromatography) and newer technique like capillary electrophoresis (CE).

In comparison, 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. Therefore, most of the SIAMs have been established using HPLC.

1.7.3 Steps involved during the development of stability-indicating analytical methods (SIAMs):

A SIAM is a quantitative analytical procedure used to detect a decrease in the amount of the active pharmaceutical ingredient (API) present due to degradation. According to FDA guidelines, a SIAM is defined as a *validated analytical procedure that accurately and precisely measures active ingredients (drug substance or drug product) free from potential interferences like degradation products, process impurities, excipients, or other potential impurities*, and the FDA recommends that all assay procedures for stability studies be stability-indicating [48]. During

stability studies, liquid chromatography (LC) is used routinely to separate and quantitate the analytes of interest. There are three components necessary for implementing a SIAM: sample generation, method development, and method validation.

Step 1: Generation of the Sample:

Stressing the API in both solutions and in solid-state form generate the sample that contains the products most likely to form under most realistic storage conditions, which is in turn used to develop the SIAM. In simplest terms, the goal of the SIAM is to obtain baseline resolution of all the resulting products (the API and all the degradation products) with no coelutions.

Samples should be stored in appropriate vessels that allow sampling at timed intervals and that protect and preserve the integrity of the sample. Thermo stated and humidity-controlled ovens should also be employed. Generally, the goal of these studies is to degrade the API by 5-20 %. Any more than this and relevant compounds can be destroyed, or irrelevant degradation products produced (for example, degradation products of the degradation products).

Any less, and important products might be missed. Experience and data obtained from studies performed previously on related compounds also should be used when developing new protocols.

Table: 1.5 lists some common conditions used in conducting forced degradation studies for drug substances [49].

Sample condition	Time / Exposure
Solid / 60 - 70ºC	7 – 10 days
Solid / 60 - 70ºC / 75% RH	10 days
Solid / simulated sunlight	2 – 3 weeks x ICH confirmatory exposure
0.1 to 2 N HCl solutions either at RT or at 60 - 70ºC	1 – 3 days
0.1 to 1 N NaOH solutions either at RT or at 60 - 70ºC	1 – 3 days
Dilute hydrogen peroxide (0.1 to 6%) at RT or at 60 - 70ºC	1 – 3 days
Solution in Water or at 60 - 70ºC	1 – 3 days

Step 2: Developing the LC Method:

After the sample is generated through the use of a properly designed and executed forced degradation, it can be used to develop the LC method. These days, LC method development often is performed on gradient systems capable of automated column and solvent switching, and temperature control. Systems and software that automate the process, some with decision making built-in, also have been reported [50]. Scouting experiments often are run, and then conditions are chosen for further optimization. Resolving power, specificity, and speed are key chromatographic method attributes to be kept in mind during method development. However, excellent resources are available to anyone not already schooled in the art [51].

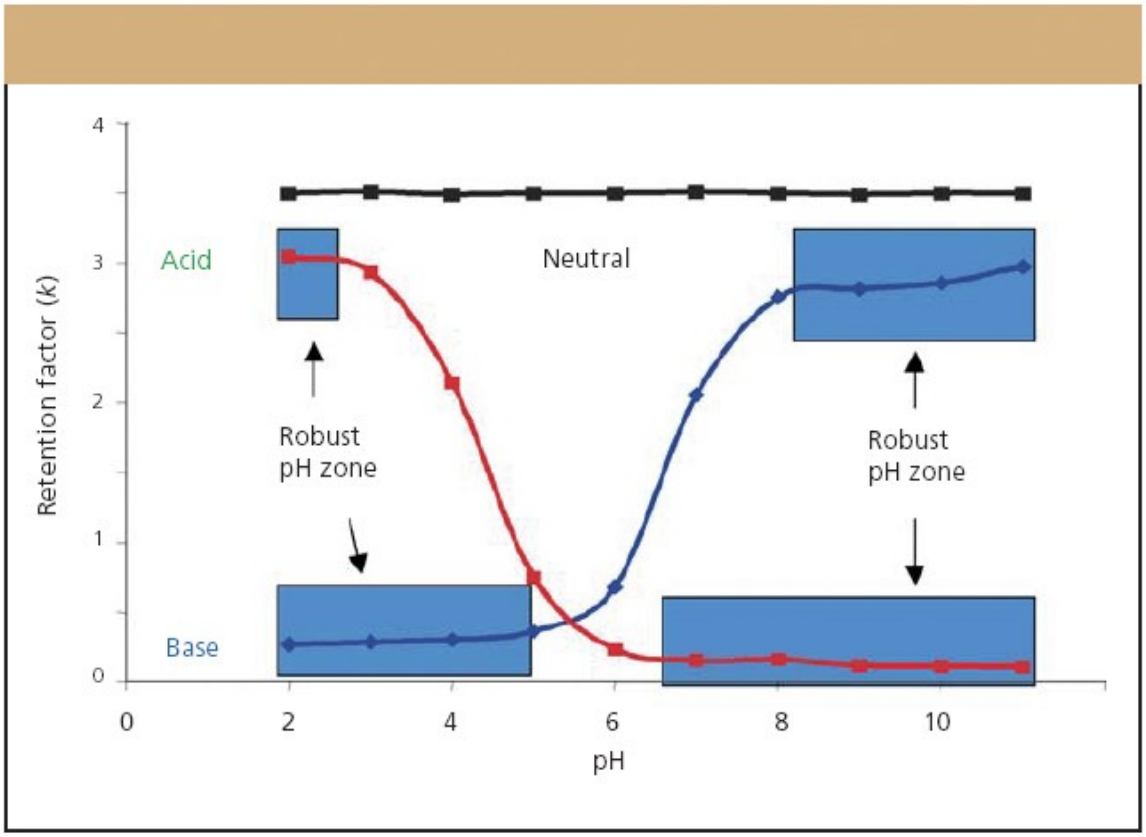
Selectivity during Method Development:

