Chapter 1

General Introduction
Driven by chemistry but increasingly guided by pharmacology and clinical sciences, drug research has contributed more to the progress of medicine during the past century than any other scientific factor. Chemistry, pharmacology, microbiology and the biochemistry helped shape the course of drug discovery and bring to a level where new drugs are no longer generated solely by the imagination of chemists but result from a dialogue between biologists and chemists. This dialogue, centered on biochemical mechanism of action, stems from the understanding of biological structure and function and gives rise to the creation of novel chemical structures.

Drug development begins with the discovery of a drug molecule that has demonstrated therapeutic value to control, combat, prevent or cure diseases. The synthesis and characterization of such drug molecules i.e. active pharmaceutical ingredients (APIs) and their testing to generate preliminary safety and therapeutic efficacy data are prerequisites to identification of drug candidates for further detailed investigations. The preclinical or early drug development stage where these preliminary data are generated usually includes single and repeat-dose animal toxicology studies.

Prior to embarking on major investigations on the drug candidate, it is prudent to understand the physical and chemical properties of API (preformulation studies) and their impact on dosage form selection and design, impurity profiles of the API, and the stability of the drug molecule. Quantification of the impurities and identification of those impurities above the established threshold is essential to evaluate the toxicity profiles of these impurities to distinguish these from that of the API, when applicable. Also critical is the information on stability of the API by itself and in formulated material to establish shelf life (expiration dates) of the API by itself and in its formulated state so that the identity, purity, and strength of the API can be
assured during all phases of clinical trials and data from human clinical studies are reliable for the evaluation of toxicity and efficacy. The investment made on early developing and validating analytical methods for their robustness can minimize the need for further revision and solidify further progress in drug development.

Specific dosage form-related investigations should translate the information available on physicochemical properties of the drug substance to the formulation of the product. The main emphasis is on understanding the properties of the API and resulting interaction or compatibility with the excipients to achieve the desired formulation that produces a consistently high quality drug product during manufacturing. For solid dosage forms, crystal properties, solubility, particle size, wettability, flow and other information should be considered for optimization of mixing, granulation, and dissolution. Stability of the drug substance and retention of the crystal properties of the active drug substance during various phases of the manufacturing process will require investigation, as well as the blend and dosage form content uniformity.

For semisolids, such as creams and ointments, similar consideration of solubility, stability, and preservative effectiveness would apply. For liquid dosage form, solubility and stability will be important consideration for oral liquids, and in addition, viscosity for suspensions. For antibacterial liquid dosage form preservative stability and effectiveness should also be considered. For liquid dosage forms with ophthalmic and opticules, the method of sterilization and its effect on stability of the API and excipients assume special significance for inhalation products, control of particle size of delivered medication (which should be < 5μ) is a significant component of preformulation development. Inhalation aerosols should be formulated
sterile. Therefore, the effect of the method of sterilization on the stability of excipients and API should be an important consideration.

Assurance of sterility for parenteral product is critical, and the effect of the method of sterilization on the excipient, drug substance, and preservative (when applicable) stability requires investigation. For dosage form where preservatives are used, in addition to chemical studies, antimicrobial properties of the preservatives should be investigated to assure the preservative effectiveness. Compendial tests (antimicrobial preservative effectiveness tests, microbial limits test and sterility test, and biological assay test for antibiotics) appropriate to as specific dosage form should be tested to evaluate the microbiological component during preformulation studies [1].

**Origin of impurities**

Impurities in the drugs are originated from various sources and phases of synthetic process and preparation of pharmaceutical dosage forms. A sharp difference between the process-related impurities and degradation products is always not possible. However, majority of the impurities are characteristic of synthetic route of the manufacturing process. Since there are several possibilities of synthesizing a drug, it is possible that the same product of different sources may give rise different impurities. For example, in some of the recent papers, the impurities profiles of orbofiban [2] trimethoprim [3], and fluoxetine HCl [4] originated from different sources have been described. Generally the origin of impurities could be from any of the following steps during synthesis.

- Last intermediate of synthesis
- Products of incomplete reaction during the synthesis
- Products of over reaction
• Impurities in the starting materials of the synthesis
• Impurities originating from the solvents of the reaction
• Impurities originating from the catalysts
• Products of the side-reactions
• Degradation product as impurities
• Enantiomeric impurities
• Residual solvents
• Inorganic impurities
• Impurities in excipients
• Polymorphs as impurities

Types of impurities

Impurities associated with the APIs are classified into three groups for regulatory purposes as organic, inorganic and residual solvents [5].

• Organic impurities may arise from the starting material, (most often form the isomeric impurities) synthetic intermediate (incomplete reaction or excess reagent used) byproducts, degradation products, reagents, ligands, and catalysts. The reagents, ligands and catalyst are less commonly found in APIs, in some cases, they may pose a problem as impurities.

• Inorganic impurities present in pharmaceutical products originate from the equipments used and from reagents, catalysts, heavy metals, drying agents and filter aids. The main sources of impurity of heavy metals are the water used in the processes and the reactors (if stainless steel reactors are used), where acidification or acid hydrolysis takes place. These impurities of heavy metals can easily be avoided using demineralized water and glass-lined reactors.
Residual solvents and other volatile impurities must be detected and assayed not only because of their potential toxicity and deleterious environmental effects but also because they can impart undesirable organoleptic characteristics to drugs. Since residual solvents arise in excipients and occasionally in the manufacturing of drug products. According to International Conference on Harmonization (ICH) guidelines, residual solvents can be grouped into three categories based on the possible risk to human health [5]. Category I includes solvents such as benzene (2ppm limit) and carbon tetrachloride (4 ppm limit). The solvents belonging to category II are methylene chloride (600 ppm limit), methanol (3,000 ppm limit) pyridine (200 ppm limit), toluene (890 ppm limit), N, N-dimethyl formamide (880 ppm limit), and acetonitrile (410 ppm limit). The solvents of the category II are most commonly used during the manufacturing process. Acetic acid, acetone, isopropyl alcohol, butanol, ethanol, and ethyl acetate are solvents of category III. The solvents have higher tolerance limits. ICH guidelines [6] have recommended daily exposure of 50 mg or less per day.

Regulatory aspect

Control is more important today than ever. Until the beginning of the 20th century, drug products were produced and sold having no imposed control. Quality was generally poor. Many products were patent medicines of dubious value. Some were harmful and addictive. In 1937, ethylene glycol was used as a vehicle for an elixir of sulphanilamide, which caused more than 100 deaths [7]. Thereupon the Food, Drug and Cosmetic act was revised requiring advance proof of safety and various other controls for new drugs. The impurities to be considered for new drugs are listed in regulatory document of the Food and Drug Administration (FDA) [8], International Conference on the Harmonization of the Technical Requirements for Registration of
Pharmaceuticals for Human Use (ICH) [9] and the United States Pharmacopoeia (USP) [10]. Nevertheless, there are many drugs in existence, which have not been studied in such detail. The USP and National Formulary (NF) are the recognized standards for potency and purity of new drugs. These compendia have become official upon adoption of the first food and drug act. They formulate legal standards of quality, purity and strength of new drugs. The good manufacturing practices provide minimum quality standards for production of pharmaceuticals as well as their ingredients [11]. The ICH, which took place in Yokohama, Japan in 1995, has released new guidelines on impurities in new drug products [12]. These guidelines have a number of advantages, both for the industry and the regulators. The most critical aspect of the elaboration of the guidelines was the definition of the levels of impurities for the identification and quantification (Table 1.1). Quantification is the process of acquiring and evaluating data for establishing the biological safety of an individual impurity or a given impurity profile at the levels specified. The level of any impurity present in a new drug substance that has been adequately tested in safety and clinical studies is considered qualified. A rationale for selecting impurity limits based on safety consideration has to be provided. The ‘Decision Tree for Safety Studies’ (Fig. 1.1) describes consideration for the qualification of impurities when threshold are exceeded. This has some consequence for method development. Analytical procedures should be able to separate all the impurities from each other and the method should be optimized to separate and quantify them in the dosage forms.

