CHAPTER-1

GENERAL INTRODUCTION
The history of drugs is shrouded in the beginnings of the human race. Many drugs in use today have been known for hundreds or thousands of years. A remarkable range of drugs, to combat infectious diseases, control cardiovascular ailments, treat cancers and provide palliatives in the case of central nervous system disorders, have completely transform the practice of medicine over the past half a century. Together with significant advances in diagnostics and vaccines for the early and definitive detection of disease and the prevention of infection, the progress of drug research has contributed to the advancement of the human health care in both the developed and developing countries of the world.

Drugs are chemical compounds that modify the way the body and mind work. Most people think that these biological activities should help or heal sick people or animals. There is, however, no known drug that is not harmful or even poisonous at high doses. In a way that neither condemns nor condones drug use, a single example will suffice to illustrate the point. In his book “Confessions of an English Opium-Eater” the man of letters Thomas De Quincey [1] described in a footnote the dilemma of his friend, a surgeon, who was suffering from a lingering and fatal malady. Yet he had a family to support and had to continue working for as long as possible. Because of his professional knowledge he saw the necessity of reducing the daily dose of opium that he took to alleviate his pain to a minimum. I now quote the words used by De Quincey: “But to do this he must first obtain the means of measuring the quantities of opium; not the apparent quantities as determined by weighing, but the virtual quantities after allowing for the alloy of varying amounts of impurity”. With the knowledge available at that time De Quincey’s friend was unable to form such an assessment. He did, however, achieve a uniform method of extracting the opium so as to give a fairly constant potency.
The gradual change from the use of natural products in their entire state to either purified extracts from those products or to synthetic chemically produced materials can be said to have been taking place between the time of Paracelsus, who lived in Basel during the first half of the sixteenth century, to that of Ehrlich, to whom the award of a Nobel prize in 1909 was a fitting reward for his remarkable researches and breakthrough during the first decade of this century. This period has been described as that leading from Quintessence to the Chemical and has been fascinatingly reviewed by H. J. Barber [2]. This transition from the Quintessence to the Chemical stimulated a very considerable amount of interest in the analysis of natural products to determine, as De Quincey had said one hundred years earlier, not the apparent quantities as determined by weighing but the virtual quantities after allowing for the alloy of impurity. Thus, there is no doubt that nearly a century of pharmaceutical research has contributed spectacularly to improvement in human health and quality of life.

The growing awareness of the need to analyze drug substances was also apparent before any synthetic materials found regular use in medicine. Higher standards for the preparation of pharmaceutical ingredients had been set, following the 1858 Medical Act's stipulation that the General Medical Council (U.K.) should produce a list of medicines and compounds and manner of preparing them together with true weights and measures by which they are to be prepared and mixed [3]. The first editions of British Pharmacopoeia and Japanese Pharmacopoeia were published in 1864 and 1886, respectively. Thereafter, pharmaceutical and fine chemical manufacturers laid greater emphasis on meeting the standards set by pharmacopoeias. In 1890, Jesse Boot established an analytical laboratory and its
staff were involved mainly for analyzing proprietary medicines of competitors in order that Boots should develop new and/or cheaper formulations [4].

The increase in use of classical analysis to quantitate and define materials used in medicine, together with the increase in the use of materials of synthetic origin and of increasing complexity, is evident from a study of pharmacopoeias of various countries issued between 1900 and 1950. Shortly after this period one can see the exciting beginnings of a completely new era in the analysis of drugs based on the introduction of new concepts of analytical methodology that found no reference in the classical analytical textbooks of the time. The introduction of complexometric and non-aqueous titrimetry in the early 1950s, gas liquid chromatography in 1952, thin layer chromatography in 1956 together with the increasing applications of ultraviolet and infra-red spectroscopy during this period, can all be followed in the pages of any national pharmacopoeia that was being published regularly during this period. This brings us to the early 1960s and at those times any regulatory interest in the quality of drugs rested almost entirely on the pharmacopoeias.

In the European Community of countries, directives have been issued to ensure that the legislation in each member state shall provide for the comprehensive examination of intended drug materials before they are allowed to enter the market. Mutagenicity, carcinogenicity, toxicity, effect of reproductive function, pharmacodynamics and pharmacokinetics are among the properties to be studied and material must be unequivocally characterized with respect to the substance itself and the amounts of impurity that are likely to arise during the course of validated production processes.

Purity, a metaphysical concept, has always been considered as an essential factor in ensuring drug quality. Absolute purity does not exist and the degree of
purity of a product is only a reflection of the analytical techniques used for its 
assessment. All pharmaceutical substances unavoidably contain impurities resulting 
from many sources like raw materials and reagents, as reaction by products, and 
through degradation during manufacture and storage or extraneous contamination.

Chemical impurities are classified into three classes for regulatory purpose as 
organic, inorganic and residual solvents.

- Organic impurities may arise from starting materials (most often from isomeric 
  impurities), synthetic intermediates (incomplete reaction or excess reagent used) 
  and degradation product which may depend on alteration in reaction conditions 
  such as temperature, pH or in storage condition (hydrolysis, ring opening etc.).

- Inorganic impurities present in pharmaceutical products originate from the 
  equipment used and from reagents, catalysts, drying agents and filter aids.

- 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 manufacture of 
  drug products, it was decided to draft a separate guideline to address appropriate 
  levels. A key component of the guideline and a fundamental concept is 
  qualification [5]. “Qualification is defined as the process of acquiring and 
  evaluating data that establishes the biological safety of an individual impurity or 
  a given impurity profile at the level (s) specified”. In view of this concept, the 
  pharmaceutical analyst must give useful thoughts to the analytical methods, 
  especially in the development phases. Therefore, it is an important task for the 
  pharmaceutical analyst to select an assay procedure, which provides the best 
  solutions to the problems. At a later stage, once the pharmaceutical product has
entered the open market, there should be every opportunity given for external independent challenge. The possibility of deterioration on storage, criminal substitution, counterfeiting and innocent mistake must all be considered. In some areas of the world these possibilities are, regrettably, very much more likely than in others and circumstantial evidence suggests that they are on the increase. However, they should be guarded against everywhere. It is for this reason that sound, publicly available specification are an essential part of the regulatory control system, notwithstanding the advance procedures of licensing and inspection in many countries.

