Chapter - 1

General Introduction
In the context of the present knowledge, the less predictive experiments, observations and interpretations of the ancient philosophers and scholars must be regarded as purely heuristic which were based on the superstitions and misunderstandings of the natural phenomena. Nevertheless, the account of those deeds of antiquity, the middle ages and even the first 350 years of the “modern age” make fascinating anecdotal reading. But even today it is a customary in good sense and justified too, to credit them who have, in fact, smoothen the pathway for the recent sophisticated and conclusive research works.

It was Aristotle who had anticipated the nature of the hormones and claimed that the humours were secreted at certain cites to act elsewhere in the body and thus gave birth to the Chemical Science, and liberated it from the preconceived ideas of mysterious forces. During the period regarded as the “Quintessence to Chemical” (Paracelsus, 16th century–Ehrlich, 19th century) [1], people had undergone major gradual change in the use of natural products in their entire states (mainly having medicinal importance) to either the purified extracts of the material of interest from those or to synthetic chemically–produced materials.

Louis Pasteur (1822–1895) had given a great breakthrough to the chemotherapy for the treatment of infectious diseases caused by the pathogenic parasites. This lead to a wave in antiprotozoal chemotherapy system around 1890, which forced to develop some hypnotic, antimalarial and anti-inflammatory drugs, and adrenergic and cholinergic hormones, followed by the discovery of antibacterial and antibiotics, pharmacodynamics of analgesics, antihistaminics, vitamins and few new hormones. The post World War–II period had given major breakthrough to the drug development and a number of antituberculous agents, the steroid hormones and contraceptives, antipsychotic, anxiolytic and
antidepressant psycho-pharmacological drugs were discovered. And finally with the enforcement of Pure Food and Drug Act by the Food and Drug Administration of the United States, the therapeutic research, drug development, introduction and pharmaceutical manufacturing entered their “Golden Age” (1940–1960).

With the cautious nature of human being and growing awareness especially for the health hazard reasons, the concept of quality emerged which lead to the interest in the work of analysis. With no name designated and no specificity given in the old age, analytical chemistry is the important part of the good quality life in every period of the development. The very basic aspect of analytical chemistry defines it as “the relationship between the so-called intrinsic chemical information of the objects and systems [2] and the information (results, reports) provided by the analytical systems (laboratories, on-site analysers etc.)”. A book written by Thomas De Quincey [3] can well document the old age relation of analytical chemistry with our daily life. In his book “Confessions of an English Opium Eater”, the man of letters penned about his surgeon friend who was suffering from a lingering and fatal malady. The surgeon was on the horns of a dilemma as he had a family to support and thus eager to continue in work. Because of his professional knowledge, he saw the necessity of reducing his daily dose of opium that he took to alleviate his pains to a minimum. I now quote the words of De Quincey in this context: “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 impurities”. With the skills of the time (about 1820) De Quincey’s surgeon friend was unable for such an evaluation. He did, however, achieve a uniform method of extracting the opium so as to give a fairly constant potency. G.D. Christian [4] has traced, in a report, the evolution of
analytical chemistry into the 20th century and the revolution in quantitative analysis using modern analytical instruments.

The chemical analysis is divided into qualitative and quantitative types of analyses. The qualitative analysis deals with the detection of constituents or components present in the analyte under investigation. The quantitative analysis determines the proportions in which the constituents or the amount of constituents are present in the matter of interest.

Undoubtedly, as far as the quality in the analytical results is concerned, the top aim of the analytical chemists is to minimise the differences between the ideal, true information and that produced in the routine work. This primary goal of analytical chemist relies heavily on two capital analytical properties: the accuracy and the representativeness. In the search of excellency in these aspects, the analytical chemists worldwide are continuously making their best efforts for developing the sophisticated (accurate and precise) and sensitive techniques. Here the technical aspect is not including the instrumentation only, but also the various types of other trial like the suitable modifications in the methods of estimation, certain changes in the traditional ways of conducting the chemical reactions and sometimes developing new theoretical concepts for the old/previous existing instruments/methods.

Not only the different theoretical aspects or the advance instrumentation (whether it is old or new) are affecting the quality in the analytical results but also it is seriously influenced by the different analytical properties. These analytical properties may be classified as the capital (accuracy and representativeness), basic (sensitivity, selectivity, precision and sampling) and accessory (expeditiousness, cost-effectiveness and personal-related considerations like personal safety and comfort). The capital analytical properties are associated with the
consistency in the results and strongly rely on the basic properties which are responsible for
the quality in the output of both inter as well as intra-laboratory analytical processes. Accessory
analytical properties, though seems to be less significant, but often have major practical
implications and sometimes affect the output heavily. Thus the basic and accessory properties
are related to the analytical methodology. The hierarchy of these important components has
been presented (Fig. 1.1) while their interdependence is shown in Fig. 1.2.