**Stability studies**

The procedure for conducting stability storage studies and testing is provided in several ICH [13-16] and FDA guidance [17] documents. The objective of stability
<table>
<thead>
<tr>
<th>Dose</th>
<th>Threshold for Identification (%)</th>
<th>Qualification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1mg</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1-10 mg</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>10-100 mg</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>100-2 g</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>&gt;2 g</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Decrease degradation product level below threshold

Yes

Above Threshold

No

Qualified

Yes

Structure elucidated?

No

Yes

Toxicity documented and sufficient?

No

Related to others with known toxicity?

Yes

Acceptable justification?

No

Qualified

Yes

Consider patient population and duration of use

Consider need for:
1. Genotoxicity studies (point mutation chromosomal aberration)
2. General toxicity studies (one species, min. 14 days, max. 90 days)
3. Other specific toxicity endpoint, as appropriate

Adverse Effects

Yes

Consider additional testing or removal / lowering level of degradation product

No

Qualified

Fig. 1.1 The ‘Decision Tree For Safety Studies’
testing is to provide evidence of how the quality of a drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light. This information is used to establish container closure specifications, storage conditions, retest periods, and shelf lives (expiration dates) for drug substance and drug product. The ICH guidelines also provide information on photostability testing of drug substance and drug products [14], stability testing of new dosage forms [15], and ways to minimize the number of samples stored and tested by bracketing and matrixing [16].

Analytical support to stability programmes begins with the stability evaluation of drug substance at the preformulation and preclinical stages of drug development. Information on the intrinsic stability of an API should be integral part of stability evaluation of the API and the drug product. To understand the intrinsic stability of the API, forced degradation or stress studies are normally performed on the drug substance. Stress testing is usually carried out on a single batch of drug substance. ICH [13] recommends conducting forced degradation studies for temperature stresses in 10°C increments above the accelerated storage temperature condition of 40°C (50°C, 60°C, etc.), at a relative humidity of 75 % or greater. In addition, stresses such as photodegradation, oxidation, hydrolysis across wide pH ranges (acid and base hydrolysis) are recommended, the data generated will help develop stability – indicating method that can unequivocally separate the API from others and help elucidate the pathways of degradation of API.

A stability-indicating method is required for the evaluation of samples from long-term and accelerated storage stability studies and can also be used for assay and impurity profiling in drug substance and drug product, stability information will
help in developing early-on strategies for formulation, packaging, and storing of the API and the drug product (e.g., light resistant packaging). The data from long term (25°C ± 2°C and 60% ± 5% relative humidity (RH) for 12 months) and accelerated (40°C ± 2°C and 75% ± 5% RH for 6 months) stability studies are necessary in defining storage conditions and establishing expiration dating for drug substance or drug product [13, 17] APIs and drug products may be assessed at higher temperatures, especially if there is a potential for exposure to uncontrolled environments such as warehouse or shipping conditions.

Ahuja [18] and Gorog [19] have published books covering different aspects of impurities, including the government regulations and guidelines and identification and monitoring of impurities found in drug products. A recent publication covered the importance of impurity analysis in pharmaceutical products [20]. A number of recent articles [21-26] have described the analysis of various drugs and their impurities using HPLC-electrospray tandem mass spectrometry, GC/MS, LC, Capillary electrochromatography, LC-MS-MS, and HPLC.

**Specifications for post approval manufacture, release, and shelf Life**

ICH guidance Q6A [27] addresses specifications (test procedures and acceptance criteria) for drug substance and drug products. Specifications are part of a total quality control strategy, proposed and justified by the applicants and approved by regulatory authorities to assure product quality and consistency of the drug substance and drug product during manufacture and through its shelf life. Release specification should be influenced by the stability profile of the drug product and statistical bounds for release established through assessment of the validated process. When tested according to the analytical test procedure listed in the application, the API, the excipients, and the drug product should all meet the listed acceptance
criteria in order to be released. The regulatory acceptance criteria for drug products are generally the same from release throughout shelf life. As stated in ICH guidance, the concept of different acceptance criteria for release versus shelf life specification applies to drug products only [27]. The following is a summary of specification for drug substance and drug product for product release and shelf life evaluation (Fig. 1.2).

Assay methods

From the beginning of official pharmaceutical analysis the aim of including assay methods in compendial monographs has been to characterize the quality of bulk drug materials by setting limits of their active ingredient content. In recent years, the assay methods included in compendial monographs are titrimetry, spectrometry, chromatography, capillary electrophoresis and electro analytical methods. The present state-of-the-art is reflected by the data in the Table 1.2 based on the recent edition of European [28] and US [29] pharmacopoeias.

Titrimetric methods

It is apparent from Table 1.2 that in majority of cases titrimetric methods are still used. Signs of some modernization are the spreading of non-aqueous titration method expanding the field of application of titrimetric methods to (very) weak acids and bases as well as potentiometric end point detection improving the precision of the methods. Advantages of these methods are saving time and labour, high precision and the fact that there is no need of using reference standards. Recently titrimetric methods have been used for the determination of captopril
Analytical Testing for Marketed Product

Drug Substance

Universal Tests
- Description—solid, liquid, color
- Identification—IR, HPLC-UV
- Dissolution—UV spectrophotometry
- Specific stability—induction period or stress testing
- Impurities—assay method

Specific Tests
- Polymorphic transition
- Enantiomeric excess
- Hygroscopicity
- Water content
- Inorganic impurities
- Microbial tests

Drug Product

Universal Tests
- Description—size, shape, color
- Identification—UV spectrophotometry
- Impurities—stability

Specific Tests
- Chirality

Dosage-Form Specific

Solid Oral Drug Product
- Tablets, Capsules, and granules
- Dissolution—single point measurement
- Disintegration— dissolution
- Hardness/Fracture
- Uniformity of Dosage Units—Weight of individual dosage units
- Microbial Limits—Excipients

Liquid Oral Drug Product
- Uniformity of dosage units
- Microbial Limits—Excipients

Parenteral Drug Products
- Uniformity of dosage units
- Microbial Limits—Excipients

Fig. 1.2: Analytical testing for marketed products
Table 1.2: Proportion of various analytical methods prescribed for the assay of bulk drug materials in Ph. Eur. 4 [28] and USP XXVII [29]

<table>
<thead>
<tr>
<th>Method</th>
<th>Ph. Eur. 4 (%)</th>
<th>USP 27 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>15.5%</td>
<td>44%</td>
</tr>
<tr>
<td>GC</td>
<td>2%</td>
<td>2.5%</td>
</tr>
<tr>
<td>Titration</td>
<td>69.5%</td>
<td>40.5%</td>
</tr>
<tr>
<td>Acid-base</td>
<td>57.5%</td>
<td>29.5%</td>
</tr>
<tr>
<td>Aqueous mixtures</td>
<td>21%</td>
<td>5.5%</td>
</tr>
<tr>
<td>Indicator</td>
<td>6.5%</td>
<td>4.5%</td>
</tr>
<tr>
<td>Potentiometric</td>
<td>14.5%</td>
<td>1%</td>
</tr>
<tr>
<td>Non-aqueous</td>
<td>36.5%</td>
<td>24%</td>
</tr>
<tr>
<td>Indicator</td>
<td>9.5%</td>
<td>14%</td>
</tr>
<tr>
<td>Potentiometric</td>
<td>27%</td>
<td>10%</td>
</tr>
<tr>
<td>Redox (Iodometry, Nitritometry, etc.)</td>
<td>6.5%</td>
<td>5.5%</td>
</tr>
<tr>
<td>Other (complexometry, argentometry, etc.)</td>
<td>5.5%</td>
<td>5.5%</td>
</tr>
<tr>
<td>UV-vis spectrophotometry</td>
<td>9.5%</td>
<td>8.5%</td>
</tr>
<tr>
<td>Microbiological assay (antibiotics)</td>
<td>3%</td>
<td>2.5%</td>
</tr>
<tr>
<td>Other (IR, NMR, polarimetry, fluorimetry, atomic absorption spectroscopy, polarography, gravimetry etc.)</td>
<td>0.5%</td>
<td>2%</td>
</tr>
</tbody>
</table>
[30], albendazole [31] and gatifloxacin [32] in commercial dosage forms. Sparfloxacin [33] was determination by non-aqueous titration method.