Over the last 30 years the very existence of searching analytical methods has contributed quite considerably to an improvement in the general quality of the drugs. We are all familiar with adage that “Quality must be built into a product - it can never be analyzed into it”. The most frequently used analytical techniques in pharmaceutical analysis are titrimetry, chromatography, electrochemical methods and spectroscopy. Sometimes these techniques are combined with other supporting/subsidiary instruments like flow injection analysis system or kinetic type of analysis. Thus making them more sensitive, selective and fast.

In the field of drug analysis volumetric methods, either direct or indirect, have their own importance due to their inherent simplicity. This is the reason why still a large number of official methods for the determination of pharmaceuticals in the pharmacopoeias are based on titrimetry. Though it is the oldest technique in the market now but still having their recognition in the field of scientific research. Recently titrimetric methods have been used for the determination of albendazole [6], gatifloxacin [7], promethazine theodate [8] and procaine hydrochloride [9] in commercial dosage forms.
Chromatographic methods have many applications in trace analysis and sometimes prove it as the only way. The different types of chromatography such as thin layer chromatography, high performance thin layer chromatography, column chromatography, high performance liquid chromatography, gas chromatography and capillary electro-chromatography have most frequent applications in the field of pharmaceutical as well as biomedical analyses.

Thin layer chromatography (TLC) has enjoyed widespread popularity in modern pharmaceutical analysis because of its sensitivity and speed of analysis. A TLC method has been developed for the determination of oxysterols in plasma using plates coated with either RP-C18 or silica gel F$_{254}$ [10]. In two other accounts, thin layer chromatography has been employed to identify an unknown compound in hydroquinine that has a higher $R_f$ value and forms during migration when dichloromethane and methanol are used as the development solvent [11] and to estimate the level of quinine, cinchonine and cinconidine in natural products and marketed formulations in combination with fluorescence enhanced detection [12]. This technique has also been used to analyse celecoxib in commercial dosage forms [13]. The contents of gestodene and cyproterone have been determined in raw material and dosage forms by thin layer chromatography [14].

High performance thin layer chromatography (HPTLC) is an Off-Line technique whose every stage of analysis can be visualized. Review papers [15,16] by Renger have discussed development and validation of thin layer chromatographic and high performance thin layer chromatographic methods in pharmaceutical quality control analyses. The advantages stated are as follows:

- simple to handle the instrument
• short analysis time to analyze complex or crude samples with minimum sample clean up
• evaluate the entire chromatogram with a wide variety of techniques and parameters without time constraints
• simultaneous but independent development of multiple sample and standards on each plate, leading to an increased reliability of results (in-system calibrations)
• robustness for easy transfer of samples.

Several drugs have been successfully investigated by HPTLC in pharmaceutical preparations [17-20].

Among all chromatographic methods, high performance liquid chromatography (HPLC) is most acceptable now a day due to its simplicity, high specificity and sensitivity for the analysis of drugs in both dosage forms and in biological fluids. For example, captopril can be measured in pharmaceutical tablets by anion exchange HPLC using indirect photometric detection [21]. HPLC is also useful in analyzing vitamin B₁₂ [22]. Dexamethasone has been estimated by HPLC using isocratic reversed-phased procedure in cream formulations [23] and a gradient method for evaluating its stability as the unformulated drug substance and in formulated products [24]. This method can also be used to measure impurities, degradation products and product preservatives at levels between 0.05 and 0.1%. HPLC assay procedures have been developed for cephalexin and cefadroxil, cefaclor and cefataxim in pharmaceuticals [25] and for cefixime trihydrate in bulk drugs [26].

Because of the specificity and sensitivity afforded by gas chromatography (GC), it has been widely used for the detection and determination of trace level contaminants in pure substances and also in pharmacokinetic studies. The use of mass spectrometer (MS) as the gas chromatographic detector provides additional
sensitivity and specificity over other analytical methods and can yield qualitative and quantitative information not available from other analytical techniques [27]. Capillary gas chromatography-mass spectrometry is utilized for monitoring anticancer drugs [28]. A number of papers have been published on the applications of gas chromatography in pharmaceutical analysis both in dosage forms and biological fluids [29-33].

Capillary electrophoresis (CE) is a relatively new analytical techniques based on the separation of charged analytes through a small capillary under the influence of an electric field. Additionally, CE is based on a separation mechanism, which differs from all types of chromatography, which makes it either a potential alternative analytical technique capable of faster analysis and higher efficiency than HPLC, or complimentary technique to HPLC to augment the information obtained from the analysis. CE is becoming popular for the analysis of drugs in pharmaceutical preparations. Several reports have appeared on the application of this technique in routine drug analysis [34-40]. Different modes of CE such as capillary zone electrophoresis (CZE) [41,42], micellar electrokinetic chromatography (MEKC) [43,44], isotachophoresis (ITP) [45,46], capillary-electrokinetic (CEC) [47,48], capillary gel electrophoresis (CGE) [49,50], iso-electric focusing (IEF) [51,52] and affinity capillary electrophoresis (ACE) [53, 54] have been developed and applied for pharmaceutical purity testing and in bioanalysis of drugs.

The use of electrochemical (EC) methods for the analysis of drugs and other compounds of biological interest has increased greatly over the last few years. The reason for this increase is simple, a wide variety of electrode materials, electrocells, electrochemical instruments, and electrochemical techniques that can be used with convenience and reliability are now commercially available.
Important electrochemical techniques such as amperometry, conductometry, potentiometry, anodic and cathodic stripping voltammetry, differential pulse voltammetry and polarography have been used in drug analysis. Potentiometric titrations are also reported in the pharmacopoeias as the standard method for the determination of certain drug substances [55-57]. Despite the wide use of spectrometry and chromatography analysts are choosing polarography and voltammetry [58-63] as an alternative due to their selectivity and sensitivity.