Selectivity can be manipulated by any one or a combination of different factors that include solvent composition, type of stationary phase in column, and mobile phase buffers and pH. Chromatographers for the most part are comfortable changing solvents and column stationary phases to generate a separation. However, advances in LC column technology recently have made possible the use of pH as a true selectivity tool for the separation of ionisable compounds [52, 53]. These hybrid chemistry columns take advantage of the best of both the silica and polymeric column worlds. They are manufactured using a classical sol-gel synthesis that incorporates carbon in the form of methyl groups, resulting in columns that are mechanically strong, with high efficiency, and operate over an extended pH range. The graphics in Figure1.1 illustrate why pH can be such a useful tool.

Acidic compounds are more retained at low pH; while basic compounds are more retained at higher pH (neutral compounds are of course unaffected). At pH values used traditionally (pH 4-8); a slight change in pH would result in a dramatic shift in retention (up-slope or down-slope of curve). However, by operating at pH extremes, not only is there a 10-30-fold difference in retention that can be exploited in method development, the method can be made more robust as well, a desirable outcome with validation in mind. Indeed, the selectivity differences

afforded by a change in pH are the equivalent to a 20% change in the organic solvent composition, and often are underutilized.

Fig1.1: Reverse – phase retention behavior as pH is varied



Evaluating Specificity during Method Development:

Another key parameter to evaluate during method development is specificity. *The United States Pharmacopoeia (USP)* and International Conference on Harmonisation (ICH) guidelines define specificity as the ability of a method to assess unequivocally the analyte of interest in the presence of potential interferences [54, 55]. In the past, it has been acceptable to evaluate resolution, peak shape, and tailing factors to measure and document specificity. However, starting with *USP 24,* and as

a direct result of the ICH process, it was recommended that a peak purity test based upon photodiode-array (PDA) detection or mass spectrometry (MS) be used to demonstrate that a given peak was pure.

Modern PDA technology is a powerful tool for evaluating specificity. PDA detectors can collect spectra across a range of wavelengths; at each data point collected across a peak, and through software manipulations involving multidimensional vector algebra, they compare each of the spectra to determine peak purity. In this manner, PDA detectors today can distinguish minute spectral and chromatographic differences not readily observed by simple overlay comparisons [56-58].To be successful, three components are required:

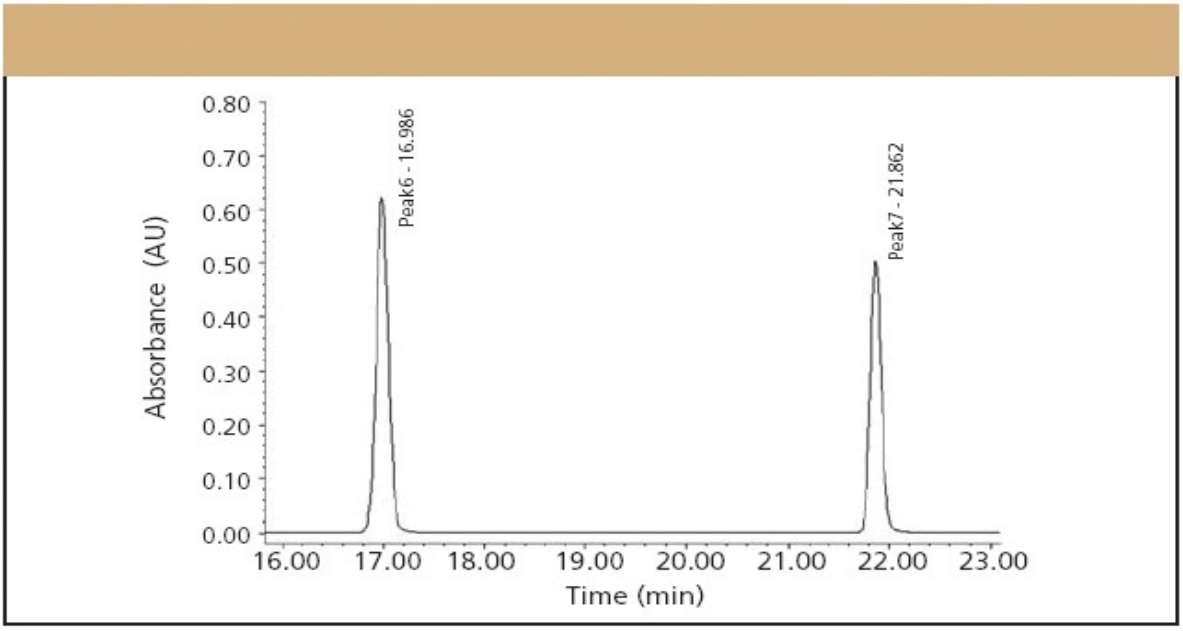
- A UV chromophore, or some absorbance in the wavelength range selected
- Some degree of chromatographic resolution.
- Some degree of spectral difference.