**Chromatographic methods**

HPLC methods appeared for the first time for the assay of bulk drug materials in 1980 [34]. As seen in Table 1.2, this has become the predominant method in USP XXVII [29] and — although to a lesser extent— it is one of the most widely used methods also in Ph. Eur. 4 [28].

The specificity of this method is excellent and at the same time sufficient precision is also attainable. However, it has to be mentioned that the high specificity, precision and accuracy are attainable only if lengthy system suitability tests are carried out prior to the HPLC assay. For the reason the price to be paid for the high specificity, precision and accuracy is also high. **Fig. 1.3** shows the pie diagram indicating the percent usage of various chromatographic techniques from which it becomes very clear that HPLC has been the main technique used for analysis of impurities in drugs. The choice of proper detection mode is crucial to ensure that all the components are detected. With UV detection, this problem could be overcome by using a multiple wavelength scanning programme which is capable of monitoring several wavelengths simultaneously. It provides assurance that all the UV-absorbing components are detected, if present in sufficient quantity. Photodiode-array detectors are useful in determining the purity of enantiomeric drugs by HPLC. Most workers used the reversed-phase mode with UV absorbance detection whenever appropriate, because this provided the best available reliability, analysis time, repeatability and sensitivity. In fact, this technique has set the standard against which others are
Fig. 1.3: Pattern of use of different chromatographic techniques employed for analysis of impurities in drugs
compared (Fig. 1.4.) Several drugs have been assayed in pharmaceutical formulations [35 - 39] and in biological fluids [40 - 42] using HPLC. Thus, HPLC provides a major service in answering many questions posed by pharmaceutical industry. However, the limitations of HPLC include the cost of columns, solvents and a lack of long term reproducibility due to the proprietary nature of column packing. Liquid chromatography combined with mass spectrometry (LC-MS) is considered as one of the most important techniques of the last decade of 20th century [43]. It became the method-of-choice for analytical support in many stages of quality control and assurance within the pharmaceutical industry [44,45]. Recently HPLC-MS has been used for assay of drugs [46 - 51].

Gas chromatography is a dynamic method for separation and detection of volatile organic compounds. Gas liquid chromatography commands a significant role in the analysis of pharmaceutical product [52]. The advent of high-molecular weight products such as polypeptides, or thermally unstable antibiotics limits the scope of this technique. Its principal limitation rests in the relative non-volatility of the drug substances. Therefore, derivatization is virtually mandatory, but the techniques for producing volatile derivatives of drugs are legion. Recently, gas chromatography has been used for assay of drugs such as isotretinoin [53], fluoxetine, fluvoxamine, clomipramine [54] and nicotine [55].

Capillary electrophoresis (CE) is a relatively new analytical technique based on the separation of charged analytes through a small capillary under the influence of an electric field. In this technique solutes are seen as peaks as they pass through the detector and the area of each peak is proportional to their concentration,
Fig. 1.4: Usage of different detectors for HPLC analysis of drugs
which allows quantitative determinations. CE separations are generally more efficient, can be performed on a faster time scale, require only nanoliter injection volumes, and in most cases, take place under aqueous conditions. These four characteristics of CE have proven to be advantageous for many pharmaceutical applications. Several reports have appeared on the application of this technique in the routine drug analysis [56 - 58]. Different modes of capillary electrophoresis such as capillary zone electrophoresis [59 - 63], miscellar electrokinetic chromatography [64-66] isotachophoresis [67,68], capillary gel electrophoresis [69,70] isoelectric focusing [71,72] and affinity capillary electrophoresis [73,74] have been developed and applied for pharmaceutical purity testing and in bioanalysis of drugs.

Thin layer chromatography is a popular technique for the analysis of a wide variety of organic and inorganic substances, because of its distinct advantages such as minimal sample clean-up, wide choice of mobile phases, flexibility in sample distinction, high sample loading capacity and low cost. TLC is a powerful tool for screening unknown materials in bulk drugs [75]. It provides a relatively high degree of assurance that all possible components of the drug are separated. The high specificity of TLC has been exploited to quantitative analytical purpose using spot elution followed by spectrophotometric measurement. The TLC method is also prescribed in USP XX VII e.g. as “single- steroid Assay (511)” [29]. TLC has been utilized for the determination of some steroids [76], pioglitazone [77], celecoxib[78] and quinapril and hydrochlorothiazide in combination tablets[79].

High performance thin layer chromatography (HPTLC) is a fast separation technique and flexible enough to analyze different kind of samples. This technique is advantageous in many ways as it is simple to handle and requires short analysis time to analyze the complex or the crude sample cleanup. HPTLC evaluates the entire
chromatogram with a wide variety of techniques and parameters without time constrains. Moreover, there is simultaneous but independent development of multiple sample and standards on each plate, leading to an increased reliability of results. HPTLC has been used to quantitate drugs as ethinloestradiol and cyproterone [80], hydrochlorothiazide [81] and vitamin C and dipyrone [82].

Laboratory automation was initiated in the second half of the XX century. Steward in the U.S. as well as Ruzicka and Hansen in Denmark, created the flow injection analysis (FIA) technique for the automation of chemical procedure [83,84]. The introduction of this technique came to revolutionise the concept of automation in chemical analysis by allowing instrumental measurement to be carried out in the absence of physical equilibrium (without homogenization of sample and carrier / reagent) and chemical equilibrium (without completing the reactions) [85 - 88].

FIA has got some clear advantages over conventional techniques:

- Fast determination and on-line sample pre-treatment permitting almost real-time monitoring.
- High sampling frequency allowing high resolution of the dissolution processes.
- Low sample consumption, introducing minimum disturbance to the volume of dissolution medium, particularly when high sampling frequency is involved.
- Parallel testing in multivessels using a single detector.
- Simultaneous determination of multicomponents in dosage form.
- High stable sample pre-treatment and detection capable of continuous operation over extended periods
- Continuous baseline monitoring and on-line recalibration of detection system.
- Low reagent consumption.
Following the general application of computers in routine laboratory a second generation of flow analysis was proposed by Ruzicka and Marshall in 1990 designated sequential injection analysis (SIA) [89]. As with the FIA, this is a non-segmented continuous flow technique based on the same principle of controlled dispersion and reproducible manipulation of the FIA concept, but whose mode of functioning is based on the concept of programmable flow.

The FIA technique has lent an important contribution to the development of automation in pharmaceutical analysis and its advantages are well documented in several review articles [90 - 95] as well as in a specialized monograph [96].

The introduction of SIA has awakened the interest of the scientific community for automation in the pharmaceutical area [97]. Many articles dedicated to pharmaceutical analysis have been published, including two review articles [98,99], applying sequential injection analysis to a wide variety of matrices, such as solid matrices (tablets, capsules), pastes (ointments, creams), liquids (emulsions, suspensions, solutions) and covering various active ingredients with different therapeutic activities. By benefiting from the advantages in the economy of reagents and the elevated sampling rates, the majority of the applications are dedicated to the determination of active ingredients for quality control in pharmaceutical formulations.