The simplicity of UV-spectrophotometry is best known and most widely accepted for qualitative and quantitative analysis and in structure identification. UV-spectrophotometry, a non-destructive technique usually does not require elaborate sample preparation step prior to assay. Therefore, the sample can be recovered for further testing or subsequent analytical procedures. However, these methods are inadequate when two or more drugs showing similar UV spectra in the presence of other components present in the drug sample like excipients or decomposition products. The use of UV-spectrophotometry for drug analysis, especially in pharmaceutical preparations has been increased rapidly in the last few years [64-67].

Photometric methods of analysis are performed in the visible region of light. These methods are usually based on the following aspects:

- complex-formation reaction
- oxidation-reduction process
- a catalytic effect

In each type of reaction the absorbance of coloured compounds is measured. Usually the analyte under investigation being colourless, they are reacted with suitable chemical reagents in order to convert them in coloured compounds. Simple colorimetric and UV methods continue to be popular for carrying out single-
component assays on a variety of formulated products. Representative examples of UV-visible methods of drug analysis for some pharmaceuticals that have been published [68-99] are given in Table 1.1.

In the course of this discussion, the importance of computer-aided spectrophotometric determination of multicomponent systems cannot by ignored. Pharmaceutical preparations are usually mixtures of the active principle and various excipients that absorb in the same region as the component of interest, thereby resulting in band overlap and impending the use of the technique with simple calibration methods. The inception of microcomputers and spectrophotometers that allow absorbance spectra to be expeditiously recorded at many wavelengths has enable the development of analytical methods based on the mathematical resolution of multivariate signals for the rapid quantitation of mixtures of analytes in control analyses. The application of multivariate calibration methods to spectral data in the biomedical and pharmaceutical fields has acquired a routine nature [100-104]. Partial least square (PLS) has become the de facto standard for multivariate calibration because of the quality of the obtained calibration models, the ease of its implementation and the availability of software [105]. It shows the advantage of using full spectra, which is critical for the spectroscopic resolution of complex mixtures of analytes. It allows for a rapid determination of components, usually with no need of a prior separation. An additional advantage of robust multivariate methods, PLS, is that calibration can be performed by ignoring the concentration of all other components except the analyte of interest. This makes these methods especially appealing for the determination of the active components in ophthalmic and nasal solutions as well as in the syrups, whose excipients may show absorption
### Table 1.1

**Assays of drugs in pharmaceutical formulations by UV-visible spectrophotometric procedures**

<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>Acetaminophen</td>
<td>m-cresol</td>
<td>640</td>
<td>68</td>
</tr>
<tr>
<td>Amiodarone HCl</td>
<td>p-chloranilic acid</td>
<td>535</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>2,3-dichloro 5,6- dicyano</td>
<td>575</td>
<td>69</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>70</td>
</tr>
<tr>
<td></td>
<td>Ninhydrin in DMF medium</td>
<td>595</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>2,3-dichloro 5,6- dicyano</td>
<td>580</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>1,4- benzoquinone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>530</td>
<td>72</td>
</tr>
<tr>
<td>Amoxicillin &amp; ampicillin</td>
<td>KIO$_3$</td>
<td>520</td>
<td>73</td>
</tr>
<tr>
<td>Ampicillin, amoxicillin &amp; carbenicillin</td>
<td>Folin ciocalteau phenol peroxide &amp; 750</td>
<td>750, 770</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>1-chloro-2,4-dinitrobenzene</td>
<td>380</td>
<td>75</td>
</tr>
<tr>
<td>Benidipine HCl</td>
<td>Methanol</td>
<td>238</td>
<td>76</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>Tris buffer</td>
<td>284, 305</td>
<td>77</td>
</tr>
<tr>
<td>Diltiazem HCl</td>
<td>Sodium metavanadate</td>
<td>750</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Bromothymol blue</td>
<td>415</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue</td>
<td>415</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Bromocresol green</td>
<td>415</td>
<td>79</td>
</tr>
<tr>
<td>Famotidine</td>
<td>KMnO$_4$ in alkaline medium</td>
<td>610</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Ninhydrin</td>
<td>590</td>
<td>81</td>
</tr>
<tr>
<td>Flunarizine</td>
<td>Iodine</td>
<td>295, 355</td>
<td>82</td>
</tr>
<tr>
<td>dihydrochloride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levodopa</td>
<td>Ce(IV) nitrate in H$_2$SO$_4$ medium</td>
<td>510</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>NaOH</td>
<td>300</td>
<td>84</td>
</tr>
<tr>
<td>Menadione</td>
<td>NaOH in the presence of amine</td>
<td>450</td>
<td>85</td>
</tr>
<tr>
<td>Methyldopa</td>
<td>Ce(IV) nitrate in H$_2$SO$_4$ medium</td>
<td>550</td>
<td>83</td>
</tr>
<tr>
<td>2-methyl-1, 4-naphto-</td>
<td>Ethyl aceto acetate &amp;</td>
<td>550</td>
<td>86</td>
</tr>
<tr>
<td>Compound</td>
<td>Reagent/medium</td>
<td>λ (nm)</td>
<td>( \varepsilon ) (M⁻¹ cm⁻¹)</td>
</tr>
<tr>
<td>----------------------</td>
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</tr>
<tr>
<td>quinone</td>
<td>ethanolic ammonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mometasone furoate</td>
<td>Methanol</td>
<td>248</td>
<td>87</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Persulfate in alkaline medium</td>
<td>320,390</td>
<td>88</td>
</tr>
<tr>
<td>Nicorandil</td>
<td>Brucine-sulfanilic acid in H₂SO₄ medium</td>
<td>410</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>3-methyl-2-benzothiazoline hydrazone HCl-metol</td>
<td>560</td>
<td>89</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>KMnO₄ in neutral medium</td>
<td>530</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>4-methyl amino phenol and K₂Cr₂O₇</td>
<td>525</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Bromocresol green</td>
<td>415</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue</td>
<td>415</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Bromothymol blue</td>
<td>415</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Eriochrome Black T</td>
<td>520</td>
<td>92</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>KMnO₄ in alkaline medium</td>
<td>603</td>
<td>93</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>Cerium</td>
<td>243</td>
<td>94</td>
</tr>
<tr>
<td>Silymarin</td>
<td>KMnO₄ in neutral medium</td>
<td>530</td>
<td>95</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Persulfate in alkaline medium</td>
<td>355</td>
<td>96</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>Nitrous acid</td>
<td>420</td>
<td>97</td>
</tr>
<tr>
<td>Verapamil HCl</td>
<td>Chloramine T</td>
<td>425</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>N-Bromosuccinimide</td>
<td>415</td>
<td>99</td>
</tr>
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</table>
spectra that are severely overlapped with those from the analytes. The complementary use of PLS multivariate calibration and artificial neural networks (ANNs) for the simultaneous spectrophotometric determination of three active components such as chlorpheniramine, nephazoline and dexamethasone in a pharmaceutical formulation has been successfully explored [106].

