CHAPTER 1

GENERAL INTRODUCTION
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In the book "Confessions of an English Opium-Eater" the man of letters Thomas De Quincey [1] described in a footnote the dilemma of his surgeon friend 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 pains to a minimum. I now quote the words used by Thomas 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 the time (about 1820) 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 16th century, to that of P. Ehrlich, to whom the award of a Nobel Prize in 1909 was 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 Chemical" and have 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.

The purpose of researches in analytical chemistry is to devise new methods of analysis or to improve the existing ones. Establishment of the mechanisms of various reactions and
the formulation of the fundamental laws of Chemistry has largely been based on the results of chemical analysis. It has extensive applications in analysis of pharmaceuticals in drug formulations and body fluids, organic compounds, biochemicals, polluted water, foods, agriculture and in many other areas. The chemical analysis is divided into qualitative and quantitative analysis. The qualitative analysis deals with the detection of constituents or components present in compound or sample. The quantitative analysis determines the proportions in which the constituents or the amount of constituents are present. The greater part of chemical analysis is quantitative.

The realm of analytical chemistry is widening day by day with the modern sophisticated instrumental techniques which made it possible to elucidate the microstructure of molecular species and to identify and determine the substance in the highest state of purity. Therefore, there have been several surveys in the past few decades that have summarized the recent evolution of analytical chemistry and is covered in modern analytical texts [3,4]. Moreover, Christian [5] has traced, in a report, the evolution of analytical chemistry into the 20th century and the revolution in quantitative analysis using modern analytical instruments.

Pharmaceutical analysis is one of the most promising branches of analytical chemistry. The chemistry dealing with pharmaceuticals reflects that, in 19th century, pharmacists working in their laboratories focussed on the extraction and purification of naturally occurring drugs. In 1876 the pharmacologist Buchheim wrote that the mission of pharmacology was to identify the active substance within the natural drug, to find out the chemical properties responsible for their action and to prepare synthetic analogs that were more effective [6], while the chemist took over the role of isolation and chemical identification of biologically active constituents from the medicinally important plants.
Since the drug development, introduction and pharmaceutical manufacturing entered their golden age. A large number of important and innovative new drugs was introduced day by day. Similarly, from the time a new pharmaceutical product is discovered, its journey through the market place can be as long as 15 years [7]. During this time, the drug substance will pass through many stages of development including laboratory and semi-scale synthesis, pilot plant scale-up, efficacy and toxicology testing, clinical trials and finally full scale production. Therefore, it was of utmost importance to analyse the drug and its pharmaceutical formulations.

The direct determination of drug in a mixture is generally performed for one or more purposes [8]. Either one or more active principles in a known substance are required to be accurately estimated for the purposes of standardization, proof of purity, commercial valuation, or in chemico-legal cases, in which case it may be properly called an assay, or, all of the essential ingredients of an unknown drug are required to be at least approximately separated and determined where this is possible for the purpose of obtaining some idea of the relative merits or demerits of the mixture and to provide a convenient and safe medicine to the public.

In pharmaceutical industry, the quality of the manufactured drug and its formulations must be carefully controlled. Slight changes in composition or in the purity of drug itself can affect the therapeutic values. Therefore, it is necessary to establish the properties and therapeutic value of a drug before it is approved and made available in the market. Establishment of the permissible level of dosage of a drug requires the determination of its composition, toxicity and its metabolite at various stages.

The availability of sub-standard medicines to the general public possess many problems, both clinically and economically. It is widely believed that such sub-standard preparations are readily available in many developing countries [9-14]. This may be due to
poor manufacturing procedures, poor storage conditions or deliberate counterfeiting of branded or generic products. Therefore, it is important to recognize that the drugs may contain impurities. These impurities may result from many sources like from raw materials and reagents, as reaction byproducts, and through degradation during manufacture and storage. Since, the impurities can have safety and efficacy implications, and are, therefore, the subject of considerable attention by both manufacturers and regulatory agencies.

Impurities can be classified into three groups namely: organic, inorganic and residual solvents. Organic impurities may arise from starting materials, intermediates and synthetic by-products or from reagents and catalyst or as a consequence of degradation. Inorganic impurities may result from reagents, ligands or catalysts as heavy metals or inorganic salts. Residual solvents are inevitable in drug substances since without solvents, purification and generation of the desired crystal morphology would be impossible. However, since residual solvents also 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. Qualification is defined as the process of acquiring and evaluating data which establish the biological safety of the individual impurity or a given impurity profile at the level(s) specified. Thus, the pharmaceutical analyst and toxicologist must work hand in hand throughout the pre-clinical and clinical development programme in order to be able to set meaningful specification requirements. The pharmaceutical analyst must give careful thought to the analytical methods, especially in the development phases, Therefore, it is an important task for the pharmaceutical analyst to choose an analytical technique with modern instrumentation which provides the best solution to the problem.