**UV-Visible spectrophotometry**

Another group of methods in pharmacopoeias are spectrophotometric methods based on natural UV absorption and chemical reactions [100]. The advantages of these methods are low time and labour consumption. The precision of these methods is also excellent. The use of UV-VIS Spectrophotometry especially applied in the analysis of pharmaceutical dosage form has increased rapidly over the
last few years [101 - 104]. The colorimetric methods are usually based on the following aspects:

- Complex-formation reaction
- Oxidation-reduction process
- A catalytic effect

It is worth mentioning that colorimetric methods are frequently used for the assay of bulk materials. For example, the blue tetrazolium assay is used for the determination of corticosteroid drug formulations [105,106] and is included in the recent edition of US Pharmacopoeia ((351) “Assay for steroids” [29]). The colorimetric method is also utilized for the determination of cardiac glycosides and is presented in recent edition of European Pharmacopoeia [28]. Several approaches using spectrophotometry for determination of active pharmaceutical ingredients in bulk drug and formulations have been reported and details of these methods are recorded in Table 1.3 [107 - 150].
<table>
<thead>
<tr>
<th>Name of drug</th>
<th>Reagents used</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetoaminophen</td>
<td>m-Cresol</td>
<td>640</td>
<td>107</td>
</tr>
<tr>
<td>Amiodarone HCl</td>
<td>p-Chloranilic acid</td>
<td>535</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>2,3-Dichloro 5,6-dicyano</td>
<td>575</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>1,4-benzoquinone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amlodipine besylate</td>
<td>p-Chloranilic acid</td>
<td>540</td>
<td>109</td>
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<tr>
<td></td>
<td>Ninhydrin in DMF medium</td>
<td>595</td>
<td>110</td>
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<tr>
<td></td>
<td>2,3-Dichloro 5,6-dicyano</td>
<td>580</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>1,4-benzoquinone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
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<td>KIO$_3$</td>
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<td>Ampicillin, amoxycillin &amp;ampicillin &amp; carbenicillin</td>
<td>Folin ciocalteau phenol &amp; 750</td>
<td>750,770</td>
<td>113</td>
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<td>Ascorbic acid</td>
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<td>Tris buffer</td>
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<td>Diltiazem HCl</td>
<td>Sodium metavanadate</td>
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<td></td>
<td>Bromothymol blue</td>
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<td>118</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue</td>
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<td>118</td>
</tr>
<tr>
<td>Substance</td>
<td>Reactant</td>
<td>λ (nm)</td>
<td>n (%)</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------------------------------------</td>
<td>--------</td>
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<td><strong>KMnO₄ in alkaline medium</strong></td>
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<tr>
<td></td>
<td><strong>p-Chloranilic acid</strong></td>
<td>525</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td><strong>Ninhydrin</strong></td>
<td>595</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td><strong>Ascorbic acid</strong></td>
<td>530</td>
<td>124</td>
</tr>
<tr>
<td>Labetalol HCl</td>
<td>Sodium nitroprusside &amp; hydroxylamine hydrochloride</td>
<td>695</td>
<td>125</td>
</tr>
<tr>
<td>Losartan potassium</td>
<td><strong>KMnO₄ in alkaline medium</strong></td>
<td>603</td>
<td>126</td>
</tr>
<tr>
<td>Levodopa</td>
<td><strong>Ce(IV) nitrate in H₂SO₄ medium</strong></td>
<td>510</td>
<td>127</td>
</tr>
<tr>
<td>Metyldopa</td>
<td><strong>Ce(IV) nitrate in H₂SO₄ medium</strong></td>
<td>550</td>
<td>127</td>
</tr>
<tr>
<td>L-dopa</td>
<td><strong>NaOH</strong></td>
<td>300</td>
<td>128</td>
</tr>
<tr>
<td>Menadione</td>
<td><strong>NaOH in the presence of amine</strong></td>
<td>450</td>
<td>129</td>
</tr>
<tr>
<td>Metoprolol tartrate</td>
<td><strong>KMnO₄ in alkaline medium</strong></td>
<td>610</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td><strong>Ninhydrin</strong></td>
<td>595</td>
<td>131</td>
</tr>
<tr>
<td>Mometasone furoate</td>
<td><strong>Methanol</strong></td>
<td>248</td>
<td>134</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Persulphate in alkaline medium</td>
<td>320, 390</td>
<td>133</td>
</tr>
</tbody>
</table>

- **Bromocresol green**: 415 nm, 118 n (%)
- **KMnO₄ in alkaline medium**: 610 nm, 119 n (%)
- **Ninhydrin**: 590 nm, 120 n (%)
- **Iodine**: 295, 355 nm, 121 n (%)
- **Potassium iodate and iodide in aqueous medium**: 352 nm, 122 n (%)
- **7,7,8,8-Tetracyanoquinodimethane**: 743 nm, 123 n (%)
- **p-Chloranilic acid**: 525 nm, 123 n (%)
- **Ninhydrin**: 595 nm, 124 n (%)
- **Ascorbic acid**: 530 nm, 124 n (%)
- **Sodium nitroprusside & hydroxylamine hydrochloride**: 695 nm, 125 n (%)
- **KMnO₄ in alkaline medium**: 603 nm, 126 n (%)
- **Ce(IV) nitrate in H₂SO₄ medium**: 510 nm, 127 n (%)
- **Ce(IV) nitrate in H₂SO₄ medium**: 550 nm, 127 n (%)
- **NaOH**: 300 nm, 128 n (%)
- **NaOH in the presence of amine**: 450 nm, 129 n (%)
- **KMnO₄ in alkaline medium**: 610 nm, 130 n (%)
- **Ninhydrin**: 595 nm, 131 n (%)
- **Methanol**: 248 nm, 134 n (%)
- **Persulphate in alkaline medium**: 320, 390 nm, 133 n (%)
<table>
<thead>
<tr>
<th>Drug</th>
<th>Reagents/Reactions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicorandil</td>
<td>Brucine-sulphanilic acid in H$_2$SO$_4$ medium</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>3-Methyl-2-benzothiazoline hydrazone HCl-metol</td>
<td>134</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>KMnO$_4$ in neutral medium</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>4-Methyl amino phenol and K$_2$Cr$_2$O$_7$</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Bromocresol green</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Bromothymol blue</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Erichrome Black T</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>KOH in dimethylsulphoxide</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>Ammonium molybdate</td>
<td>138</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>KMnO$_4$ in alkaline medium</td>
<td>139</td>
</tr>
<tr>
<td>Carboxamine</td>
<td>Cu (II) &amp; eosin</td>
<td>140</td>
</tr>
<tr>
<td>Pantoprazole sodium</td>
<td>Potassium ferricyanide and ammonium ferric sulphate</td>
<td>141</td>
</tr>
<tr>
<td>Perindopril erbumine</td>
<td>1-Chloro-2,4-dinitrobenzene in dimethyl sulphoxide</td>
<td>142</td>
</tr>
<tr>
<td>Ramipril</td>
<td>Potassium iodate and potassium iodide in aqueous medium</td>
<td>143</td>
</tr>
<tr>
<td>Silymarin</td>
<td>KMnO$_4$ in neutral medium</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>3-Methyl-2-benzothiazoline hydrazone &amp; potassium persulphate</td>
<td>145</td>
</tr>
<tr>
<td>Compound</td>
<td>Reaction/Compound</td>
<td>Mol. Wt.</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Persulphate in alkaline medium</td>
<td>355</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>Nitrous acid</td>
<td>420</td>
</tr>
<tr>
<td>Verapamil HCl</td>
<td>Chloramine T</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td>N-Bromosuccinimide</td>
<td>415</td>
</tr>
<tr>
<td></td>
<td>Potassium metaperiodate</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td>Tropaeolin 000 No.1</td>
<td>400</td>
</tr>
</tbody>
</table>
The derivative method has found applications not only in UV-Spectrophotometry but also in infrared [151], atomic absorption, flame spectrophotometry [152,153], and fluorimetry [154,155]. The use of derivative spectrometry is not restricted to special cases, but may be of advantage whenever quantitative study of normal spectra is difficult. Its disadvantage is that the differential degrades the signal-to-noise ratio, so that some form of smoothing is required in conjunction with differentiation [156].