Figure 1.2 shows an example of a partial reversed-phase LC separation, where, by the appearance, the peaks certainly are well-resolved, sharp, and symmetrical.

An examination of peak 2 indicated the peak was pure. However, a close examination of the spectral information related to peak one reveals a different situation. In Figure 1.3, the calculated peak purity (in green) is plotted against the noise threshold (in blue), both superimposed on the red chromatographic trace. The purity plot clearly indicates a co-elution

in the front of the peak as the purity plot exceeds the threshold, requiring more method development. PDA detectors can be limited on occasion in evaluating peak purity, governed by the three required components mentioned previously, as well as the noise of the system and disparate levels of absorbance responses.

Fig 1.2: Chromatogram showing the separation of two components seemingly pure

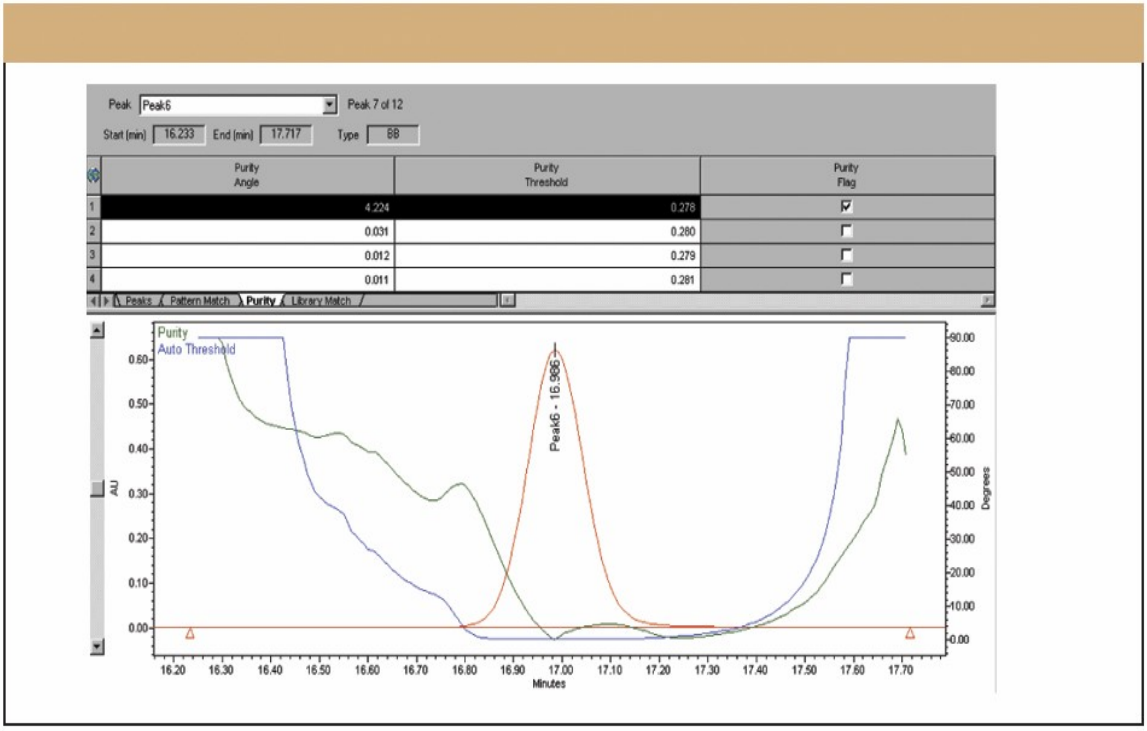


That is, the more similar the spectra, and the lower the relative absorbances, the more difficult it can be to distinguish co-eluted compounds. Mass spectrometric detection overcomes many of these limitations and in most laboratories; it has become the detection method of choice for even routine method development.