Difference spectrophotometry is an important and useful technique used in the determination of medicinal substances by eliminating specific interference from the degradation products, co-formulated drugs and non-specific irrelevant absorption from the formulation matrix. The technique involves reproducible attraction of the spectral properties of the absorbance difference between two solutions, provided that the absorbance of the other absorbing substance is not affected by the reagent(s) used to alter the spectral property [107].

Derivative spectrophotometry is an analytical technique of great utility for both qualitative and quantitative information from spectra composed of unresolved bands. Although it was introduced more than thirty years ago [108-110] and has advantages for the solution of specific analytical problems. The derivative method has found applications not only in the UV-visible spectrometry but also in infrared [111], atomic absorption [112], flame emission spectrometry [113] and fluorimetry [114,115]. The use of derivative spectrophotometry is not restricted to special cases, but may be of advantage whenever quantitative study of normal spectra is difficult. Its disadvantage is that the differentiation degrades the signal-to-noise ratio, so that some form of smoothing is required in conjunction with the differentiation [116].

As an analytical tool for the pharmaceutical analytical laboratory, near-infrared spectroscopic methods have the potential to dramatically improve the
quality of the drug manufacturing process from assessing incoming raw materials to the final drug product. There are several advantages associated with this technique such as reduction in the cost of testing, require no reagents, associated reagent preparation steps, sample preparation steps and generally require only one working analyst day to complete testing. The instrumentation used is eminently suited to being used in production facilitates and on-line measurements are routinely used in various industries [117,118]. When introducing NIR analysis into a regulated industry, such as pharmaceutical industry, special attention must be paid to satisfying the regulatory requirements for analytical procedures. The US pharmacopoeia has proposed guidelines [119,120] for this technique.

Fluorimetry and phosphorimetry find wide applications in quantitative studies of rates of degradation, metabolism, and excretion of drugs where other analytical techniques are not sufficiently sensitive. A gradual increase in the number of papers on the potential applications of fluoimetry and phosphorimetry in the field of pharmaceutical analysis has been noticed during the last few years [121-124].

The analytical measurement of elemental concentrations is important for the analysis of the major and minor constituents of pharmaceutical products. Metals are the major constituents of several pharmaceuticals such as dialysis solutions, lithium carbonate tablets, antacids and multi-vitamin and mineral tablets. The metal ions in pharmaceuticals are determined more accurately and conveniently by atomic absorption spectrometry. The use of atomic absorption spectrometry in this regard has been the subject of several reviews and papers [125-130].

Nuclear Magnetic Resonance (NMR) spectroscopy has been mainly used for the elucidation and confirmation of structures. For the last decade, NMR methods have been introduced to quantitative analysis in order to determine the impurity
profile of a drug, to characterize the composition of drug products in body fluids, in solid state measurements to provide the information about polymorphism of drug powders, quantification of drugs in tablets [131-137] and for micro-imaging to study the dissolution of tablets [138-140].

One of the significant analytical developments in the last two decades has been the widespread utilization of Flow Injection Analysis (FIA) assay of drugs in pharmaceutical laboratories. It has several advantages:

- reduced reagent consumption,
- high sampling frequency
- safety in applying toxic reagents because the whole analysis proceeds in a closed system.

An additional advantage observed in flow injection analysis is increased selectivity when the analyte is accompanied by more slowly reacting compound. This technique has been utilized successfully in the determination of some compounds of pharmaceutical interest [141-146].

Whenever the question of mathematical and statistical treatment arises, the role of chemometrics cannot be ignored. Chemometrics is the chemical discipline concerned with the application of mathematical, statistical methods as well as those other methods based on mathematical logic, to chemistry, in order to design or select optimal procedures and experiments, and provide maximum chemical information by analyzing chemical data [147,148]. Several reviews have been reported on applications of chemometric methods in biomedical and pharmaceutical analysis [149-153].
The principles and applications of the kinetic methods have been reviewed [154,155,156]. 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. The sample and reagent can be mixed manually or automatically. Only slow enough reaction tolerate manual mixing and even so, they are better handled automatically, not only to obtain more rapid and reproducible results, but also to increase the reaction rate in some cases. There are several approaches that can be used for the determination of single components in the absence of any kinetic interference (Table 1.2). However fixed-time and initial rate methods have been used more frequently for the determination of drugs in pharmaceutical formulations and biological fluids [157-160]. Kinetic automatic techniques are generally based on open systems among the most popular of which are stopped flow (SF) system [161] and the continuous addition of reagent (CAR) technique [162-164]. Several drugs have been determined by using the CAR technique with photometric [165,166] and fluorimetric detection [167]. On the other hand, the CAR technique has been extended to chemiluminescence (CL) reaction as CAR chemiluminescence spectrometry (CARCL), a new approach that have proved outstanding for the analysis of drugs and other substances of analytical interest. 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 improves the sensitivity and selectivity which in turn reduce the analysis time for the analyte [168-170]. Multicomponent kinetic determinations, often called as differential rate methods, are also receiving popularity in the field of
Table 1.2  
**Single-component methods without error compensation**

<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>
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</tr>
</tbody>
</table>
pharmaceutical research [171,172]. Two new approaches i.e. kinetic wavelength pair method [173] and H-point standard addition method [174] have been proposed for dealing with overlapping spectra of components in the binary mixtures.