For a single-peak spectrum, the first-derivative is a plot of gradient $\frac{dA}{d\lambda}$ of the absorption envelop versus wavelength and features maximum and minimum; the vertical distance between these is the amplitude, which is proportional to the analyte concentration; theoretically, $\frac{dA}{d\lambda}$ is zero at $\lambda_{\text{max}}$ for the band in the normal spectrum. The second-derivative spectrum, $\frac{d^2 A}{d\lambda^2}$ versus wavelength, has two maxima with a minimum between them, at $\lambda_{\text{max}}$ of the normal absorption band [157]. In principle, both peak-heights (measured from $\frac{d^2 A}{d\lambda^2} = 0$) are proportional to the analyte concentration but the amplitude can also be measured by the so-called tangent method, in which a tangent is drawn to the maxima and amplitude is measured vertically from tangent to the minimum [158]. The differentiation discriminated against broad bands, emphasizing sharper feature to an extent that increases with increasing derivative order, because for bands (Gaussian or Lorentzian) the amplitude $D_n$ of the $n$th derivative is related to the $n^{th}$ power of the inverse of the band width, $W$, of the normal spectrum [159]:

$$D_n \propto (1/W)^n$$

Thus, for two bands A and B of equal absorbance but different width, the derivative amplitude of the sharper band (A, for example) is greater than that of
the broader (B) by a factor that increases with increasing derivative order [160,161]:

\[ D_n / D_{n-1} \propto (W_B / W_A)^n \]

For the reason, the use of derivative spectra can increase the detection sensitivity [162-164] of minor spectral features. For quantitative analysis, if Beer’s law is obeyed for normal spectrum, the following equation can be obtained:

\[ d^n A / d \lambda^n = d^n \epsilon / d \lambda^n \cdot lC \]

Where \( A = \) Absorbance, \( \epsilon = \) molar absorptivity, \( l = \) cell path-length and \( C = \) concentration of the analyte and this forms the basis of analytical determinations [165]. Derivative spectrophotometry has been applied for quantification of drugs in pharmaceutical preparations [166-169].

Near infrared spectroscopy (NIRS) is a fast and non-destructive technique that provides multi constituent analysis of virtually any matrix. In recent years, NIR spectroscopy has gained wide acceptance within the pharmaceutical industry for raw material testing, product quality control and process monitoring. The growing pharmaceutical interest in NIR spectroscopy is probably a direct result of its major advantages over other analytical techniques, namely, an easy sample preparation without any pretreatments, the possibility of separating the sample measurement position and spectrometer by use of fiber optic probes, and the prediction of chemical and physical sample parameters from one single spectrum. The major pharmacopoeias have generally adopted NIR techniques. The European [170] and United States pharmacopoeia [171] address the suitability of NIR instrumentation for use in pharmaceutical analysis. NIR spectroscopy combined with multivariate data analysis opens many interesting perspectives in pharmaceutical analysis, both qualitatively and quantitatively. Within the last 10 years the number of publications...
describing quantitative NIR measurements of active ingredient in intact tablets has increased tremendously [172-186].

Since the first report appeared in 1996 [187] describing the use of NMR spectroscopy to screen for potential drug molecules, the field of NMR based screening has evolved rapidly. Over the last few years, a variety of novel approaches have been introduced and found widespread application in both pharmaceutical and academic research settings. Recently NMR finds its application in quantitative analysis in order to determine the impurity profile of the drug [188], in the characterization of the composition of the drug products and in quantitation of drugs in pharmaceutical formulations and biological fluids [189,190].

A gradual increase in the number of articles on the application of Fluorimetry and Phosphorimetry in quantitative analysis of various drugs in dosage forms and biological fluids have been noticed in recent past [191-193].

The application of electrochemical techniques to the analysis of drugs and pharmaceuticals has increased greatly over the last few years. The renewed interest in electrochemical techniques can be attributed in part to more sophisticated instrumentation and to increased understanding of the technique themselves. Here the application of the various electrochemical modes in the analysis of drugs and pharmaceuticals are presented in Table 1.4 [194-207].
Table 1.4: Determination of drug by various electrochemical techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Drugs determined</th>
<th>Remark</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltammetry</td>
<td>Isoniazid</td>
<td>Using overoxidized poly pyrrole glassy carbon modified electrode</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>Danazole</td>
<td>Square wave adsorptive stripping voltammetry</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>Ethinylestradiol</td>
<td>Performed at a mercury electrode</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Lamotrigine</td>
<td>Experimental parameters optimized</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>Rabeprazole</td>
<td>At glassy carbon electrode</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>Clozapine</td>
<td>Performed at a mercury electrode</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>Atenalol</td>
<td>Using nanogold modified indium tin oxide electrode</td>
<td>200</td>
</tr>
<tr>
<td>Polarography</td>
<td>Artemether</td>
<td></td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>Josamycin</td>
<td></td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>Ciclopirox olamine</td>
<td></td>
<td>203</td>
</tr>
<tr>
<td>Amperometry</td>
<td>Salbutamol</td>
<td></td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>Verapamil</td>
<td></td>
<td>205</td>
</tr>
<tr>
<td>Potentiometry</td>
<td>Chlorpromazine</td>
<td></td>
<td>206</td>
</tr>
<tr>
<td></td>
<td>Clobutinol HCl</td>
<td></td>
<td>207</td>
</tr>
</tbody>
</table>
The field of kinetic method of analysis has been developing since the late 1950s. Although the field is now mature, it has recently been undergoing a major resurgence in activity. The renewed interest can be attributed to advances that have been made in principles, in automated instrumentation, in understanding chemical and instrumentation, in understanding chemical and instrumental systems, in data treatment methods, and in analytical application. Also, it is becoming clear that the kinetic approach to analytical chemistry is rather general with several advantages over traditional equilibrium approach [208 - 210]. Essentially, kinetic methods rely on the measurements of concentration changes (detected via signal changes) in a reactant (which may be the analyte itself) with time after the sample and reagents have been mixed manually or automatically. There are several approaches that can be used for the determination of single components in the absence of any kinetic interference (Table 1.5). However, fixed-time and initial rate methods have been used more frequently for the determination of drugs in pharmaceutical formulations and biological fluids [211-213]. Kinetic automatic techniques are generally based on open systems among the most popular of which are stopped flow system [214] and the continuous addition of reagent (CAR) technique [215,216]. Several drugs have been determined by using the CAR technique with photometric [217] and fluorimetric detection [218]. The use of catalysts to accelerate analytical reactions is feasible with both reaction rate and equilibrium determinations. In this concern, the use of micellar media in kinetic method is recently encouraged to enhance the rate of reaction (through micellar catalysis) and may additionally improve the sensitivity and selectivity which in turn reduce the analysis time for the analyte [219 - 221].
Table 1.5: Several kinetic approaches for determination of single component

<table>
<thead>
<tr>
<th>A. Direct-computation methods</th>
<th>B. Curve-fitting methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Integral methods</td>
<td>1. Integral methods</td>
</tr>
<tr>
<td>a. Fixed-time</td>
<td>a. Linear responses</td>
</tr>
<tr>
<td>i. One-point</td>
<td>b. Non-linear response</td>
</tr>
<tr>
<td>ii. Two-point</td>
<td>i. Direct computation</td>
</tr>
<tr>
<td>iii. Multipoint</td>
<td>ii. Predictive (extrapolation)</td>
</tr>
<tr>
<td>b. Variable-time</td>
<td>2. Rate methods</td>
</tr>
<tr>
<td>i. One-point</td>
<td></td>
</tr>
<tr>
<td>ii. Two-Point</td>
<td></td>
</tr>
<tr>
<td>2. Rate methods</td>
<td></td>
</tr>
<tr>
<td>a. Initial-rate</td>
<td></td>
</tr>
<tr>
<td>b. Intermediate-rate</td>
<td></td>
</tr>
<tr>
<td>3. Integrated-signal methods</td>
<td></td>
</tr>
</tbody>
</table>
Multicomponent kinetic determinations, often called as differential rate methods, are also receiving popularity in the field of pharmaceutical research [222,223]. Two new approaches i.e. kinetic wavelength pair method [224] and H-point standard addition method [225] have been proposed for dealing with overlapping spectra of components in the binary mixtures.