MS has come a long way from the days in which many companies had a dedicated central MS lab and staff. Modern mass spectrometers are

smaller, simpler, and operate from the same software used to operate the chromatographic system or other detectors commonly utilized, decreasing the learning curve. MS can provide unequivocal peak purity information, exact mass, and structural and quantitative information depending upon the type of instrument used.

Fig: 1.3 The calculated peak purity (in green) is plotted against the noise threshold (in blue), both super imposed on the red chromatographic trace.



MS is also a very useful tool to track peaks as they move around in response to selectivity manipulations in method development. However, only the combination of both PDA and MS on a single instrument and software platform provides the type of valuable orthogonal information required when evaluating specificity and developing SIAMs.

Step 3: Validation of SIAMs

Different methods have different requirements when it comes to validation. The *USP* recognizes four method categories and defines the analytical performance characteristics that must be measured to validate each method type [59]:

- Category 1 : Analytical methods for the quantification of major components of bulk drug substances or active ingredients.
- Category 2 : Analytical methods for the determination of impurities in bulk drug substances or degradation compounds.
- Category 3 : Analytical methods for the determination of performance characteristics.
- Category 4 : Identification tests

Table 1.6 summarizes each category and the analytical performance characteristics that must be investigated.

SIAM falls into the quantitative division of Category 2, and as such, all analytical performance parameters must be determined, except for the limit of detection, limit of quantification would apply instead, because SIAMs need to be quantitative. According to ICH guideline on validation of analytical methods [60], *the objective of an analytical procedure is to demonstrate that it is suitable for its intended purpose.* One should keep in mind that stress testing methods are screening methods used to help in understanding the degradation chemistry of the drug and therefore, do

not need to (nor, in general, can they) be validated to the extent of final control methods.

Table 1.6: Data elements required for assay validation (as per *USP*)

Analytical performance characteristics	Assay Category I	Assay Category II		Assay Category III	Assay Category IV
		Quantitative	Limit tests		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
Detection limit	No	No	Yes	*	No
Quantitation limit	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

*****May be required, depending on the nature of the specific test

The concepts in the ICH guideline on validation of analytical methods are a good starting point for validation of stress testing methods, however, the overall validation should be significantly abbreviated when compared to the validation of final control methods, as stress testing methods are investigational methods. Accuracy normally should not be a problem with stress testing methods as long as the response of the detector is linear and samples are completely dissolved prior to analysis. The specificity of the methods can not be fully validated because one does not know all of the possible degradation products during initial stress testing. Specificity can be addressed by using any known impurities and degradation products produced in the method development samples.

Precision (repeatability) of the assay of the main component can be evaluated by preparing a limited number of assay samples and using simple statistics to estimate the standard deviation. Estimation of intermediate precision and reproducibility should normally not be necessary for stress testing methods. Detection and quantitation limits for degradation products can be determined by using parent compound and assuming that the response of all degradation products will be similar. Although there is no requirement to reach any specific detection limit, a reasonable is 0.1% since the aim of stress testing is to detect the major degradation products in samples which is approximately 10 – 20% degraded. The linearity of the method should be validated over ranges for both assay and impurity determination. A typical assay range might be from 50% to 150% of nominal sample concentration, while a typical range for impurity determination might cover a range from the quantitation limit to a few percent. If one wishes to quantitate impurities vs. the parent peak, then linearity (range) should be demonstrated from the quantitation limit to at least 120% of nominal sample concentration.

One of the most important aspects of stress testing is the analysis of samples using a suitable analytical method, which, in many cases, is reverse-phase HPLC. This necessitates the development of an HPLC method capable of measuring both the loss of parent compound as well the levels of degradation products or impurities formed in stress conditions.

1.7.4 Role of Mass Balance during SIAM development:

Mass balance correlates the measured loss of a parent drug to the measured increase in the amount of degradation products. It is a good quality control check on analytical methods to show that all degradation products are adequately detected and do not interfere with quantitation of the parent drug (*i.e.,* stability-indicating methods). Regulatory agencies use mass balance to assess the appropriateness of the analytical method as a stability-indicating method and determine whether all degradents have been accounted for [61].

In mass balance calculations, the loss of parent drug or the amount of drug remaining is determined from a sample assay, and the measured increase in degradation products is determined by a related substances method. The fundamental approach for determining mass balance is to quantitate the decomposition peaks using degradation methods and then reconcile the measured loss in the parent drug with the amount of degradation products. If the loss in potency can be reasonably accounted for by the amount of degradants measured, then mass balance is achieved.