METHOD VALIDATION

The ability to provide timely, accurate, and reliable data is central to the role of analytical chemists and is especially true in discovery, development and manufacture of pharmaceuticals. Analytical data are used to screen potential drug candidates, aid in the development of drug synthesis, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products, and test final products for release. The quality of analytical data is a key factor in the success of a drug development program. The process of method development and validation has a direct impact on the quality of these data.

The initial step in the analytical method development procedure is to establish what is to measure and how accurately it should be measured. Quite often method validation evolves from method development and so the two activities are often closely tied, with the validation. The process for the development, validation and use of an analytical method is shown in Fig. 1.1.

Once an analytical method is advent. It is important to validate the method before it should be recommended for the routine analysis. Method validation of analytical procedure, i.e. the process of defining an analytical requirement and confirms that the method under consideration has performance capabilities consistent with what the application requires in determining the reliability and reproducibility of the method with respect to regulatory requirement in order to get the reliable data by incorporating statistical analysis to evaluate its performance.
Fig. 1.1: The process of development, validation and routine use of an analytical method
Several International Organization and Regulatory Authorities, which are involved in fixing the criteria for the validations are listed in Table 1.3. In the field of analysis, it is very clear that the definitions cover the entire field of analytical chemistry from bioanalysis to substance and product analysis.

It has been agreed that the key for evaluation of method reliability and overall performances are:

- confirmation of identity
- analyte stability
- selectivity/specificity
- precision and accuracy
- limit of detection and quantitation
- recovery
- robustness/ruggedness

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

The strategy is most effective if statistical design is used in all stages of development and not only for screening or optimizing the process.

**Prevalidation**

During method optimization some performance parameters should be investigated in order to avoid serious problems during the validation step. The performance parameters to evaluate, before actually beginning the validation process are accuracy, precision and robustness, and evaluation of these parameters can be called prevalidation [175]. In contrary to, validation is defined as a process of demonstrating that analytical procedure is suitable for its intended purpose [176,177], prevalidation is defined as the formal evidence that an analytical system
<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
does what it is supposed to do so. This definition has some implications by analogy with Tranter [178], as it presupposes that there is a description of what the analytical system should do.

The aim of prevalidation is to obtain knowledge about analytical procedure and validation characteristics. This process serves to test validation parameters with the purpose of proving suitability of analytical procedure. The basis for useful prevalidation procedure is to obtain the most appropriate calibration function (often linear calibration function) covering at least a working range of one power of ten. The efficiency of prevalidation procedure is given by characteristic data such as constants of calibration and as analytical evaluation function, standard deviation of procedure, limit of quantitation, metrological characteristics of analytical procedure and other. It should be emphasized that for prevalidation process, the complete analytical procedure is very important, i.e. procedure that is defined by specifying all conditions under which the measurements must be made [179-181].

Moreover, even though the prevalidation concept is originally developed for pharmaceutical applications, it can reasonably be applied to many analytical procedures in different fields with similar specifications (toxicology, environmental, food chemistry etc.).

Validation Proper

Among all of the International Organization, the ICH guidelines achieved a great deal in harmonising the definitions of the required validation characteristic and their basic requirements.

The International Conference on Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has harmonized the requirements in two guidelines [176, 177]. 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 industry 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. Normally, evaluated validation characteristic and their minimum number of determinations required if applicable are given in Table 1.4 [177].

**Stability-Indicating Assay (SIAM)**

With the advent of ICH [182-185], US-FDA [186,187], WHO, European committee for Directorates [188-190] guidelines, the requirement of establishment of stability indicating assay (SIAM) become more clearly mandated. These guidelines explicitly require conduct of forced decomposition studies under a variety of conditions like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. In definition “A stability-indicating assay (SIAM) is a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product.” A stability-indicating assay (SIAM) accurately measures the active ingredients, without interferences from degradation products, process impurities, excipients, or other potential impurities. Overall there are two stages in the validation of SIAM. First stage is early in the development cycle when drug substance is subjected to forced decomposition studies and the SIAM is established based on the knowledge of drug degradation behaviour. The main focus of validation at this stage is on establishment
Table 1.4

Validation characteristics normally evaluated for the different types of test procedure [176] and the minimum number of determinations required (if applicable) [177].

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

^aIncluding dissolution, content potency

^bLack of specificity of one analytical procedure could be compensated by other supporting analytical procedure (s).

^cIntermediate precision sufficient for submission.

^dMay be needed in some cases.
of specificity/selectivity, followed by other parameters like accuracy, precision, linearity, range, robustness etc. This validated method finds application in the analysis of stability samples of bulk drug for determination of its retest or expiry period. In the second stage, when the SIAM so developed is extended to formulations or other matrices. The emphasis gets limited to just prove the pertinence in the presence of excipients or other formulation constituents. Here only parameters of critical importance like specificity, selectivity, accuracy and precision are revalidated.

There are several other reports in literature on the development of stability indicating assays of specific drugs [191-196]. If one critically evaluates the literature reports, derivatives spectroscopy [197-200] and chromatographic techniques [201-205] have been commonly employed for this purpose.