Modern approaches to pharmaceutical and biomedical analysis is due to the application of increasingly sophisticated methods of structural analysis like spectroscopy, isotopic
labeling, automated quantitative analysis, separation by chromatography and other partition methods. Laboratory automation through modern instruments open a new era for the study of minute amounts of biochemicals up to parts-per-billion level or lower. They also shorten the analysis time in order to achieve high-quality routine analysis.

The methods for determination of drugs and their formulations are divided into physical, chemical, physico-chemical, and biological ones. Physical methods of analysis involve the studying properties of substance. They include the determination of solubility, transparency, and the degree of turbidity; colour; density or specific gravity; melting, boiling and freezing points. Chemical methods of analysis for determination of drugs are based on chemical reactions. They include the determination of ash content, the hydrogen ion concentration (pH) of medium and characteristics numeral indices of oils and fats. Qualitative and quantitative analysis of drugs are generally carried out with respect to functional groups. Biological methods of analysis characterize the pharmaceutical effect of the drug. Biological tests are conducted on animals and less frequently on separate isolated organs or part of organs of animals. The effect of drug is expressed in activity units and is determined by comparison with the activity of a reference substance.

The most commonly used analytical techniques in pharmaceutical analysis are described below. The chromatographic methods include paper chromatography, thin layer chromatography, column chromatography, capillary electrochromatography, high performance thin layer chromatography, high performance liquid chromatography, and gas chromatography. The electrochemical methods mainly used for this purpose are polarography, voltammetry, and amperometry. The radiotracer techniques include mainly the isotopic dilution and the activation analysis. The spectroscopic techniques include nuclear magnetic resonance, infrared spectrophotometry, flame photometry, fluorometry, UV-visible spectrophotometry, derivative spectrophotometry and difference
spectrophotometry. Kinetic automatic methods are good choices for drug analysis as they permit the sensitive, selective determination of many drugs within a few seconds with no sample pretreatment in many cases. Flow injection analysis is also a very fast, simple sensitive and inexpensive method.

Chromatographic techniques have many applications in trace analysis. There are a number of analysis that could not have been accomplished in any other ways except the use of chromatographic techniques. High performance liquid chromatographic methods have been successfully applied in drug analysis and have now appeared in the dissolution monographs of pharmacopoeias for the assay of drug in dissolution fluids [15, 16]. It is becoming increasingly popular because of its sensitivity and specificity in multicomponent analysis and widely used for the identification of drugs in plasma and drug formulations [17-30].

High performance thin layer chromatographic technique is flexible enough to analyse different kinds of samples. It is an off-line technique whose every stage of the analysis can be visualized. It can be used in pharmaceuticals, biomedical and environmental analysis. Several drugs have been investigated by high performance thin layer chromatography in pharmaceutical preparations and biological fluids [31-37].

Capillary electrochromatography is a method in which a liquid mobile phase is driven through a stationary phase in a packed capillary column by the electro-osmotic flow generated by a large difference in potential across the column [38-41]. Capillary electrochromatography has been applied to the separation of several pharmaceutical compounds. A mixture of diastereoisomers not previously separated on a chiral HPLC column, was separated by capillary electrochromatography [42].

Flow injection analysis is a widely used analytical technique [43,44] and has several advantages: (1) reduced reagent consumption [45]; (2) high sampling frequency [46]; and
(3) 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 components. Flow injection analysis has been applied in the analysis of several pharmaceutical compounds [47-56].

Electrochemistry can be used at the early stage of drug researches for screening the pharmacological activity of a homologous series of newly synthesized molecules. There are several examples in the literature showing the relationship between the electrochemical data and the pharmacological properties of drugs [57-60]. Simple and rapid investigation technique such as cyclic voltammetry [61] can be advantageously applied for predicting the psychotic activity of the phenothiazine derivatives (from the differences in the stability of the cationic radical), the anti-inflammatory activity of organoselenenides (from the differences in the oxidation potential) [58] and the anthelmintic activity of arylethenylypyridinium salts (from differences in the reducing potential) [59-60]. In drug research, electrochemical techniques may have application to drug-protein [62] and drug-DNA binding studies [63] giving results useful in drug bioavailability and toxicity tests, respectively.

As far as the assay of drugs in a pure form is concerned, following the pharmacopoeia official monographs, electrochemical investigation modes are mainly concerned with potentiometric titration in non-aqueous media. Amperometric titrations are reported and few examples of polarography have been mentioned in The United States Pharmacopoeia since 1975. From review articles dealing with polarography and voltammetric analysis of drugs [64-66] it appears, however, that many drugs are electroactive. By compiling a list of most frequently occurring drugs (Table 1.1) and checking the electrochemical literature data [64-66], it was found that most of the drugs listed in Table 1.1 can be studied by polarography and/or voltammetry. For the investigation of drug formulations, cyclic
voltammetry [67], potentiometric and amperometric titration, constant current and potential
coulometry [64,65,68], ion-selective electrodes [68-71] and various voltammetric
techniques [64-66] have been successfully applied with minor sample treatments.