The assessment of degradation in pharmaceutical products involves two aspects of analytical measurement. Firstly, a specific or selective analytical method must be available for accurate assay of parent drug compound, in order to measure any loss. Second, methodology should be in place for quantification of the degradation products formed. Ideally,

when degradation occurs, the measured amount of parent drug lost should correlate well with the measured increase in degradation products. This correlation is referred to as “mass balance” [62]. More recently, the International Conference on Harmonization (ICH) has provided definition of “mass balance; material balance” as follows:

The process of adding together the assay value and levels of degradation products to see how closely these add up to 100% of initial value, with due consideration of the margin of analytical precision. The concept is useful scientific guide for evaluating data, but it is not achievable in all circumstances. The focus may instead be on assuring the specificity of the assay, the completeness of the investigation of route of degradation, and the use, if necessary, of identified degradents as indicators of the extent of degradation via particular mechanism [63]. The analyst must balance time and resource demands to provide the information necessary to understand degradation without going to extreme measures of quantify components of little interest.

Mass balance in pharmaceutical analysis is very important for several reasons. By demonstrating the degardative losses of parent drug correlate well with the measured increase in degradation products unaccounted for. Conversely, if one observes, for example, a 20% loss of parent drug but only measures a 5% increase in degradation products, it is likely that additional degradation products formed are not accurately determined by the given method(s). Because unknown degradation products could

potentially be toxic or otherwise compromise the safety of drug, it is important to have methods that detect all major degradation products. Thus, safety is the major reason for the study of mass balance.

Mass balance is also useful in method validation [64]. In order to demonstrate that analytical methods are stability-indicating, unstressed and stressed materials are often compared. Any increase in degradation product that correlates well with loss of parent drug, aids in demonstrating that the methods can accurately assess degradation. Mass balance is also important in understanding alternative degradation pathways [65]. For example, consider a situation where both acid-catalyzed and oxidative degradation produces a substantial loss of parent compound in stress-testing studies. If good mass balance is achieved for the acid-catalyzed degradation, but not for the oxidative degradation, further work to better understand the oxidative degradation pathway(s) is warranted. It may be that the poor mass balance in the latter case results from important oxidative products that are unaccounted for or from structures, which need to be more fully elucidated to understand response factor differences. Mass balance is an important consideration in assessing degradation pathways of pharmaceutical products. Often, response factor differences between degradation products and the parent compound are responsible for mass balance problems. RRFs should, therefore, be incorporated, when possible, in the quantification of degraded samples.

1.7.5 Application of SIAMs:

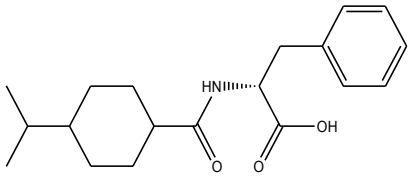
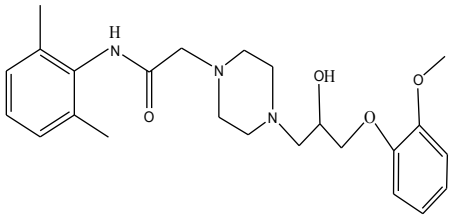
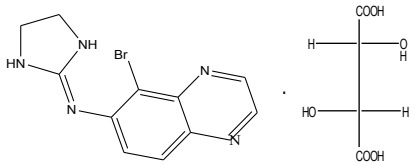
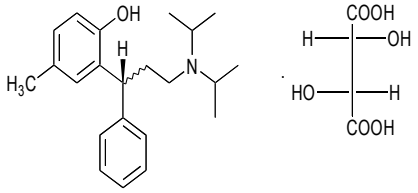
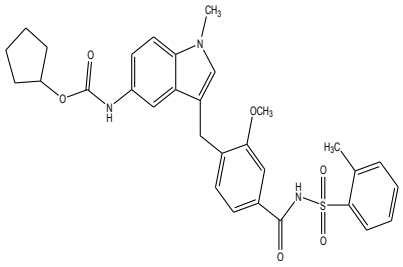
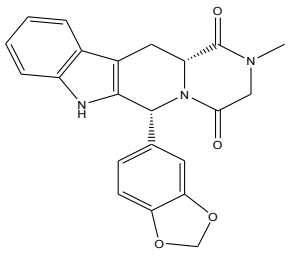
Stability studies are used to establish the re-test period for the active ingredient – that is the length of time it can be stored and used without analyzing immediately before use – and the shelf life of the finished product. The release and shelf life specifications for the product may differ to accommodate degradation of active ingredient or other acceptable changes, which may occur on storage. The International Conference on Harmonization (ICH) drug stability test guideline Q1A (R2) requires that analysis of stability samples should be done through the use of validated stability-indicating analytical methods (SIAMs). Additional guidance is given only for photo stability testing. It also recommends carrying out the stress testing on drug substance to establish its inherent stability characteristics and to support the suitability of proposed analytical procedure. The validated SIAMs will be used extensively for testing the stability samples of both drug substance as well as drug product.