**Figure 1.1.** Goals of analytical chemistry and their relationships to the analytical quality and
analytical properties.

**Figure 1.2.** Analytical tetrahedra showing interdependence of the analytical properties.
An investigation of Fig. 1.1 shows that these are the basic properties, which are mainly
deciding with the top goal of the analytical methodology, i.e. accuracy and representativeness.
In fact these basic properties are themselves interdependent and affect each other strongly.
Due to this strong relation, the excellency or even a simple enhancement in any of the basic
property can only be achieved at the expense of another one. The present generic objective of
the analytical chemistry is to get the high quality analytical information by expending minimum
material, less time with least number of the man-power and minimum risks at the lowest cost of
expense.

As far as the pharmaceutical analysis is concerned, it always needs for the sensitive
and sophisticated techniques. The most frequently used analytical techniques in pharmaceutical
analysis are titrimetry, chromatography, electrochemical analysis 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 of analyses, 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.

Chromatographic methods have many applications in trace analysis and sometimes
prove it as the only way. The different kind of it like paper chromatography, thin layer
chromatography, column chromatography, capillary electrochromatography, high performance
thin layer chromatography, high performance liquid chromatography and gas chromatography,
have most frequent applications in the field of pharmaceutical as well as the biomedical analyses. Thin layer chromatography (TLC) is a simple, in-expensive and rapid technique, having wide applications for the separation and identification of impurities and degraded products [5]. Capillary electrochromatography (CEC) is a method in which 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 [6–9]. CEC previously has been utilised for the separation of certain pharmaceuticals; an interesting example is that of a diastereoisomeric mixture not successfully separated on a chiral HPLC column [10], which clearly shows its considerable advantage. Among all the chromatographic methods, high performance liquid chromatography is most acceptable nowadays due to its sensitivity and selectivity even in a multicomponent mixture. It is widely used for the identification of drugs in plasma and dosage forms [11–24], and creating its own position in the dissolution monographs of pharmacopoeias for the assay of drugs in dissolution fluids [25, 26].

Electrochemical methods are characterised by high sensitivity, selectivity and accuracy. These methods are based on the basic electrical parameters like current (I), resistance (R) and voltage (V) which has been utilised alone or in combination for analytical purposes. Pharmaceuticals containing phenolic, amino, heterocyclic nitrogen, ketonic or aldehyde group undergo oxidation at their characteristic potentials and hence selectivity of the technique is increased. Important electrochemical techniques such as amperometry, conductometry, potentiometry, anodic and cathodic stripping voltammetry, differential pulse voltammetry and polarography have been used in drug analysis [27]. Ion-selective electrodes are electrochemical sensors that allow potentiometric measurements of the activity of particular species in aqueous and mixed solvents, or partial pressures of dissolved gases in water [28].
Potentiometric titrations are also reported in the pharmacopoeias as the standard method for the determination of certain drug substances [29–31]. Despite the wide use of spectrometry and chromatography, analysts are choosing polarography and voltammetry due to their sensitive and characteristic qualitative as well as quantitative nature of analysis. Bersier has reviewed the importance of these techniques in pharmaceutical analysis [32].

Spectroscopic methods are widely used for the assay of drugs in pharmaceutical formulations. The most commonly used spectroscopic techniques for quantitative analysis are ultraviolet (UV) and visible methods. UV–spectrophotometry is best known and most widely applied in structure identification and quantitative analysis. It has become an established and fundamental technique in pharmaceutical research and analysis as well as in the broad area of organic and inorganic analysis. Conventional UV–spectrophotometric methods are simple, fast and economical and do not need any elaborate preparatory step for the samples prior to assay. Endriz [33] has described an UV–spectrophotometric method for the determination of heroin hydrochloride, methapyrilene hydrochloride, and quinine hydrochloride mixtures. The spectra for heroin, methapyrilene, and quinine show points of maximum absorption at 280 nm, 313 nm and 348 nm, respectively. Methapyrilene and quinine do not seriously interfere with the heroin maximum, and methapyrilene in dilute acid solution absorbs only slightly at the quinine maximum of 348 nm. The procedure is fast and accurate enough for forensic purposes. Analyses of chloramphenicol and tetracycline hydrochloride [34] and phenobarbital and pentobarbital in pharmaceutical formulations [35] are further examples of the utility of UV–spectrophotometry in this area. Because of the sensitivity of the UV method, the amount of sample required for the spectral identification is very small; drug levels and metabolites have been determined in biological tissues and fluids after separation and extraction. Since most
materials are not photosensitive, the technique is non-destructive, and the metabolite or drug can be recovered for further testing. However, these methods are inadequate in the presence of other components (like any drug of combination, excipients or decomposition products) showing similar UV spectra [36].