Table 1.1

Electroactive drugs in drug formulations as the only active ingredient

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>Nitrofurantoin</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Phenoxybenzylpenicillin</td>
</tr>
<tr>
<td>Acebutalol</td>
<td>Prednisolone</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>Prednisone</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>Retinol</td>
</tr>
<tr>
<td>Lidoflazine</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Triamcinolone</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>Vincamine</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Felodipine</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Isosorbide dinitrate</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Benidipine</td>
</tr>
<tr>
<td>Cyanocobalamine</td>
<td>Amlodipine</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Atenolol</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Nimodipine</td>
</tr>
<tr>
<td>Heparin</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td></td>
</tr>
</tbody>
</table>

The use of spectroscopic methods has been made on the largest scale in the drug
analysis. However, visible spectrophotometric methods of analysis are among the earliest
instrumental techniques, but even today they are considered to be one of the top ranking
methods because of their overall utility. These methods of analysis are based on measuring
the absorption of electromagnetic radiation by coloured compounds in the visible part of
the spectrum. If the analytes are colourless, they are converted into coloured compounds by
reaction with suitable reagent. In the last few decades, the technique has been extensively
used for determination of drugs.

UV spectrophotometric methods are simple, quick, economical, and usually do not
require an elaborate preparation step prior to assay. However, a UV method is not adequate
when two or more drugs with similar UV spectra are present in the sample or excipients or
decomposition product exhibits UV interference, as is the case with dissolution testing of
phenytoin capsules [72].

For spectrophotometric determinations, certain requirements must be fulfilled. The
important one are: application of Beer's law limit, stability of the colour, sensitivity and
selectivity of the method. Beer's law relates the absorbance with concentration by relation
A = εbc where A is the absorbance, ε is molar absorptivity, b is path length of the
absorbing medium (expressed in cm), C is the concentration of the absorbing solute. In a
general way, a straight line is obtained on plotting absorbance against the concentration.
The deviation from the straight line is also observed beyond the range of applicability of
Beer's law.

Simple colorimetric and UV methods continue to be popular for carrying out single-
component assays on a variety of formulated products. Representative examples of some of
the many assays that have been published are given in Table 1.2.