1.8 Scope and Objectives of research work:

The present research work focuses on the development of novel stability-indicating analytical methods for some active pharmaceutical ingredients and few of their dosage forms. The work also includes the validation of the developed methods as per ICH requirements and demonstrates the suitability of developed methods to asses the stability of

Table 1.7 APIs, its chemical name, structure and therapeutic activity

S.No	*API* and its chemical	Structure	Therapeutic activity

	name		
1	**Nateglinide** N-(trans-4-isopropylcyclohexyl-carbonyl)-D-phenylalanine		Anti-diabetic drug
2	**Ranolazine** (RS)-N-(2,6-dimethylphenyl)-2-[4-[2-hydroxy-3-(2-methoxyphenoxy)propyl] piperazin-1-yl] acetamide		Anti –anginal and anti-ischemic agent
3	**Brimonidine tartarate** 5-bromo-6-(2-imidazolidinylideneamino) quinoxaline tartarate		Treatment of open-angle glaucoma or ocular hypertension
4	**Tolterodine tartarate** (R)-N, N-di isopropyl-3-(2-hydroxy-5-methylphenyl)-3-phenyl)-3-Phenyl propanamine L-hydrogen tartarate		Urinary urge incontinence
5	**Zafirlukast** {3-[2-Methoxy-4-(toluene-2-sulfonylaminocarbonyl)-benzyl]-1-methyl-1H-indol-5-yl}-carbamic acid cyclo pentyl ester		Treatment of asthma
6	**Tadalafil** (6R, 12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylene dioxyphenyl) pyrazino(1′, 2′:1,6) pyrido-(3,4-b)indole-1,4-dione		Enhances erectile function

active pharmaceutical ingredient (API). The list of active pharmaceutical compounds taken for research study was listed in Table 1.7.

Novel stability indicating LC methods were developed to determine the related components in different classes of pharmaceutical compounds which includes Nateglinide (anti-diabetic), Ranolazine (Anti anginal), Brimonidine Tartrate (used in treatment of open-angle glaucoma), Tolterodine tartrate (used in treatment of urinary urge incontinence and other symptoms related to unstable bladder), Zafirlukast (used in treatment of asthma) and Tadalafil (used in treatment of erectile dysfunction). The developed methods were validated according to regulatory norms. Stress testing was conducted according to ICH. Mass balance and stability studies were also conducted. The developed methods can be successfully implemented during the quality monitoring and also well employed for the assessment of quality during its storage and stability.

References:

1. Impurities in New Drug Substances, ICH Guidelines (Q3A).

2. Gorog.sandor, chemical and analytical characterization of related organic impurities in drugs, Anal Bioanal. Chem, vol.377, 2003, pp. 852-862.
3. Rao. R. Nageswara , Nagaraju.V, An overview of the recent trends in development of HPLC methods for determination of impurities in drugs, Journal of Pharmaceutical and Biomedical Analysis, vol.33, 2003, pp. 335-377.
4. Grekas. Nikolaos, organic impurities in chemical drug substances, pharmaceutical technology Europe, October 1- 2005.
5. Rousseau. Guy, international conference on harmonization impurity guidelines- the industry perspective, drug information journal, vol.34, 2000, pp. 903-907.
6. Roy.Jiban, Pharmaceutical impurities–A mini review, AAPS Pharmscitech,vol.3, no2, 2000. http://www.aapspharmscitech.org.
7. Qiu, F. and Norwood, D.L., Identification of pharmaceutical impurities, Journal of liquid chromatography & related technologies, vol.30, 2007, pp. 877-935.
8. Gorog, S. Identification and determination of impurities in drugs, Pharm. Biomed. Anal, 4; Elsevier: Amsterdam , 2000, 12–13.
9. British Pharmacopoiea 2001, Pharmacopoieal norms for enantiomeric impurities.
10. Gavin, P.F. & Olsen, B.A. A quality evaluation strategy for multi-sourced active pharmaceutical ingredient (API) starting materials.

J. Pharm. Biomed. Anal. 41 (4), 2006, pp. 1251–1259.

11. Muehlen, E. Impurities in starting materials and drugs. Pharmazeut. Ind. 54 (10), 1992, pp. 837–41
12. ICH, harmonized tripartite guidelines on impurities in new drug products (Q3B).
13. High beam encyclopedia, Organic impurities in chemical drug substances (Cover Story).
14. USP Guideline for submitting a request for revision of the USP/NF. Chapter 1, non-complex drug substances and products.
15. General chapters <466>, "Ordinary impurities" and <1086>, "Impurities in official articles," in USP 28-NF 23 (US Pharmacopoeia, 12601, Twin brooks Parkway, Rockville, Maryland 20852, USA. 2004).
16. CPMP Guideline on control of impurities of pharmacopoeial substances: compliance with the European pharmacopoeia general monograph “substances for pharmaceutical use” and general chapter “control of impurities in substances for pharmaceutical use”, 2004.
17. General chapter 5.10, control of impurities in substances for pharmaceutical use and general monograph 01/2005: 2034, Substances for pharmaceutical use, in European pharmacopoeia, 5th edition, pp. 559-561 and pp.586-587.
18. Vishweshwar. S & Gupta. R. M. FAQ on impurity profile for the

bulk drugs, Pharma Times, vol. 34, 2002.