Photocolorimetric methods of analysis are performed in the visible region of light. They are based on the measurement of the absorbance of the coloured compounds. Usually, the analyte being colourless, they are reacted with the 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 some of the many assays that have been published are given in Table 1.1.

In the course of this discussion, the importance of computer-aided spectrophotometric determination of multicomponent systems can not be ignored. The quantitative spectrophotometric analysis of active ingredients in a solution containing more than two substances can be difficult to achieve by means of the traditional methods. In order to resolve this problem, several papers were published on the application of least squares method and orthogonal polynomials for the analysis of multicomponent systems [64–69].

Impurities present on the particle surfaces, e.g. from residual mother liquor, may be low in terms of percentage but they may change in a significant way the behaviour of the powder in the manufacturing process or in the final medicinal product. Therefore, a methodology is needed to detect such deficiencies at the surfaces or in the upper layers of the powder. Spectroscopic reflectance technique of sufficient sensitivity and precision could be of
<table>
<thead>
<tr>
<th>Compound</th>
<th>Method</th>
<th>Conditions</th>
<th>Measurement (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>Colorimetric</td>
<td>By oxidative coupling with m-cresol</td>
<td>640</td>
<td>37</td>
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<tr>
<td>Ascorbic acid</td>
<td>Colorimetric</td>
<td>With 1-chloro 2,4-dinitrobenzene</td>
<td>380</td>
<td>38</td>
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<tr>
<td>Amlodipine besylate</td>
<td>Colorimetric</td>
<td>With p-chloranilic acid in chloroform-dioxane mixture</td>
<td>540</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>With ninhydrin in DMF</td>
<td>595</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>With bromothymol blue</td>
<td>405</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>With 3-methyl 2-benzothiazolinone hydrazone hydrochloride</td>
<td>630</td>
<td>41</td>
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<tr>
<td></td>
<td></td>
<td>With sodium hydroxide</td>
<td>456</td>
<td>42</td>
</tr>
<tr>
<td>Astemizol</td>
<td>Colorimetric</td>
<td>With p-chloranilic acid</td>
<td>540</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>With suprachen Violet 3B</td>
<td>590</td>
<td>44</td>
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<tr>
<td></td>
<td></td>
<td>With tropaeolin 000</td>
<td>500</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>With iron(III) and 1,10-phenanthroline</td>
<td>515</td>
<td>44</td>
</tr>
<tr>
<td>Azapropazone</td>
<td>Colorimetric</td>
<td>With N-bromosuccinimide</td>
<td>488</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>With N-chlorosuccinimide</td>
<td>451</td>
<td>45</td>
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<tr>
<td>Benidipine hydrochloride</td>
<td>UV</td>
<td>In methanol</td>
<td>238</td>
<td>46</td>
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<tr>
<td>Bromazepam</td>
<td>Colorimetric</td>
<td>With Mohr-salt</td>
<td>584</td>
<td>47</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>UV</td>
<td>In Tris buffer</td>
<td>284, 305</td>
<td>48</td>
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<tr>
<td>Diltiazem hydrochloride</td>
<td>Colorimetric</td>
<td>With sodium metavanadate in sulphuric acid</td>
<td>750</td>
<td>49</td>
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<td></td>
<td></td>
<td>With bromothymol blue</td>
<td>415</td>
<td>50</td>
</tr>
<tr>
<td>Compound</td>
<td>Method</td>
<td>Condition</td>
<td>Wavelength (nm)</td>
<td>Absorbance (nm)</td>
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<tr>
<td>Flurazepam</td>
<td>UV</td>
<td>In HCl solution between pH 6 and pH 0</td>
<td>230</td>
<td>51</td>
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<tr>
<td></td>
<td>UV-difference</td>
<td></td>
<td>225</td>
<td>51</td>
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<tr>
<td>Loratadine</td>
<td>Colorimetric</td>
<td>With bromophenol blue</td>
<td>415</td>
<td>50</td>
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<tr>
<td>L- dopa</td>
<td>UV</td>
<td>With sodium hydroxide</td>
<td>300</td>
<td>53</td>
</tr>
<tr>
<td>Menadione</td>
<td>Colorimetric</td>
<td>With sodium hydroxide</td>
<td>450</td>
<td>54</td>
</tr>
<tr>
<td>Methyldopa</td>
<td>Colorimetric</td>
<td>With Ce(IV) nitrate in 2M sulphuric acid medium at 80 °C</td>
<td>550</td>
<td>55</td>
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<tr>
<td></td>
<td></td>
<td>With barbituric acid at 100 °C</td>
<td>540</td>
<td>56</td>
</tr>
<tr>
<td>2-methyl 1,4- naphthoquinone</td>
<td>Colorimetric</td>
<td>With ethylacetoacetate and Ethanolic ammonia</td>
<td>550</td>
<td>57</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Colorimetric</td>
<td>With potassium permanganate at neutral pH</td>
<td>530</td>
<td>58</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Colorimetric</td>
<td>With potassium persulphate in alkaline medium</td>
<td>390</td>
<td>59</td>
</tr>
<tr>
<td>Oxprenolol</td>
<td>Colorimetric</td>
<td>With cerium (IV) in sulphuric acid</td>
<td>480</td>
<td>60</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>Colorimetric</td>
<td>With chromotrope 2B</td>
<td>530</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>With chromotrope 2R</td>
<td>546</td>
<td>61</td>
</tr>
<tr>
<td>Thyroxin</td>
<td>Colorimetric</td>
<td>With nitrous acid in ice bath</td>
<td>420</td>
<td>62</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>UV</td>
<td>With potassium persulphate in alkaline medium</td>
<td>355</td>
<td>63</td>
</tr>
</tbody>
</table>
help to discriminate between apparently identical materials from different sources or to detect inconsistencies between the batches from the same source.