**METHOD VALIDATION**

Validation of analytical procedure is a vital aspect not just for regulatory purposes, but also for their reliable long-term application. However, a sensible validation is also essential from a business perspective because analytical data are the basis of many decisions such as batch release, establishment / verification of shelf life, etc.

ICH guidelines should be regarded as basis and philosophy of analytical validation, not as a check list. "It is the responsibility of the applicant to choose the validation procedure and protocol most suitable for their product" [226]. Suitability is strongly connected with the requirement and design of the given analytical procedure, which obviously varies and must, therefore, be reflected in the analytical validation. This includes the identification of the performance parameters relevant for the given procedure, the definition of appropriate acceptance criteria, and the appropriate design of validation studies. In order to achieve this, the analyst must be aware of the fundamental meaning of these performance parameters, calculations, and tests and their relationship to his specific application. A lack of knowledge or (perhaps) a wrong understanding of “efficiency” will lead to validation results that address the real performance of the analytical procedure only partly or insufficiently. The process for the development, validation and use of analytical method is shown in Fig. 1.5.
Calibration model, range and linearity

Define performance specifications

Devise development experiments

Execute and evaluate results

Plan method validation experiments

Precision and accuracy

Analyte stability

Collate results

Write validation report

Apply validated method

Fig. 1.5: The process of development, validation and routine use of an analytical method.
Several International Organizations and Regulatory Authorities are involved in fixing the criteria for the validations and are listed in Table 1.6. It has been agreed that the parameters for evaluation of method reliability and overall performances are:

- confirmation of identity
- solution stability
- selectivity/ specificity
- linearity
- accuracy and precision
- limits of detection and quantitation
- recovery
- robustness/ ruggedness
- equivalence testing

The overall validation strategy consists of four components, which are prevalidation [227,228], validation proper, study proper and statistical analysis.

Among all of the International Organizations, the ICH guidelines achieved a great deal in harmonizing the definitions of the required validation characteristics and their basic requirements. The International Conference on Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has harmonized the requirements in two guidelines [229,230].
Table 1.6 Validation of analytical methods: International definitions

<table>
<thead>
<tr>
<th>Organization</th>
<th>Applicability</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>IUPAC</td>
<td>Worldwide</td>
<td></td>
</tr>
<tr>
<td>ILAC</td>
<td>Worldwide</td>
<td></td>
</tr>
<tr>
<td>WELAC</td>
<td>Europe</td>
<td></td>
</tr>
<tr>
<td>ICH</td>
<td>Europe, Japan, USA</td>
<td>Only pharmaceutical products</td>
</tr>
<tr>
<td>ISO</td>
<td>Worldwide</td>
<td>Lack definition of selectivity and specificity</td>
</tr>
</tbody>
</table>

Abbreviations:
IUPAC, International Union of Pure and Applied Chemistry
ILAC, International Laboratory Accreditation Conference
WELAC, Western European Laboratory Accreditation Co-operation
ICH, International Conference on Harmonisation
ISO, International Organization for Standardization
The first one summarizes and defines the validation characteristics needed for various types of test procedure. The second one extends the previous test to include the experimental data required and some statistical interpretation. These guidelines serve as a basis worldwide both for regulatory authorities and industries and bring the importance of a proper validation to the attention of all those involved in the process submission. In order to fulfill the validation responsibilities properly, the background of the validation parameters and their consequences must be understood. The validation characteristics and their minimum number of determinations required if applicable are given in Table 1.7 [230].

**Confirmation of identity**

In general analytical methods consist of a measurement stage, which may be preceded by an isolation stage. It is necessary to establish that the signal or reaction produced at measurement stage is only due to the analyte and not due to something chemically or physically similar or arising as a coincidence. This is the confirmation of identity. Whether or not other compounds interfere with the measurement of the analyte will depend on the effectiveness of the isolation stage if it was part of the method, as well as the specificity / selectivity of the measurement stage.

**Selectivity / Specificity**

Selectivity of a method refers to the extent to which it can determine particular analyte(s) in a complex mixture without interference from other component in the mixture. The term selectivity and specificity have often been used interchangeably. The term specific generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that
Table 1.7: Validation characteristics normally evaluated for the different types of test procedure [229] and the minimum number of determinations required (if applicable) [230]

<table>
<thead>
<tr>
<th>Validation characteristics</th>
<th>Minimum number</th>
<th>Test procedure</th>
<th>Impurities</th>
<th>Assay&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Identity</td>
<td>Quantitative</td>
<td>Limit</td>
</tr>
<tr>
<td>Specificity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Linearity</td>
<td>5 concentrations</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Range</td>
<td>-</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Accuracy</td>
<td>9 determinations over 3 concentration levels (e.g. 3 × 3)</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Precision Repetability</td>
<td>6 determinations at 100% or 9 determinations over 3 concentration levels (e.g. 3 × 3)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Intermediate Precision/ reproducibility&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2-series</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Detection limit</td>
<td>-</td>
<td>No</td>
<td>No&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Yes</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>-</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup>Including dissolution, content potency

<sup>b</sup>Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure (s).

<sup>c</sup>Intermediate precision sufficient for submission.

<sup>d</sup>May be needed in some cases.
provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is set to be selective. Since very few analytical methods respond to only one analyte, the use of the terms selectivity is more appropriate than specificity. The International Union of Pure and Applied Chemistry (IUPAC) has expressed the view that "specificity is the ultimate of selectivity". The IUPAC discourages use of the term specificity and instead encourages the use of term selectivity.

The selectivity of the analytical method must be demonstrated by providing data to show the absence of interference with regard to degradation products, synthetic impurities and the matrix (excipients present in the formulated product at their expected levels).

**Linearity and calibration range**

Evaluation of the linearity of the relationship between the actual analyte concentration and the test result from the method, however, is required for quantitation testing for impurities, and for assay methods. The requirement for linearity is independent of the technology used to ascertain the analyte concentration. Many analytical methods are known where the relationship between the raw measured data and the analyte concentrations are non-linear. Electrochemical measurements, for example, rely on the Nernst equation, which indicates a logarithmic relationship between the cell voltage and the analyte concentration. Spectroscopic measurements rely on Beer's law, which also expresses a logarithmic relationship between the measured transmittance data and absorbance, absorbance being the quality that theoretically is proportional to the analyte concentration.
Using spectroscopy as the basis for the discussion here, we find that while Beer's law shows that the measured absorbance is proportional to concentration, in practice many sources of interference can occur which will cause deviation from the theory. For example, stray light will cause deviations from linearity at low transmittance levels, as will excessive bandwidth of the monochromator. Saturation of the detector or operation at too high a signal level will make the detector response become non-linear with respect to the optical energy, which will make the computed absorbance non-linear (with respect to concentration) at high energy levels (i.e. high transmittance of the sample). Other effects also exist.

"Linearity is defined in section 7 of ICH guidelines [231] as "The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample". The following quote is found in section III of [232]:

"In some cases, to obtain linearity between assays and sample concentrations, the test data may have to be subjected to a mathematical transformation prior to the regression analysis".

This quote clearly indicates that if raw data is not itself linearly related to the analyte concentrations, and then it may be made linear through a mathematical transformation. As indicated above, any suitable mathematical function may be used for the linearization process. The guidelines also contain the following passage (in section III of [232]):

"If there is linear relationship, test results should be evaluated by appropriate statistical methods, for example, by the calculation of a regression line by the method of least squares".
Fig. 1.6 illustrates the definition of the dynamic range of an analytical method, which extends from the lowest concentration at which quantitative measurements can be made (limit of quantitation or LOQ) to the concentration at which the calibration curve departs from linearity (limit of linearity). To be very useful, an analytical method should have a dynamic range of at least two orders of magnitude.