19. Dantus.Mauricio.M and Wells.L.Margaret, regulatory issues in chromatographic analysis in the pharmaceutical industry, J. of liquid chromatography & related technologies, vol. 27, no.7-9, 2004, pp.1413-1442.
20. Florey K, Brittain HG, eds. Analytical Profiles of Drug Substances. New York: Academic Press, Vol. 1-25, 1972-1998.
21. Singh S, Bakshi M. Guidance on conduct of stress tests to determine inherent stability of drugs, Pharma Technol Online April, 2000, pp.1-14.
22. Bridle JH, Brimble MT. A stability indicating method for dipyridamole. Drug Dev Ind Pharm, vol 19, 1993: pp.371-381.
23. Padmanabhan G, Becue I, Smith JB. Cloquinol. In: Florey K, ed. Analytical Profiles of Drug Substances. New York: Academic Press Vol. 18, 1989: pp. 76-77.
24. Gröningsson K, Lindgren J-E, Lundbeg E, Sandberg R, Wahlén A. Lidocaine base and hydrochloride. In: Florey K, ed. Analytical Profiles of Drug Substances. New York: Academic Press. Vol.14 1985: pp. 226-227.
25. Lagu AL, Young R, McGonigle E, Lane PA. High performance liquid chromatographic determination of suprofen in drug substance and capsules. J Pharm Sci; 71(1): 1982, pp. 85-88.
26. Muhtadi FJ. Analytical profile of morphine.In: Florey K, ed. Analytical

Profiles of Drug Substances. New York: Academic Press, Vol. 17, 1988: pp. 309.

27. Maron N, Wright G. Application of photodiode array UV detection in the development of stability-indicating LC methods: determination of mefenamic acid. J Pharm Biomed Anal vol .8, 1990; pp. 101-105.
28. Nassar MN, Chen T, Reff MJ, Agharkar SN. Didanosine. In: Brittain HG, ed. Analytical Profiles of Drug Substances and Excipients. New York: Academic Press Vol. 22, 1993: pp. 216-219.
29. Johnson BM, Chang P-TL. Sertraline hydrochloride. In: Brittain HG, ed. Analytical Profiles of Drug Substances and Excipients. New York: Academic Press Vol. 24, 1996: pp. 484.
30. See Table V in Singh S, Bakshi M. Guidance on conduct of stress tests to determine inherent stability of drugs, Pharma Technol Online April, 2000; 1-14.
31. Anderson NH, Johnston D, McLelland MA, Munden P. Photostability testing of drug substances and drug products in UK Pharmaceutical laboratories. J Pharm Biomed Anal, 9(6) 1991; pp.443-9.
32. ThomaK. Survey of twenty German Manufacturers (1995). In: Tønnesen H, ed. Photostability of Drugs and Drug Formulations. London: Taylor & Francis, 1996: pp.136-137.
33. Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi, SW. Pharmaceutical photostability: a technical and practical interpretation of the ICH guideline and its application to

pharmaceutical stability: Part I, Pharmaceutical Technology, 25(3) March 2001; pp. 98-110.

34. Thatcher SR, Mansfield RK, Miller RB, Davis CW, Baertschi SW. Pharmaceutical photo stability: a technical and practical interpretation of the ICH guideline and its application to pharmaceutical stability: Part II, Pharmaceutical Technology, 25(4): April 2001; pp.50-62.
35. Boccardi G, Deleuze C, Gachon M, Palmisano G, Vergnaud JP. J Pharm Sci, 81(2):1992; pp.183-185.
36. Boccardi G. II Farmaco, 49(6):1994; pp.431-435.
37. Baertschi SW. The Role of Stress Testing in Pharmaceutical Product Development, presented at the American Association of Pharmaceutical Scientists Midwest Regional Meeting, Chicago, IL, May 20, 1996.
38. Weiser WE. Developing analytical methods for stability testing. Pharma Technol 1998: pp. 20-29.
39. Alsante KM, Friedmann RC, Hatajik TD, Lohr LL, Sharp TR, Snyder KD, Szczesny EJ. Degradation and impurity analysis for pharmaceutical drug candidates. In: Ahuja S, Scypinski S, eds. Handbook of Modern Pharmaceutical Analysis. Vol.3. Separation Science and Technology. Academic Press, 2001: pp. 85-172.

40. Reynolds DW, Facchine KL, Mullaney JF, Alsante KM, Hatajik TD, Motto MG. Available guidance and best practices for conducting forced degradation studies. Pharma Technol February, 26(2), 2002.