**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**

There has been some controversy regarding the technical term for this validation characteristic i.e. specificity vs selectivity [206]. The terms selectivity and specificity are often used interchangeably. A detailed discussion of this term as defined by different organizations has been made by Vessamann [207].
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, which 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 said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.

In addition, where it is unclear whether or not the interferences are already present, the selectivity of the method can be investigated by studying its ability to measure compared to other independent method/techniques. These parameters apply to both qualitative and quantitative analysis.

**Accuracy and Precision**

Accuracy expresses the closeness of a result to a true value. Method validation seeks to quantify the likely accuracy of the results by assessing systematic and random effects on results. Accuracy is therefore, normally studied as two components: ‘trueness’ and ‘precision’. The trueness (of a method) is an expression of how close the mean of a set of results (produced by the method) is to the true value. Trueness is normally expressed quantitatively in terms of “bias” which provides a measure of systematic or determinate error of an analytical method. Precision is a measure of how close results are to one another, and is usually expressed by measures such as standard deviation which describe the spread of results, relative standard deviation, coefficient of variation, and variance.

Generally, the precision of an analytical method is readily obtained by simply repeating the measurements. The two most common precision are:

- repeatability (Intra day assay)
- reproducibility (Inter day assay)
They represent two extreme measures of precision, which can be obtained. Repeatability, also termed ‘Intra day assay’ precision (the smallest expected precision) will give an idea of the sort of variability to be expected when a method is performed by a single analyst on one piece of equipment over a short time scale by applying the whole analytical procedure to the sample i.e. the sort of variability to be expected between the results when a sample is analysed in duplicate. If a sample is to be analysed by a number of laboratories for comparative purposes then a more meaningful precision measure is reproducibility (this is the largest measure of precision normally encountered). Sometime also termed as ‘Inter day assay’. Both repeatability and reproducibility are generally dependent on analyte concentration, and should be determined at different concentration levels and if relevant, the relationship between precision and analyte concentration should be established.

Repeatability and intermediate precision can be calculated by an analysis of variances [208, 209]. No acceptance limit for accuracy and precision for an assay method is reported in the main regulatory guidelines [210, 177, 211], and only a document of the European community commission deals with the performance of analytical methods and the interpretation of results [212]. Several approaches discussed in the ICH guideline are given in Table 1.5.

**Linearity and calibration curve**

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformation, proportional to the concentration of analytes in samples within a given range, is widely used in pharmaceutical analysis [213].

Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus
Table 1.5
Quantitative approaches to demonstrate accuracy according to ICH [177]

<table>
<thead>
<tr>
<th>Drug substance</th>
<th>Application of the analytical procedure to a reference material.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Comparison of the results with those of a second, well characteristic 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 analyt to drug product.</td>
</tr>
<tr>
<td></td>
<td>Comparison of the results with those of a second, well characterized procedure.</td>
</tr>
<tr>
<td>Impurities</td>
<td>Spiking of the impurity to drug substances or product.</td>
</tr>
<tr>
<td>(quantitative)</td>
<td>Comparison of the results with those of a second, well characterized procedure.</td>
</tr>
</tbody>
</table>
concentration plot. A correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level. The requirements and relevant parameter for the various calibrations are given in Table 1.6.

Limit of detection, Limit of quantification and Sensitivity

The limit of detection (LOD) of a technique can be conceived of as the smallest concentration, or amount of analyte that can be established as being different, at a reasonable statistical confidence level, from a blank (a material similar is composition to the sample except that the analyte is absent) [214-216]. One common definition of LOD is the concentration (or quantity) of analyte that produces a signal that exceeds the signal observed from a blank by an amount equal to three times the standard deviation for the measurement on the blank [216, 217].

At analyte concentrations at or near the LOD, the precision of the measured data is usually poor. Thus, it is inadvisable to attempt to quantify the analyte unless its concentration is well above the LOD.

A limit of quantification (LOQ), defined as the analyte concentration for which the signal exceeds that for a realistic analytical blank by 10 times the standard deviation, is often specified as the smallest analyte concentration that one should attempt to quantify [216]. Several approaches have been given in the ICH guideline to determine the detection and quantitation limit (Table 1.7.).

Methods that can detect small concentration of analytes often are called "sensitive" methods and the manner in which a change in analyte concentration produces a change in the resulting signal is known as sensitivity [218]. It is therefore possible for a method to be very sensitive but to exhibit relatively poor limits of
<table>
<thead>
<tr>
<th>Quantitation</th>
<th>Requirements</th>
<th>Relevant parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single-point calibration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>External standard</td>
<td>Linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
</tr>
<tr>
<td>Non-significant ordinate intercept</td>
<td></td>
<td>Inclusion of zero in confidence interval of the ordinate intercept, magnitude of the intercept (as percent of the signal 100% test concentration).</td>
</tr>
<tr>
<td>Homogeneity of variances*</td>
<td></td>
<td>F-test of the variances at the lower and upper limit of the range.</td>
</tr>
<tr>
<td><strong>Multiple-point calibration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear, unweighted</td>
<td>Linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
</tr>
<tr>
<td>Homogeneity of variances*</td>
<td></td>
<td>F-test of the variances at the lower and upper limit of the range.</td>
</tr>
<tr>
<td>Linear, weighted</td>
<td>Linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
</tr>
<tr>
<td>Non-linear</td>
<td>Continuous function</td>
<td>Regression</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------</td>
<td>------------</td>
</tr>
<tr>
<td>For main peak: linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
<td></td>
</tr>
<tr>
<td>Non-significant ordinate intercept</td>
<td>Inclusion of zero in confidence interval of the ordinate intercept, magnitude of the intercept (as percent of the signal 100% test concentration).</td>
<td></td>
</tr>
<tr>
<td>Homogenity of variances*</td>
<td>F-test of the variances at the lower and upper limit of the range.</td>
<td></td>
</tr>
<tr>
<td>For impurities: linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
<td></td>
</tr>
</tbody>
</table>