Table 1.2

Assay of drugs in drug formulations by spectrophotometric procedures

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conditions</th>
<th>Measurement(nm)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>With 1-chloro-2,4-dinitrobenzene</td>
<td>380</td>
<td>[73]</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>By oxidative coupling with m-cresol</td>
<td>640</td>
<td>[74]</td>
</tr>
<tr>
<td>Menadione</td>
<td>With sodium hydroxide</td>
<td>450</td>
<td>[75]</td>
</tr>
<tr>
<td>Substance</td>
<td>Reaction</td>
<td>Temperature</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------------------------------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>With potassium persulphate in alkaline medium</td>
<td>355</td>
<td>[76]</td>
</tr>
<tr>
<td>Thyroxin sodium</td>
<td>With nitrous acid in an ice cold bath</td>
<td>420</td>
<td>[77]</td>
</tr>
<tr>
<td>2-methyl-1,4-naphthoquinone</td>
<td>With ethylacetocetate and ethanolic ammonia</td>
<td>550</td>
<td>[78]</td>
</tr>
<tr>
<td>L-dopa</td>
<td>With sodium hydroxide</td>
<td>300</td>
<td>[79]</td>
</tr>
<tr>
<td>Methyl dopa</td>
<td>With cerium(IV) nitrate in 2M H₂SO₄ at 80 °C</td>
<td>550</td>
<td>[80]</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>With potassium persulphate in alkaline medium</td>
<td>390</td>
<td>[81]</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>With chloranil at pH 9.5</td>
<td>346</td>
<td>[82]</td>
</tr>
<tr>
<td>Thonzylamine</td>
<td>With dimethylbarbituric acid in presence of dicyclohexyl carbodiimide</td>
<td>492</td>
<td>[83]</td>
</tr>
<tr>
<td>Imipramine</td>
<td>With ammonium metavanadate</td>
<td>618</td>
<td>[84]</td>
</tr>
<tr>
<td>Desipramine</td>
<td>With ammonium metavanadate</td>
<td>618</td>
<td>[84]</td>
</tr>
<tr>
<td>Amlodipine besylate</td>
<td>With bromothymol blue</td>
<td>405</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>With 3-methyl-2-benzothiazolinone hydrazone hydrochloride</td>
<td>630</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>With NaOH</td>
<td>456</td>
<td>[86]</td>
</tr>
<tr>
<td>Aspirin</td>
<td>After hydrolysis with NaOH</td>
<td>302</td>
<td>[87]</td>
</tr>
<tr>
<td>Astemizol</td>
<td>With chloranilic acid</td>
<td>540</td>
<td>[88]</td>
</tr>
<tr>
<td></td>
<td>With suprachen violet 3B</td>
<td>590</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td>With tropaeolin 000</td>
<td>500</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td>With iron (III) and 1.10-phenanthroline</td>
<td>515</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td>With acetic acid and N-bromosuccinimide</td>
<td>520</td>
<td>[90]</td>
</tr>
<tr>
<td></td>
<td>With HCl and celestine blue</td>
<td>540</td>
<td>[90]</td>
</tr>
<tr>
<td></td>
<td>With Folin-Ciocalteau reagent and NaOH</td>
<td>720</td>
<td>[90]</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>With glycine buffer and azocarmine G</td>
<td>540</td>
<td>[90]</td>
</tr>
<tr>
<td></td>
<td>With alizarin yellow G</td>
<td>405</td>
<td>[91]</td>
</tr>
<tr>
<td>Drug</td>
<td>Reagent/Mixture</td>
<td>Wavelengths</td>
<td>Notes</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------------------------------------</td>
<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>gluconate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisapride</td>
<td>With chromotropic acid</td>
<td>530</td>
<td>[92]</td>
</tr>
<tr>
<td></td>
<td>With phloroglucinol</td>
<td>450</td>
<td>[92]</td>
</tr>
<tr>
<td></td>
<td>With N-(1-naphthyl) ethylene-diamine dihydrochloride</td>
<td>540</td>
<td>[92]</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>In Tris buffer</td>
<td>284,305</td>
<td>[93]</td>
</tr>
<tr>
<td>sodium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>With p-dimethylaminocinnamaldehyde</td>
<td>538</td>
<td>[94]</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>In HCl solution</td>
<td>230</td>
<td>[95]</td>
</tr>
<tr>
<td></td>
<td>Between pH 6 and pH 0</td>
<td>225</td>
<td>[95]</td>
</tr>
<tr>
<td>Loperamide</td>
<td>With bromothymol blue</td>
<td>414</td>
<td>[96]</td>
</tr>
<tr>
<td>hydrochloride</td>
<td>With bromophenol blue</td>
<td>415</td>
<td>[96]</td>
</tr>
<tr>
<td></td>
<td>With naphthol blue black B</td>
<td>627</td>
<td>[96]</td>
</tr>
<tr>
<td>Loratadine</td>
<td>With bromophenol blue</td>
<td>415</td>
<td>[97]</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>With p-dimethylaminocinnamaldehyde</td>
<td>665</td>
<td>[94]</td>
</tr>
<tr>
<td>Niclosamide</td>
<td>With zinc and p-benzoquinone</td>
<td>506</td>
<td>[98]</td>
</tr>
<tr>
<td>Oxprenolol</td>
<td>With Ce(IV) in H2SO4</td>
<td>480</td>
<td>[99]</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>With nitrating agent</td>
<td>410</td>
<td>[100]</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>With potassium dichromate</td>
<td>370</td>
<td>[101]</td>
</tr>
<tr>
<td>Verapamil</td>
<td>With chromotrope 2B</td>
<td>530</td>
<td>[102]</td>
</tr>
<tr>
<td>hydrochloride</td>
<td>With chromotrope 2R</td>
<td>546</td>
<td>[102]</td>
</tr>
<tr>
<td>Benidipine</td>
<td>With bromocresol green</td>
<td>408.5</td>
<td>[103]</td>
</tr>
<tr>
<td>hydrochloride</td>
<td>With bromophenol blue</td>
<td>404</td>
<td>[103]</td>
</tr>
<tr>
<td></td>
<td>With thymol blue</td>
<td>570</td>
<td>[103]</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>With zinc and HCl, followed by</td>
<td>555</td>
<td>[104]</td>
</tr>
<tr>
<td></td>
<td>diazotisation and coupling with N-(1-naphthyl) ethylenediamine-2HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atenolol</td>
<td>With chloranilic acid</td>
<td>534</td>
<td>[105]</td>
</tr>
</tbody>
</table>
Colorimetric methods based on charge-transfer reaction of certain $\pi$-acceptors have been successfully utilized in pharmaceutical analysis. The $\pi$-acceptors such as p-chloranilic acid, dichlorophenyl-indophenol and 2,3-dichloro 5,6-dicyano p-benzoquinone have been used to carry out the spectrophotometric assay of certain cardiovascular drugs: carbochromen hydrochloride, verapamil hydrochloride, acebutalol hydrochloride, carazolol and propranolol hydrochloride [108]. Spectrophotometric procedures have been presented for determination of two commonly used antidepressant drugs, fluoxetine and sertraline hydrochloride. The methods are based on charge-transfer complexation reaction of these drugs with either $\pi$-acceptors, chloranil and 2,3-dichloro-5,6-dicyano p-benzoquinone or $\sigma$ acceptor iodine [109]. The cardiovascular drugs, nifedipine and diltiazem hydrochloride have also been assayed in dosage forms by charge-transfer complexation reactions with chloranil [110], chloranilic acid and 2,3-dichloro-5,6-dicyano p-benzoquinone [111], respectively.