41. Trabelsi H, Bouabdallah S, Bouzouita K, Safta F. Determination and degradation study of haloperidol by high performance liquid chromatography. Pharm Biomed Anal, 29, 2002: pp. 649-657.

42. Caviglioli G, Valeria P, Brunella P, Sergio C, Attilia A, Gaetano B. Identification of degradation products of Ibuprofen arising from oxidative and thermal treatments. J Pharm Biomed Anal, 30, 2002; pp. 499-509.

43. Breil S, Martino R, Gilard V, Malet-Martino M, Niemeyer U. Identification of new aqueous chemical degradation products of isophosphoramide mustard. J Pharm Biomed Anal, 25, 2001: pp. 669-678.

44. Xu X, Barlett MG, Stewart JT. Determination of degradation products of sumatriptan. J Pharm Biomed Anal, 26, 2001; pp.367-377.

45. Feng W, Liu H, Chen G, Malchow R, Bennett F, Lin E, Pharmanik B, Chan T-M. Structural characterization of the oxidative degradation products of an antifungal agent SCH56592 by LC-NMR and LC-MS. J Pharm Biomed Anal, 25, 2001:pp. 545-557.

46. Bakshi M, Singh A, Singh S. The ICH guidance in practice: stress degradation studies on ornidazole and development of a validated

stability-indicating assay. J Pharm Biomed Anal, 26, 2001: pp. 891-897.

47. Ojha T, Bakshi M, Chakraborti AK, Singh S. The ICH guidance in practice: stress decomposition studies on three piperazinyl quinazoline adrenergic receptor-blocking agents and comparison of their degradation behavior. J Pharm Biomed Anal, 31, 2003: pp. 775-783.
48. Guidance for Industry, Analytical Procedures and Method Validation, U.S. Department of Health and Human Services FDA, August 2000 (www.fda.gov/guidance/index.htm).
49. D.W. Reynolds, *Am. Pharm. Rev.*, May/June, 56–61 (2004).
50. M.E. Swartz, M.D. Jones, and M.A. Andrews, LCGC Application Notebook, 66–67 (June 2002).
51. L.Snyder, J.J. Kirkland, and J. Glajch. Practical HPLC Method Development, 2nd ed. (Wiley-Interscience Publishers, New York, 1997).
52. U.D. Neue, T.H. Walter, B.A. Alden, Z. Jiang, R.P. Fisk, J.T. Cook, K.H. Glose, J.L. Carmody, J.M. Grassi, Y.-F. Cheng, Z. Lu, and R.J. Crowley, Am. Lab. 31, 1999: pp.36.
53. Y.-F. Cheng, T.H. Walter, Z. Lu, P. Iraneta, C. Gendreau, U.D. Neue, J.M. Grassi, J.L. Carmody, J.E. O'Gara, and R.P. Fisk, LCGC, 18(10), 2000: pp.1162.
54. The United States Pharmacopeia 25/National Formulary 20, Ch.

1225, pp. 2256–2259. (The United States Pharmacopeial Convention, Inc., Rockville, Maryland, 2002).

55. ICH Q2B: Validation of Analytical Procedures: Methodology (International Conference on Harmonization of Technical Requirements for the Registration of Drugs For Human Use, Geneva, Switzerland, May 1997).
56. M.V. Gorenstein, J.B. Li, J. Van Antwerp, and D. Chapman, LCGC 12 (10), 1994: pp. 768–772.
57. P.M. Young and M.V. Gorenstein, LCGC 12(11), 1994: pp. 832–838.
58. W.J. Warren, W.A. Stanick, M.V. Gorenstein, and P.M. Young, BioTechniques 18(2), 1995: pp. 282–297.
59. International Conference on Harmonization, “Text on Validation of Analytical Procedures”, Q2A, October 1994.
60. Patrick Lukulay, Gerard Hokanson Reconcilling Mass Balance in Forced Degradation studies. Pharma Technol October 2005.
61. Kirschbaum JJ. Synergistic use of multiple assays and achievement of mass balance to validate analytical methods. Trends Anal Chem 7, 1988: pp. 16-20.
62. International Conference on Harmonization Tripartite Guideline: Stability Testing of New Drug Substances and Products, ICH Q1A, September, 1994.
63. Riley CM, Rosanske TW, eds. Development and validation of analytical methods. In: Prog Pharm Biomed Anal. Pergamon Press, 1996.

64. Priestner AA. Use of radiolabeled drug substance to investigate mass balance during validation of a high-performance liquid chromatographic method for impurities. Anal Proc, 30, 1993: pp. 374-377.

65. Conner KA, Amidon GL, Stella VJ. In: Chemical stability of Pharmaceuticals – A Handbook for Pharmacists. 2nd ed. New York: John Wiley and Sons, 1986.