* May be presumed for a limited range (factor 10-20).
### Table 1.7

**Approaches for determining the detection and quantitation limit [177]**

<table>
<thead>
<tr>
<th>Approach</th>
<th>Detection limit</th>
<th>Quantitation limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual evaluation</td>
<td>Minimum level detectable</td>
<td>Minimum level quantifiable</td>
</tr>
<tr>
<td>Signal-to-noise</td>
<td>3:1 or 2:1</td>
<td>10:1</td>
</tr>
<tr>
<td>Standard deviation of</td>
<td>$3.3 \times S_o / b$</td>
<td>$10.0 \times S_o / b$</td>
</tr>
<tr>
<td>the response ($S_o$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>slope (b)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

*a verification with a suitable number of samples.

*b Standard deviation of the blank, residual standard deviation of the calibration line, or standard deviation of the intercept.
detection. On the other hand, an insensitive method is very unlikely to exhibit a low LOD. Thus, sensitivity is a necessary but not sufficient condition for the achievement of low LOD.

**Range and dynamic range**

The range of analytical method is the concentration interval over which acceptable accuracy, linearity, and precision are obtained. In practice, the range is determined using data from the linearity and accuracy studies.

Fig. 1.2 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, or LOL). To be very useful, an analytical method should have a dynamic range of at least two orders of magnitude. Some methods have applicable concentration range of five to six orders of magnitude.

**Robustness/ ruggedness**

Robustness testing is now a days best known and most widely applied in the pharmaceutical world because of the strict regulations in that domain set by regulatory authorities which require extensively validated methods. International conference on Harmonisation of technical requirements for Human use (ICH) [219] defines “the robustness/ruggedness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [220]. The term ruggedness is frequently used as a synonym [221-224]. The ICH guidelines [220] also recommend that “one consequence of the evaluation of
Fig. 1.2: Useful range of an analytical method: LOQ = Limit of quantitative measurement and LOL = Limit of linear response.
robustness should be that a series of system suitability parameters (e.g. resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used". Several definitions for robustness or ruggedness exist which are, however, all closely related [225-228].

The Dutch pharmacists guideline [225], the ICH guidelines [229] as well as some authors working in bio-analysis [230] consider robustness a method validation topic performed during the development and optimization phase of a method, while others [231] consider it as belonging to the development of the analytical procedure.

Therefore, the robustness test can be viewed as a part of method validation that is performed at the end of the method development or at the beginning of the validation procedure.

**STUDY PROPER AND STATISTICAL ANALYSIS**

Daily standard curves are generated to determine the sample concentrations. All calibrators and quality control samples are analyzed in duplicate. Sample concentrations are based on single determination. The quality control sample sequence is carefully monitored for systematic errors. For each standard curve, the slope, intercept, variance, correlation coefficient and the interpolated calibrated concentrations are reported.

Acceptance of the assay results are determined by monitoring the quality control results. If the interpolated concentrations are within the control charts confidence limits, established during the method validation, the data are considered valid. Upon completing a study proper and accepting the analytical runs, the quality control results are incorporated into their respective data basis to update their confidence limits.
Correlation and regression

When using instrumental methods it is often necessary to carry out a calibration procedure by using a series of samples (standards) each having a known concentration of the analyte. Two statistical procedures should be applied to a calibration curve:

- test whether the graph is linear or in the form of a curve.
- find the best straight line (or curve) through the data points.

Linearity is often tested by the correlation coefficient, ‘r’, which can be calculated for a calibration curve to ascertain the degree of correlation between the measured instrumental variables and the sample concentration.

\[
r = \frac{\Sigma x_i y_i - n \bar{x} \bar{y}}{\sqrt{(\Sigma x_i^2 - n \bar{x}^2)(\Sigma y_i^2 - n \bar{y}^2)}}
\]

where

\[
\bar{x} = \text{Mean of all the values of } x,
\]

\[
\bar{y} = \text{Mean of all the values of } y,
\]

\[
n = \text{Number of data points}
\]

The maximum value of r is 1. When this occurs there is exact correlation between the two variables (x and y). When the value of r is zero (xy = 0), there is complete independence of the variables. The minimum value of r is -1, indicates that the assumed dependence is opposite to what exists. As a general rule, 0.90 < r < 0.95 indicates a fair curve, 0.95 < r < 0.99 as a good curve, and r > 0.99 includes excellent linearity.
Linear least squares

Once a linear relationship has been shown to a high probability by the value of the correlation coefficient, \( r \), then the best straight line through the data points has to be evaluated by linear regression (the method of least square). The equation of the straight line is \( A = a + b \, C \) where \( A \) is usually the measured variable that is absorbance, plotted as a function of changing \( C \) that is the concentrations of the standards in a spectrophotometric calibration. To obtain the regression line \( A \) on \( C \), the slope \( b \) of the line and the intercept \( 'a' \) on the \( Y \)-axis are given by following equations.

\[
b = \frac{\Sigma x_i y_i - [(\Sigma x_i \Sigma y_i)/n]}{\Sigma x_i^2 - [(\Sigma x_i)^2/n]} \]

\[
a = \bar{y} - b \bar{x}
\]

Errors in the slope and the intercept

\[
S_o = \sqrt{\frac{\sum(y_i - \bar{y})^2}{(n - 2)}}
\]

where \( \bar{y} \) values are obtained from calculated regression line for given values of \( x \); once the value \( S_o \) has been obtained, both the standard deviations of the slope \( S_b \) and the intercept \( S_a \) can be obtained from the following equations

\[
S_b = S_o \sqrt{\sum(x_i - \bar{x})^2}
\]

\[
S_a = S_o \sqrt{\frac{\sum x_i^2}{n \Sigma(x_i - \bar{x})^2}}
\]

Error in the estimation of concentration

\[
S_c = \frac{S_o}{b} \left[ 1 + \frac{1}{n} + \frac{(y - \bar{y})^2}{b^2 \Sigma(x - \bar{x})^2} \right]^{1/2}
\]

where \( \bar{x} \) and \( \bar{y} \) are the average concentration and absorbance values, respectively for \( n \) standard solutions.
Confidence limit

The confidence limit defined by the experiment means \( \bar{x} \) of 'n' replicate measurements and the standard deviation (s) within the range where the true value falls is given by

\[
\text{C.L. for } \mu = \bar{x} \pm \frac{ts}{\sqrt{n}}
\]

where

\( \mu = \) true mean

\( t = \) statistical factor that depends on the number of degrees of freedom and the confidence level desired.