Acid dyes, solochrome dark blue, solochrome black T, bromothymol blue, bromophenol blue, bromocresol green, bromocresol purple, sunset yellow and indigocarmine form ion-pair complexes with basic drugs which were quantitatively extracted into chloroform. Colorimetric methods based on dye pairing continue to be important and have been used to assay such compounds as amlodipine besylate [112-114], verapamil hydrochloride [115], phenothiazine derivatives [116] and famotidine [117] in drug formulations.
Derivative spectrophotometry presents a greater selectivity and offers a convenient solution to the problem of resolving spectral overlap in the analysis of multicomponent systems [118-131]. Peak to peak and base line measurements and zero crossing measurements are the most common techniques used to prepare analytical working curves. The zero-crossing method involves the measurement of the absolute value of the total derivatives spectrum at an abscissa value (wavelength) corresponding to the zero-crossing point of the derivatives spectrum of the interfering component. At this wavelength, the amplitude of the derivative signal of one of the two components passes through zero, measurements of the value of the derivative spectrum of a mixture, made at the zero-crossing point of the derivative spectrum of one of the components is, therefore, a function only of the concentration of other component [132]. O'Haver [133] discussed the potential of the derivative spectrophotometry in clinical chemistry. Fell [134] has demonstrated the possibilities offered by this technique for the analysis of pharmaceutical formulations.

Second derivative approaches have been described for antidepressants clomipramine [135] and imiprmine [136] hydrochlorides, the flavanoids chrysin and quercetin [137,138] and the anticholinergic homoatropine hydrobromide [139]. A second-derivative procedure also has been used to measure 3-chloro-N-chloro-N-(3,4-dimethyl-5-isoxazolyl)-4-amine-1,2-naphthoquinone in the presence of its degradation product which was reported not to interfere [140]. In the case of esculine and routine, not only have the first and second-derivative spectra been used but also higher-derivative curves [141]. Differences in the measurement accuracy using these approaches are considered. Other applications of derivative spectrophotometry includes its use to measure acyclovir and diloxanide furoate in the presence of degradation products and impurities [142] as well as to determine amiloride [143], benazepril hydrochloride [144], enalapril maleate [135], and metoprolol and propranolol [136] in combination products containing hydrochlorothiazide.
Difference spectrophotometry has been proved particularly useful in the determination of medicinal substances by eliminating specific interference from degradation products, co-formulated drugs and also the non-specific irrelevant absorption from the formulation matrix. Its advantages for selective analysis have been described by several workers [145-149]. The technique involves the reproducible alteration of the spectral properties of the absorbance difference between two solutions, provided that the absorbance of the other absorbing substances is not affected by the reagents used to alter the spectral properties. Simple aqueous acids, alkalis and buffers are most frequently used for inducing spectral alteration since many drugs are weak acids or bases whose state of ionization and absorptivity depend on the pH of the solution [150].

Kinetic methods are good choices for drug analysis as they permit the sensitive and selective determination of many drugs within a few seconds with no sample pretreatment in many cases. They seem to be more attractive and superior to the other colorimetric methods due to the formation of unstable colours in some of these colorimetric ones. Moreover, the instrumentation required is generally very simple. The principles and applications of the kinetic methods have been reviewed in papers [151,152] and books [153,154]. Essentially, kinetic methods rely on 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 reactions tolerate manual mixing and, even so, they may be better handled automatically, not only to obtain more rapid and reproducible results, but also to increase the reaction rate in some cases. Kinetic automatic techniques are generally based on open systems, among the most popular of which are stopped flow (SF) system and the continuous addition of reagent (CAR) technique [155-158]. Several drugs have been determined by using CAR technique with photometric [159,160] and
fluorimetric [161] detection. These techniques are mandatory for fast reactions but can also be extended to slow reactions. In both cases, the kinetic curve (variation of the analytical signal with time) can be recorded immediately. The slope of the straight initial portion of the kinetic curve gives the reaction rate, which is proportional to the analyte concentration (initial-rate method). The fixed-time method, also frequently used to derive such a concentration, involves measuring the signal (the value of which depends on the analyte concentration) at a preset time.

These determinations can be implemented by using combinations of the SF technique with (a) fluorescence polarization immunoassay (FPIA), (b) micelle-stabilized room-temperature phosphorimetry (MSRTP) and (c) a sensitized energy-transfer fluorescence reaction, among other, all are novel strategies which have so far yielded excellent results in drug analysis. On the other hand, the CAR technique has been extended to chemiluminescence (CL) reactions as CAR chemiluminescence spectrometry (CARCL), a new approach which have proved outstanding for the analysis of drugs and other substances of analytical interest.

The use of kinetic methods [162-165] in micellar media is another recent approach to kinetic-based determinations that has proved useful in drug analysis. Micelles increase the reaction rate (through micellar catalysis) and may additionally increase the sensitivity and selectivity for the analyte. On the other hand, new kinetic chemometric (kinetometric) approaches such as the Kalman filter have been developed and applied to the simultaneous determination of various compounds of pharmaceutical interest.

Before an analytical method can be used for routine analysis, it must first be demonstrated that the method fulfils certain performance criteria. When this has been documented, the method is said to be validated. The parameters that require validation and the approach adopted for each particular case are dependent on: (a) the purpose of the
method, and (b) the sample matrix. With respect to the purpose of the method, one should consider for example whether it is to be applied to the determination of a major or minor component, or for evaluation of performance characteristics (e.g. dissolution test for a solid dosage form) and whether the test will then be used to support a release specification or a stability study.