**Accuracy**

The ICH guideline recommends the demonstration of accuracy over the whole working range. However, if only a narrow range is required (e.g. assay or impurities with a low specification limit), a six fold determination at a 100 % test concentration as described for the precision studies may also be used.

Several approaches discussed in the ICH guidelines are given in Table 1.8.

If the analytical test to be validated is compared with another procedure or applied to a reference substance, the probably different specificities must be taken into account. Therefore, statistical tests should be performed only if the systematic bias based on these differences can be quantified and thus corrected or are negligible. Otherwise, the comparison should be performed as a qualitative verification of plausibility or an acceptable maximum difference should be defined (e.g. 2.0 % for an LC assay).

Spiking experiments for recovery investigation should be performed as closely to the authentic condition as possible so that possible interferences between the analyte and matrix can be recognized. This ranges, for example, from the direct preparation of the drug product with various contents of active ingredient to which the whole analytical procedure is applied to the addition of drug substance stock solution to a placebo solution.
Fig. 1.6. Useful range of an analytical method: LOQ = Limit of quantitation and LOL = Limit of linear response.
### Table 1.8: Quantitative approaches to demonstrate accuracy according to ICH [230]

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug Substance</td>
<td>Application of the analytical procedure to a reference material</td>
</tr>
<tr>
<td></td>
<td>Comparison of the results with those of a second, well characterised procedure</td>
</tr>
<tr>
<td>Drug Product</td>
<td>Application of the analytical procedure to synthetic mixtures of drug product components</td>
</tr>
<tr>
<td></td>
<td>Spiking of analyte to drug product</td>
</tr>
<tr>
<td></td>
<td>Comparison of the results with those of a second, well characterised procedure</td>
</tr>
<tr>
<td>Impurities (quantitative)</td>
<td>Spiking of the impurity to drug substance or product</td>
</tr>
<tr>
<td></td>
<td>Comparison of the results with those of a second, well characterised procedure</td>
</tr>
</tbody>
</table>
For the quantitation of the analyte, the same calibration mode as described in the final test procedure must be used. Again, statistical tests should be used carefully, especially with complex matrices and low concentrations of impurities. Alternatively, acceptable deviations from the theoretical recovery of 100% can be defined based on the application, experiences or general statistical considerations.

**Precision**

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of experiments obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogenous, authentic samples. However, if it is not possible to obtain a homogenous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of experiments.

**Repeatability**

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. Repeatability should be assessed using:

a) a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations / 3 replicates each); or

b) a minimum of 6 determinations at 100% of the test concentration.
Intermediate precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analyst, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not the part of marketing authorization dossier.

Robustness / Ruggedness

International Conference on Harmonization of technical requirement for human use (ICH) [233] defines “robustness/ruggedness” of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variation in method parameters and provides an indication of its reliability during normal usage [234]. Ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis like small changes of pH values, mobile phase composition, temperature etc. Full validation must not necessarily include ruggedness testing; it can, however, be very helpful during the method development / prevalidation phase, as problems that may occur during validation are often detected in advance. Ruggedness should be tested, if a method is supposed to be transferred to another laboratory [235].
Significance of testing

An important property of an analytical method is that it should be free from the systematic error (bias). Determining bias involves analyzing one or more standard reference materials whose analyte concentration is known. However, random errors make it unlikely that, the measured amount will equal the known amount even when no systematic errors are present. In order to decide whether the difference between the observed and standard values can be accounted for by random variation, a statistical test, i.e., a significant test is used for the interpretation of analytical data.

- **Student's t-test:** Here comparison is made between two sets of replicate measurements made by two different methods; one is the test method while other is accepted (reference method).

\[
\pm t = \frac{\bar{x}_1 - \bar{x}_2}{S_p} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}
\]

where,

\[\bar{x}_1 = \text{mean from the test method}\]

\[\bar{x}_2 = \text{mean from the accepted (reference) method}\]

\[n_1 \& n_2 = \text{number of measurements}\]

\[S_p = \text{pooled standard deviation of the individual measurements of two sets is given by}\]

\[S_p = \sqrt{\frac{\sum(x_1 - \bar{x}_1)^2 + (x_2 - \bar{x}_2)^2}{n_1 + n_2 - 2}}\]

A statistical t-value is calculated and compared with a tabulated value for the given number of tests at the desired confidence level. If \(t_{\text{cal}} > t_{\text{tab}}\) then there is
significant difference between the results obtained by the two methods at the given confidence level. But if, \( t_{\text{cal}} < t_{\text{tab}} \) then there is no significant difference between the methods. It is an accuracy-indicating test.

- **F-Test:** This test indicates whether there is a significant difference between the two methods (i.e. the new method and the accepted reference method). It can be represent as,

\[
F = \frac{S_1^2}{S_2^2}
\]

Where,

\( S_1 \) and \( S_2 \) = standard deviation of method 1 & 2 and \( S_1 > S_2 \)

The calculated value of \( 'F' \) is compared with the tabulated standard value at the selected confidence level and degree of freedom. If \( F_{\text{cal}} > F_{\text{tab}} \) then there is significant difference between the two methods. It is a precision indicating test.

**Interval hypothesis:**

For practical purposes, the acceptable bias can be calculated statistically [236].

For example, a test method (method 2) is considered acceptable if its true mean value is within ± 2.0 % of that of the reference method (method 1), i.e,

\[-0.02 \mu_1 < (\mu_2 - \mu_1) < 0.02 \mu_1\]

this can be written as

\[0.98 < \mu_2/\mu_1 < 1.02\]

which can be generalized to

\[\theta_L < \mu_2/\mu_1 < \theta_U\]
where $\theta_L$ and $\theta_U$ represents the lower and the upper acceptance limits, respectively when $\mu_2$ is expressed as the proportion of the reference mean $\mu_1$. Statistically, $\theta_L$ and $\theta_U$ can be calculated from the relation:

$$\theta^2\left(\frac{x_1^2 - S_{p}^2 t_{tab}^2}{n_1}\right) - 2\theta x_2 x_1 + \left(\frac{x_2^2 - S_{p}^2 t_{tab}^2}{n_2}\right) = 0$$

The lower limit $\theta_L$ and the upper limit $\theta_U$ of confidence interval are obtained as

$$\theta_L = \frac{-b - \sqrt{b^2 - 4ac}}{2a}$$

$$\theta_U = \frac{-b + \sqrt{b^2 - 4ac}}{2a}$$

where,

$$a = \frac{-2}{x_1 - \frac{S_{p}^2 t_{tab}^2}{n_1}}$$

$$b = -2x_1 x_2$$

$$c = \frac{-2}{x_2 - \frac{S_{p}^2 t_{tab}^2}{n_2}}$$

where $\bar{x}_1$ and $\bar{x}_2$ estimates of $\mu_1$ and $\mu_2$ based on $n_1$ and $n_2$ measurements respectively. $S_p^2$ is the variance of pooled measurements. $t_{tab}$ is the tabulated one sided $t$-value, with $n_1+n_2-2$ degrees of freedom at the specified level of significance.

**Testing for outliers**

When a series of replicate measurements of same quantity are made, one of the results will appear too different markedly from other. There is then a great temptation to discard this “outliers” before calculating the mean and the standard deviation of the data or applying statistical tests to compare the data with other measurements. The best known method for this purpose is Dixon’s Q-test.
CLASSIFICATION OF DRUGS

All the drugs according to their chemical nature can be divided into organic and inorganic compounds. They can be prepared synthetically or reconstituted from natural sources product. All the drugs having medicinal importance can be broadly divided into two classes.

Chemical classification

The drugs are classified according to their chemical structure and properties without taking the pharmacological action. In this class most of the drugs are having at least an organic substrate; further classification is done in the relevant manner.