Significance of testing

An important property of an analytical method is that it should be free from the systematic error (bias). Determining bias involve 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 known as a significance test is used in the interpretation of analytical data.

- Student t-test (Null hypothesis)

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).

\[
\pm t = (\bar{x} - \mu) \frac{\sqrt{n}}{s}
\]
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 a 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.

- **Paired t-test**

\[
\pm t = \frac{\bar{x}_1 - \bar{x}_2}{S_p} \sqrt{\frac{n_1n_2}{n_1 + n_2}}
\]

where

- \( \bar{x}_1 \) = Mean from the test method
- \( \bar{x}_2 \) = Mean from the accepted (reference) method
- \( n_1 \) and \( n_2 \) = Number of measurements.

\( S_p \) = Pooled standard deviation of the individual measurements of two sets is given by

\[
S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}
\]

- **F-test**

For comparing the variance that is the square of the standard deviation of the two methods. If \( S_1 \) and \( S_2 \) are the standard deviations, then

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

where

\( S_1^2 > S_2^2 \)

If \( F_{\text{cal}} > F_{\text{tab}} \) at the selected confidence level, then there is a significant difference between the variances of the two methods.
• Interval hypothesis

In statistical terms, the hypothesis can be tested as

$$\theta_L < \mu / \mu < \theta_U,$$

where $\theta_L$ and $\theta_U$ represent the lower and the upper acceptance limits respectively, when $\mu$ is expressed as a portion of the reference method $\mu_1$. The lower limit $\theta_L$ and the upper limit $\theta_U$ of the confidence interval are obtained as:

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

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

where

$$a = x_1 - \frac{S_p^2 t_{lab}^2}{n_1},$$

$$b = -2x_1x_2,$$

$$c = x_2 - \frac{S_p^2 t_{lab}^2}{n_2}.$$

• Testing for outliers

Frequently, when a series of replicate measurements of same quantity are made, one of the results will appear too different markedly from the other. There is then a great temptation to discard this “outlier” 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 used for this purpose is Dixon’s Q test.

$$Q = \frac{|\text{Suspected value} - \text{Nearest value}|}{(\text{Largest value} - \text{smallest value})}$$

If $Q_{\text{cal}} > Q_{\text{tab}}$ at a given confidence level, then the outlier can be rejected.
CLASSIFICATION OF DRUGS

All the drugs according to their chemical nature can be divided into organic and inorganic compounds. They can be prepared synthetically (from chemicals) or can be directly obtained 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 under consideration. In this class most of the drugs are having at least an organic substrate, the 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 named like antianginal, narcotics, analgesics, antibiotics, diuretics, anesthetics etc. Further classification of each group is done according to the therapeutic/pharmacological specificity with the relevant organ. A detailed classification of drugs based on pharmacological action on human organs has been given in Scheme 1.1.

The present thesis deals with the determination of the following drugs:

NORFLOXACIN

Norfloxacin, 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid is a synthetic broad spectrum antibacterial drug, which is very much active against many gram positive and gram negative bacteria. It is mainly used in the treatment of urinary tract infections.
Scheme 1.1
Nicorandil, N-[2-(nitroxy) ethyl] 3-pyridine carboxamide, is a relatively new antianginal drug belongs to potassium channel activators. It is a potent vasodilator of the coronary and peripheral and vascular beds. The nitrate moiety in the chemical structure may in part contribute to the anti-ischaemic action. In human and animals, nicorandil is metabolized to several metabolites with inactive denitrated nicorandil as major metabolite. The main route of elimination from the plasma appears to be denitration of the drug by the liver to the pharmacologically inactive alcohol metabolite, denitrated nicorandil (N-(2-hydroxyethyl)-nicotinamide) followed by further side chain degradation to nicotinamide and related metabolites, including nicotinic acid and N-methyl-nicotinamide (Scheme 1.2).

SILYMARIN

Silymarin i.e. 3,5,7-trihydroxy-2-[3-(4-hydroxy-3-methoxy phenyl)-2-(hydroxy-methyl)-1,4-benzodioxan-6-yl]-4-chromanone is a free radical scavanger and an important antihepatotoxic drug. It has been used for the treatment of liver diseases of different etiology due to its hepatoprotective activity that is considered to involve antioxidation, inhibition of lipid peroxidation and the membrane stabilizing effects.
Scheme 1.2 Nicorandil and its metabolites
Ramipril is an angiotensin converting enzyme (ACE) inhibitor that lowers peripheral vascular resistance without affecting heart rate. It is used in the treatment of hypertension and congestive heart failure. The role of this drug is to inhibit last step of the biosynthesis of angiotensin II, a potent vasoconstrictor, and therefore, it causes a general vasodilatation and lowers blood pressure. The drug is chemically known as 2-[N-[(S)-L-ethoxy carbonyl-3-phenyl propyl]-L-alanyl]-(1S, 3S, 5S)-2-azabicyclo[3,3,0]octane-3-carboxylic acid.
REFERENCES


[208] DIN ISO 5725-2; Accuracy (trueness and precision) of measurement methods and results a basic method for the determination of repeatability and reproducibility of a standard Measurement method, 1990.