In the field of drug analysis, it is very clear that the definitions cover the entire field of analytical chemistry from bioanalysis to substance and product analysis. There are a number of definitions [166] given by several international organizations which are listed below:

Validation of analytical methods – international definitions

<table>
<thead>
<tr>
<th>Organization</th>
<th>Applicability</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>IUPAC</td>
<td>Worldwide</td>
<td></td>
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<tr>
<td>ILAC</td>
<td>Worldwide</td>
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<tr>
<td>WELAC</td>
<td>Europe</td>
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</tr>
<tr>
<td>ICH</td>
<td>Europe, Japan, USA</td>
<td>Only pharmaceutical products</td>
</tr>
<tr>
<td>ISO</td>
<td>Worldwide</td>
<td>Lacks definitions of selectivity and specificity</td>
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In the last few years harmonization efforts have been rather intensive in the pharmaceutical field and also for the validation process. Unfortunately, for analytical chemists, this effort from the regulatory bodies side as exemplified by the ICH, has only focussed on validation of analytical procedures used for the control of substances and
formulations. Similar requirements have, however, also to be applied for analysis of drugs in body fluids. Most of the definitions recommended by the ICH are concordant with those of other organizations such as IUPAC, ILAC and WELAC. Terms and definitions should have the same meaning all over the world, whatever kind of analysis is performed.

Method validation is an important component in determining the reliability and reproducibility of a bioanalytical method, and is a requirement of any regulatory submission [167]. The policy of Food and Drug Administration (USA) states that for each analytical method used to quantitate drug concentrations from biological fluids, specific analytical parameters must be determined with respect to accuracy, linearity, precision, sensitivity, specificity and recovery.

The overall validation strategy consists of four components, which are the prevalidation, validation proper, study proper and statistical analysis. These components constitute the platform upon which to evaluate the reliability and ruggedness of an analytical method.

**Prevalidation**

Before validation proper can be contemplated, an authenticated analytical reference standard must be available to prepare solutions of known concentrations. This standard should be of a known form, e.g. free base or salt and of known purity, if used over a time, should be monitored to ensure no decomposition or contamination has taken place. Prevalidation provides the analyst an opportunity to obtain some practical experience with the method and helps to identify the optimum experimental conditions. It is also recommended that the following studies be conducted prior to initiating the validation proper. The appropriate 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 optimum standard curve range and the number of
calibrators should be established. The appropriate regression model, which best fits the data is then selected. The extraction scheme and its recovery should be optimized to give insight into the limit of quantitation and to help determine if the extraction procedure is reproducible.

**Validation proper**

The fundamental parameters for assessing the reliability and overall performance of an analytical method which should be addressed in a validation exercise include: analyte stability, selectivity/specificity, limits of detection and quantitation, accuracy, precision, linearity and recovery.

**Analyte stability**

Published literature should be investigated, or laboratory tests should be conducted to determine whether pure analyte and/or solutions of the analyte (e.g. drug) are stable under normal laboratory conditions of heat, humidity, light and air exposure.

**Accuracy**

A reasonable definition is provided in the United States Pharmacopoeia XXII [168]:

"The accuracy of the analytical method is the closeness of test results obtained by that method to the true value." Some details of how accuracy should be determined are provided in The USP XXII and Committee for Proprietary Medicinal Product (CPMP) guidelines [168,169].

In bulk drug analysis, the approach adopted depends on the assay method to be adopted. For titrations the expected equivalence point may be calculated on theoretical grounds, taking into account the number of titratable functions in the analyte and the molecular weight, but it is recommended that this should then be verified by carrying out the intended titration procedure on a well characterized reference standard.
For light absorption assays based on specific absorbance values, the validation would require that this value be well selected. It should be noted that these values for closely related compounds may vary considerably, so it would also be appropriate to demonstrate that this is not significantly influenced by the presence of likely impurities at their intended maximum limits. For chromatographic assays, the mass balance approach of Krischbaum et al. is recommended [170]. This is based on the comparison of total peak response with and without the chromatographic column in place; a variance of not more than ± 2% should normally be achieved.

Precision

This describes the closeness of replicate determinations of an analyte by an assay procedure. Precision can be further sub-divided into within day precision, intra-assay precision or repeatability (which is an assessment of precision during a single analytical run) and between-day precision, Inter-assay precision or reproducibility (which shows the variation of precision with time and may also include different analytical staff, equipment and reagents).

Intermediate Precision Conditions

The M-Factor for different intermediate precision conditions \([M=1, 2 \text{ or } 3]\) are:

(a) \(M=1\) where only one of the three factors (operator, equipment or time) is different or where the instrument is recalibrated between successive determinations, (b) \(M=2\) where two of the three factors are different, and (c) \(M=3\) where all the three factors are different between successive determinations.