Near infrared (NIR) spectroscopy is a technique which has found its way into pharmaceutical control laboratories in recent years for raw material identification, water analysis and other pharmaceutical analyses [70–73]. Nuclear magnetic resonance (NMR) spectroscopy is a well-known spectroscopic technique specially used for the qualitative characterisation of the chemicals. In the recent attempts, NMR is also used as the sensitive quantitative method; a mini review has been presented in this context [74]. Moreover, NMR spectroscopy is favoured by chemists as a powerful technique for molecular structure determination.

Chemiluminescence is a powerful tool for drug analysis since its detection limits are extremely low, its instrumentation is very simple and of low cost. In combination with the derivatisation techniques in order to increase sensitivity, it has a wide range of applications. The excellent sensitivity and the versatility of the chemiluminometric methods of analysis are the main reason for the recent surge of interest in chemiluminescence. Certain drugs have been analysed based on the phenomena of chemiluminescence in liquid phase [75–77].

Flow injection analysis (FIA) is characterised by its simplicity, speed and the use of inexpensive equipment. In FIA, as a general principle [78], a quantity of accurately measured dissolved sample is injected or introduced into the carrier stream flowing through the system tubing, with or without additional changes like chemical reaction etc., occurring between the sample and the carrier. As the analyte (or its reaction product) passes through the continuous detector, a transient signal is generated and recorded. Therefore, FIA has certain clear
advantages like (i) reduced reagent consumption [79]; (ii) high sampling frequency [80]; (iii) safety in applying toxic reagents because the whole analysis proceeds in a closed system; and (iv) increased selectivity when the analyte is accompanied by more slowly reacting components. This technique has been utilised successfully in the determination of some compounds of pharmaceutical interest [27,81,82]. In the recent years, atomic absorption and emission spectrophotometry is also frequently used in the field of pharmaceutical analysis [83–85].

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 alterations 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 [86].

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 [87–89] and has advantages for the solution of specific analytical problems. The derivative method has found applications not only in the UV–visible spectrophotometry, but also in infrared [90], atomic absorption [91], flame emission spectrometry [92] and fluorimetry [93,94]. The use of derivative spectrometry is not restricted to special cases, but may be of advantage whenever quantitative study of normal spectra is difficult. Its disadvantage is that the differentiation degrades the signal-to-noise ratio, so that some form of smoothing is required in conjunction with the differentiation [95].
Even though many pharmaceuticals show native fluorescence, there is also an important group of compounds, which is not fluorescent, can be determined by using fluorescent probes through derivatisation reactions. An account of the fluorescent probes more widely used for the determination of drugs and related compounds have been reviewed [96].