Pharmacological classification

In this class the drugs are divided according to their action on the organism’s organ (viz. heart, brain, lymphatic system, respiratory system, endocrine system, central nervous system etc.). Hence, these drugs are called cardiovascular, narcotics, analgesics, antibiotics, diuretics, and anesthetics etc. Further classification of each group is done according to the therapeutic / pharmacological specificity with the relevant organ. This thesis deals with the determination of the cardiovascular drugs namely losartan potassium, irbesartan, lisinopril and nicorandil. A detailed classification of drugs based on pharmacological action on human organs has been given in Scheme 1.1.
DRUGS

- Infections and Infestations
  - Antibiotics and Antibacterials
    - Penicillins
    - Cephalosporins
    - Fluoroquinolones
    - Aminoglycosides
    - Sulfonamides
    - Other drugs
  - Antitubercular Drugs
  - Antifungals

- Central Nervous System

- Endocrine System

- Genitourinary System

- Cardiovascular System
  - Antianginals
  - Antiarrhythmics
  - Antihypertensives
    - Nitrates
    - β-Adrenergic Blockers
    - Calcium Channel Blockers
    - Potassium Channel Activator
      - Nicorandil
    - Other Drugs

- Gastrointestinal System
  - Antiulcer
  - Laxatives
  - Antihepatic

- Nutrition

Scheme 1.1
LOSARTAN POTASSIUM

Losartan potassium, chemically known as 2-butyl-4-chloro-1[(2′-(1H-tetrazol-5-yl)[1,1′-biphenyl]-4-yl)methyl]-1H-imidazole-5-methanol, is an orally active, non-peptide angiotensin II (type AT₁) receptor antagonists for the treatment of hypertension. Following administration the angiotensin-II blocking activity of the losartan is predominantly based on its major active metabolite EXP 3174 (Fig. 1.7), a carboxylic acid, to which losartan is converted mainly by CYP 3A4 and 2C9 isoenzymes [237-239]. The carboxylic acid metabolite EXP-3174 is about five times more potent and has longer elimination half-time (t₁/₂) [240]. Losartan and to a higher degree EXP 3174 have proven to be effectively lower elevated arterial blood pressure upto 24 h post dose and furthermore, to exert a beneficial effect on the regression of vascular and myocardial hypertrophy associated with hypertension [237]. The active ingredient losartan potassium has one isomeric process impurity (compound A) and two primary degradates previously identified as the acid dimers, compound E and F, which form thermally under acid conditions [241, 242] (Fig. 1.8). The drug is very much confined to cardio protective effects, therefore it is important to assay losartan potassium in drug formulations.
Fig. 1.7 Structure of compounds (a) Losartan Potassium; (b) EXP 3174
Fig. 1.8 Structure of process impurity (compound A) and degradates (compound E and F)
IRBESARTAN

Irbesartan is chemically known as 2-butyl-3-[[2’-(1H-tetrazole-5-yl) (1,1′-biphenyl)-4-yl] methyl]-1,3-diazaspiro [4,4] non-1-en-4-one, which is a selective angiotensin II subtype I (AT₁) receptor antagonist, having no agonist activity and no affinity for the AT₂ receptor. The drug had no affinity for α₁- or α₂ – adrenoceptors or serotonergic receptors. Irbesartan is indicated for the treatment of hypertension in adults, either alone or in combination with other hypertensive agents [243,244]. A fixed dose combination of irbesartan plus hydrochlorothiazide is also available, but is not indicated for initial therapy in patients with hypertension [245]. The recommended starting dosage of irbesartan is 150 mg administered once daily. In patients who are volume or salt depleted as a result of vigorous treatment with diuretics or haemodialysis, a lower starting dosage (75 mg / day) is recommended [243]. The maximum recommended dosage of irbesartan is 300 mg once daily. Irbesartan is not presently indicated for the treatment of heart failure [243-245]. A clinical study in the hypertensive subjects has demonstrated that irbesartan effectively lowers blood pressure with once-daily administration. Irbesartan has a bioavailability of 60-80% and this is not affected by concomitant food intake.

Irbesartan was found to undergo N-glucuronidation on tetrazole moiety during in vitro incubations with hepatic microsomes [246]. In addition to the glucuronide conjugate of irbesartan the following oxidized metabolites were identified in human urine.

1) a tetrazole N²–β-glucuronide conjugate of irbesartan, (2) a monohydroxylated metabolite resulting from ω-1 oxidation of butyl side chain, (3,4) two different monohydroxylated metabolites resulting from oxidation of the spirocyclopentane ring, (5) a diol resulting from ω-1 oxidation of butyl side
chain and oxidation of the spirocyclopentane ring, (6) a keto metabolite resulting from further oxidation of ω-1 monohydroxy metabolite, (7) a keto-alcohol resulting from further oxidation of ω-1 hydroxyl of the diol, and (8) a carboxylic acid metabolite resulting from the oxidation of the terminal methyl group of the butyl side chain.

A Scheme for the biotransformation of irbesartan in humans is presented in

Scheme 1.2
Scheme 1.2: Biotransformation of irbesartan in man.
NICORANDIL

Nicorandil, N - [2-(nitroxy)ethyl] 3-pyridne carboxamide, is a nicotinamide derivative with a nitrate group in its chemical structure. The drug belongs to a class of compound known as potassium channel activators, which are characterized by their arterial vasodilator properties. In addition, nicorandil has vesodilating properties, which are attributed to nitrate group. Therefore, by combining these two vasodilator mechanisms, nicorandil represents a novel type of compound for use in the treatment of angina pectoris. Nicorandil induces vascular smooth muscle relaxation by increasing intracellular cyclic guanosine monophosphate (cGMP) levels. Potassium channel opening hyperpolarises vascular cell membrane, causing calcium (Ca\(^{2+}\)) ion channel to close and a consequent reduction in intracellular Ca\(^{2+}\) concentration (i.e., indirect calcium antagonist) that results in further smooth muscle relaxation and vasodilation. Nicorandil is potent vasodilator of the coronary and peripheral vascular beds. Because of its effects in reducing preload and afterload, it influences two of the main determinants of oxygen demands without imparting myocardial contractility and thus, reduces cardiac workload. The drug is rapidly absorbed from the gut. The drug is weakly bound to plasma protein. Nicorandil is almost entirely metabolised in the liver. There are two predominant biotransformation pathways for nicorandil (Scheme1.3) (a) oxidation to nicorandil-\(N\)-oxide and hydroxy nicorandil; and (b) denitration of nicorandil to pharmacologically inactive alcohol metabolite \([\text{\textit{N}} - (2\text{-hydroxyethyl})\text{-nicotinamide}]\), followed by further side-chain degradation to nicotinamide and related metabolites, including nicotinic acid and \(\text{\textit{N}}\)-methyl- nicotinamide. Nicorandil is excreted mainly as metabolite in urine.
Scheme 1.3: Some identified urinary metabolites of nicorandil in animals and man
Lisinopril, N²-[(1S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline dihydrate, is an effective angiotensin converting enzyme inhibitor. The main action of lisinopril is to inhibit conversion of angiotensin I (inactive) to angiotensin II (active). Hence angiotensin II production is inhibited. The drug has several advantages. It does not have direct sympatholytic activity. Hence cardiovascular reflexes are intact and postural hypotension is rare. It can be administered in patients of hypertension with diabetes mellitus or bronchial asthma. It is well tolerated and is used in the treatment of hypertension. Lisinopril is a white crystalline powder, soluble in water and sparingly soluble in methanol and practically insoluble in acetone and ethanol. It is currently supplied as 2.5, 5, 10 and 20 mg tablets. The primary degradation product of lisinopril is lisinopril diketopiperazine which occurs through an intramolecular condensation. (Scheme 1.4)
Scheme 1.4: Structures of lisinopril and lisinopril diketopiperazine degradation product
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