Linearity

A reasonable definition is provided in CPMP Guidelines [169]: “The linearity of a test procedure is its ability (within a given range) to produce results which are directly proportional to the concentration of analyte in the sample”.
Limit of detection (LOD)/ quantitation (LOQ)

Limit of detection (LOD) is a parameter of limit tests and may be defined as the smallest quantity of analyte which may be expected to produce a response which is significantly different from that of a blank.

Limit of quantitation (LOQ) is a parameter of determination test and may be defined as the smallest quantity of analyte which can be determined with acceptable accuracy and precision.

According to IUPAC [171] for spectrochemical methods these values may be determined for the smallest concentration \((c_i)\) or amount \((q_i)\) from: \(c_i(q_i) = kS_B/S\), where \(k\) is a constant, \(S_B\) is the standard deviation of analytical blank signal, and \(S\) is the slope of response versus concentration curve (slope sensitivity). Recommended values for \(k\) are: for LOD, \(k = 3\); and for LOQ, \(k = 10\). Justification of these values has been provided by Long and Winefordner [172] and by the Analytical Methods Committee [173]. For this to be used in practice then, requires determination of the value of \(S_B\), which is related to baseline noise. For spectrochemical methods this is not a problem as the analyst can conduct a series of absorbance determinations with blank solutions, using instrumental conditions intended for sample determinations. The standard deviation may then be calculated.

Selectivity and Specificity

One of the key definitions for analytical chemist is, without doubt, selectivity. This can be considered as the hallmark of the analyst. If it is not clear about the effect of potential interferences in the method, all other attributes such as accuracy, precision, linearity, etc. are meaningless. The definition by WELAC [174] clearly states that selectivity of a method refers to the extent to which it can determine particular analyte (s) in a complex mixture without interference from other components in the mixture. A method which is perfectly selective for an analyte or group of analyte is said to be specific. A clear distinction
between the terms specific and selective has been made by Christian in his well-known treatise, Analytical Chemistry [175]. He stated that a specific reaction or test is one that occurs only with the substance of interest, while a selective reaction or test is one that can occur with other substances but exhibits a degree of preference for the substance of interest. Few reactions are specific but may exhibit selectivity.

To visualize the difference between specificity and selectivity, a graphical representation is given in Fig. 1.1 Selectivity is something that can be graded or scaled but specificity is an absolute characteristic.

![Graphical demonstration of selectivity and specificity.](image)

**Fig. 1.1.** Graphical demonstration of selectivity and specificity. The percentage of the measured signal attributable to the analyte alone (on the Y-axis) is given as a function of the degree of selectivity (b). A perfectly selective method is said to be specific (a).

**Study Proper and Statistical Analysis**

Daily standard curves are generated to determine the sample concentrations. The quality control sample sequence is carefully monitored for systematic errors. For each
standard curve, the slope, intercept, variance and correlation coefficient are reported. From the data generated, specific analytical parameters are reported including linearity, accuracy, precision, sensitivity and recovery. Acceptance of the assay results is determined by monitoring the quality control results. If the concentrations are within the control chart’s 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 databases to update their confidence limits.

As part of an on-going study looking at the quality of selected pharmaceuticals in developing countries, it was necessary to assay the pharmaceutical substances in all kinds of formulations. It is also necessary to establish methods, which are simple, sensitive, accurate and rapid and can be used for routine analysis in the laboratories of limited resources in these countries.

This thesis deals with the spectrophotometric determination of some antianginal drugs with special reference to calcium channel blockers namely nifedipine, diltiazem hydrochloride, amlodipine besylate and verapamil hydrochloride in pharmaceutical formulations.

**ANTIANGINALS**

Angina pectoris is a symptom of ischaemic heart disease [176]. Drugs used in angina pectoris are those that reduce cardiac work and myocardial oxygen need by (a) unloading the heart (b) dilating capacitance and resistance vessels (c) dilating coronary arteries and (d) blocking β-adrenoceptors. Anginal pain occurs when the coronary blood flow is insufficient to meet the heart’s metabolic requirements, it can be counteracted by drugs that either improve myocardial perfusion or reduce the metabolic demand, or both. Several antianginal drugs are available for treatment of angina. These include (a) Organic nitrates
(b) beta adrenergic blockers (c) calcium channel blockers (d) potassium channel activators and (e) antiplatelet drugs.

**Calcium channel blockers**

Calcium channel blockers are heterogeneous group of drugs whose main pharmacological effect is to prevent or slow the entry of Ca$^{2+}$ into cells via specialized calcium channels. Other names used for this class of drugs include calcium entry blockers, calcium antagonists and slow channel blockers, since the entry of calcium into cells is slower than sodium entry after stimulation.

Ca$^{2+}$ enters cells through specialized pores in the membrane wall called calcium channels. Some channels are activated by membrane depolarization (voltage operated) and others by activated receptors for neurotransmitters and/or sundary hormones and tissue factors (receptor-operated). Calcium channel blockers describe Ca$^{2+}$ entry in both the voltage-operated and receptor operated channels, but the voltage-operated channel is the more sensitive to drug blockade.