In the recent period, analysts are much interested in coupling the chromatographic techniques with that of the spectroscopic techniques. The advent of liquid chromatography with mass spectrometry, gas chromatography with tandem mass spectrometry and liquid chromatography–electrospray tandem mass spectrometry are good examples. Hirsch et al. have determined the antibiotics using chromatography–electrospray tandem mass spectrometry[97]. Dielectric relaxation spectroscopy and x-ray powder diffractometry are also utilised for the identification of pharmaceuticals [98,99]. The safety of a drug product is dependent not only on the toxicological properties of the active drug substance itself, but also on the impurities that it contains. Monitoring of drug substance’s impurities is routinely accomplished using HPLC. However, HPLC retention times can vary, resulting in uncertainty as to whether a peak at a new retention time is a new impurity. Because standards of the minor impurities (less than 0.1% by area) are not usually available, some method is needed to characterise each of these peaks without isolating them. This on-line characterisation might be accomplished using UV diode array spectral matching [100].

Whenever the question of mathematical and statistical treatments arises, the role of chemometrics can not be ignored. Chemometrics is defined as “the chemical discipline that uses mathematical, statistical and other methods of formal logic to design or select optimal procedures and experiments, and provide maximum chemical information by analysing chemical
data” [101,102]. Chemometrics is the science that helps to make good use of information technology in the chemical analysis and to develop intelligent analysers, i.e. that automatically select the correct method for a given problem, carry it out, validate it and interpret the results.

Kinetic method of analysis, which is in fact, the study of the measurement 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, is developing since late 1950s. They are not only the good choices for the drug analysis but very relevant to analytical chemistry in at least four respects: (a) it allows the elucidation of the physical, chemical and physico-chemical mechanisms on which analytical processes are based and hence their rational optimisation; (b) it facilitates the development of new analytical methods and techniques that are otherwise unattainable if the dynamic aspects are not dealt with; (c) it is the foundation of reaction rate methods (kinetic determinations); and (d) it contributes as sensitivity, selectivity and precision. The principles and applications of the kinetic methods have been reviewed [103–105]. Kinetic methods are generally rapid, reproducible and involve simple instrumentation. Though there are different modes of calibration in kinetic method, but the reaction rate methods involve two general aspects, which endow them with higher selectivity than equilibrium methods, namely the scarcity of parasitic blank signals and the possibility of using kinetic discrimination. In case of very fast reactions, the automatic handling is preferred for better and more accurate results; so the automation of the instrumentation is required. They can also be used to enhance the reproducibility and rapidity of slow reactions. Since kinetic methods of analysis require accurate timing, careful control over experimental conditions, precise sample and reagent preparation, proportioning and mixing, and accurate measurements of dynamic signals, hence they are well suited to intelligent automation. Kinetic
automatic techniques are generally based on open systems, among the most popular of which are stopped flow (SF) [106] system and the continuous addition of reagent (CAR) technique [107–110]. Several drugs have been determined by using the CAR technique with photometric [111,112] and fluorimetric detection [113]. 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 catalysts to accelerate analytical reactions is feasible with both reaction rate and equilibrium determinations. In this concern, the use of micellar media in kinetic methods is recently encouraged to enhance the rate of reaction (through micellar catalysis) which in turn reduces the time of analysis and also improves the sensitivity and selectivity [114–117]. Multicomponent kinetic determinations, often called as differential rate methods, are also receiving a good deal of current attention in research laboratories. The various approaches to multicomponent determinations have been reviewed recently by Perez–Bendito [118]. One new approach that has been proposed for dealing with overlapping spectra is the kinetic wavelength-pair method [119]. For a mixture of two components, the method relies on measuring the difference in the rate of change of absorbance with time at two pre-set wavelength pairs (four wavelengths). Another method called as the H–point standard addition method [120] proposed by Bosch–Reig et. al. is capable of kinetic determination of components with overlapping spectra in their binary mixture.