Three different calcium channels have been characterized. L-(long opening) and T-(transient) channels have been found in neurosecretory cells and cardiac, smooth and skeletal muscle cells, and N-(neuronal) channels have been located only in neurons. Each channel type has its own characteristic activation and inactivation voltage ranges, channel conductance, sensitivity to blockade by organic and inorganic compounds, etc, such that each has a unique pharmacoresponsive profile. Also, calcium channel blockers exhibit both frequency- and voltage-dependence in their ability to block Ca$^{2+}$ movements. Verapamil, for example, binds to open channel and hence selects cells that are stimulated frequently; in contrast, dihydropyridines bind more strongly to inactivated channels and hence show little frequency-dependence. However, all calcium channel blockers are voltage-dependent in that they are much better blockers when the tissue is depolarized. None of the calcium
channel blockers actually occlude the channel directly but rather act at allosteric sites on channel proteins to evoke conformational changes that cause occlusion. Calcium channel blockers have been approved for the oral treatment of variant (vasospastic) and chronic stable and unstable exertional angina pectoris. They are useful in the therapy of these diseases for three reasons: (a) they directly dilate coronary arteries and increase myocardial blood flow (b) they decrease myocardial oxygen demand by peripheral arteriolar dilation which increases afterload and (c) they exert negative chronotopic and inotropic actions which also decreases oxygen demand.

The nine calcium channel blockers that have been approved for clinical use have diverse chemical structures. Five classes of compounds have been examined [177]: (a) phenylalkylamines (b) dihydropyridines (c) benzothiazepines (d) diphenylpiperazines and (e) diarylaminopropylamine.

At present, verapamil hydrochloride (a phenylalkyl amine); diltiazem hydrochloride (a benzothiazepine); nifedipine, amlodipine besylate, nicardipine, isradipine, felodipine and nimodipine (dihydropyridines); and bepridil (a diarylaminopropylamine ether) are approved for clinical use.

**Nifedipine**

Nifedipine, i.e. dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl) pyridine-3,5 dicarboxylate, is the prototype compound of dihydropyridine class of calcium channel blockers with peripheral and coronary vasodilator properties. It reduces peripheral resistance, afterload and blood pressure. It is given by mouth in the management of hypertension and angina pectoris. In human, nifedipine is rapidly metabolized by oxidative mechanisms to dehydronifedipine which is further metabolized to more polar compounds [178-181] and their structures are given below:
where (A) nifedipine, (B) dehydronifedipine, (C) nitroso-analogue of dehydronifedipine and (D) nisoldipine.

Nifedipine is highly sensitive to photo-oxidation, changing in colour from yellow to brown upon exposure to light [182]. Nifedipine photodegradation products have a little or no pharmacological activity [183,184]. Several studies in the past have been conducted to determine the photostability of nifedipine in solution and in the solid state, including photostability of pulverized nifedipine tablet powders [185-187].

Official USP guidelines for nifedipine capsules state that the measured amount of nifedipine in commercial product be within 90–110% of the labelled quantity [188]. Manufacturers of nifedipine product utilize light resistant coatings and / or packaging to minimize photodegradation of oral nifedipine preparations from inadvertent light exposure. Few quantitative studies on the light transmissive properties of different light protective tablet film coatings or protective packaging have been done [189]. Thus, differences in
degree of light protection may exist between different brands and/or formulation types of nifedipine products.

**Diltiazem hydrochloride**

Diltiazem, (+)-5-[2-(dimethylamino)ethyl]-cis,2,3-dihydro-3-hydroxy-2-(p-methoxyphenyl)-1,5-benzothiazepine-4(5H)-one acetate monohydrochloride developed in Japan in the early 1970's [190] is a benzothiazepine calcium channel blocking agent and its structure is shown as:

![Structure of Diltiazem Hydrochloride](image)

It is a peripheral and coronary vasodilator with limited negative inotropic activity but its vasodilator properties are less marked than those of the dihydropyridine calcium channel blocker (nifedipine). Unlike nifedipine, diltiazem hydrochloride inhibits cardiac conduction, particularly at the sino-atrial and atrioventricular nodes. It is used in the management of angina pectoris and hypertension.

**Amlodipine besylate**

Amlodipine besylate is a dihydropyridine calcium channel blocking agent and chemically it is 3-ethyl 5-methyl2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl pyridine-3,5-dicarboxylate monobenzene sulphonate. The structure of amlodipine is shown as:
It is used in the management of hypertension and angina pectoris. Its actions are similar to those of nifedipine.

**Verapamil hydrochloride**

Verapamil, 5-[N-(3,4-dimethoxyphenylethyl) methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropyl valeronitrile monohydrochloride is a calcium channel blocker belonging to the phenylalkylamine class of compound and its structure is shown as:

It is a vasodilator primarily indicated for anginas and arrhythmias. Verapamil is less potent vasodilator in vivo than are the dihydropyridines.
REFERENCES