Pharmaceutical industry is one of the fast growing industries next to the information technology. Pharmaceutical analysis is a well-established section in the field of analytical chemistry whose advancements and works have been impressive in the last decades.
As mentioned above, the safety of drug products are dependent not only on the toxicological properties of the active drug substances itself but also on the impurities that it contains. Such impurities are associated with the drugs right from the manufacturing stages in the form of organic, inorganic and residual solvents (used to purify and generate the desired crystal morphology). The sources of such impurities may be the starting materials, intermediate and or synthetic by-products, reagents and catalysts. Furthermore, after getting the desired final product, the style of storage and class of packing may affect the stability of the drug substances, hence again leading to impurities, which raises a question mark on the recommended dosage amount supplied in the form of pharmaceutical formulations. Unlike other consumable products in the daily life, either the impurities or any abnormality in the recommended dosage amount of drug may lead to more adverse toxicological effects on the human life which may be life threatening.

The requirements of quality, quantity, purity and safety of pharmaceutical formulations warrants a careful thought by every one associated with this area. Due to easy availability of sub-standard drugs and medicines in the markets of the developing countries, it is crucial to assay drugs in dosage forms. Hence development of rapid, simple and cost-effective analytical methods for the analysis of drugs are the need of the day in public defence.

The increasing emphasis on the pharmacokinetic, bioavailability and therapeutic monitoring of drugs has placed a heavy burden on drug analysts. Reliable measurements of very low levels of drugs in complex matrices are frequently required. Because of the vast number of drugs introduced every year, more and more methods for drugs and metabolite determination are being developed either for routine or research use. Analytical procedures are
used throughout the drug development and the manufacturing of drug substances and drug products. Important decisions such as the establishment of the shelf-life from stability studies, the need for additional toxicological trials if new impurities appear or if known impurities exceed the qualified levels, the reworking of batches or batch release or rejection are based on analytical results. In order to make the right decisions and to avoid additional work, an appropriate performance of the analytical procedure is essential and this needs the “suitability of the method for its intended use”.

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 proof of its suitability for the intended purpose, is an important component in determining the reliability and reproducibility of the methods and is required for any regulatory submission. The method should be defensible with respect to regulatory requirements and reliable by incorporating statistical analysis to evaluate its performance. Validation of analytical methods has been the subject of discussion in recent years [121-124]. The procedure of validation requires the calculation and fitting of the experimental data within a standard fixed criteria. Statistical techniques are used to evaluate the linearity, precision, accuracy, robustness/ruggedness, sensitivity (limit of detection and quantitation) and specificity etc. Other aspects needing to be considered include identification and handling of samples prior to analysis, stability of samples under various storage conditions and the continuous assessment (quality control) of the method during its routine use. Before an analytical method is used for routine analysis it must be demonstrated first that the method fulfils certain performance criteria, when this has been documented, the method is said to be validated.
There are several international organisations and regulatory authorities, which are involved, in fixing the criteria for the validation. Some of them are listed below (Table 1.2). 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.

Table 1.2. Validation of analytical methods — international definitions.

<table>
<thead>
<tr>
<th>Organisation</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>
<td></td>
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<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>Lacks definitions of selectivity and specificity.</td>
</tr>
</tbody>
</table>


It has been agreed that the key for evaluation of method reliability and overall performance are: (i) analyte stability, (ii) method selectivity/ specificity, (iii) limit of detection and quantitation, (iv) accuracy and precision, (v) relationship between the response and concentration, (vi) recovery and (vii) ruggedness. The overall validation strategy consists of four components, which are prevalidation, validation proper, study proper and statistical analysis.

Prevalidation

Prevalidation requires the availability of an authenticated analytical reference standard to prepare solutions of known concentrations. This standard should be of 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 the reagents have been mixed. The optimum standard curve range and the number of calibrators should be established. The appropriate regression models which best fit the data is then selected. The extraction scheme and its recovery should be optimised to give insight into the limit of quantitation.

Sampling and storage is an important initial part during the analytical studies. The quality of analytical data depends critically on the validity of the sample and the adequacy of the sampling procedure. Drugs may be lost to the container or be degraded chemically, photochemically or enzymetically during storage. Hence, an investigation of the stability of the drug during sample storage (temperature and duration) is a crucial part of the method validation. The lack of information on stability may jeopardise subsequent investigations. In general, the samples that will be analysed within a few days should be stored at 4 °C; those that are to be stored for longer should be kept at –20 °C. For very long-term storage, freeze-drying should be considered. Provided it is available in sufficient quantity, the sample should be divided into aliquots before freezing, to minimise precipitation or degradation due to repeated freezing and thawing. Stock solutions of photodegradable drugs should be stored in amber-coloured containers.
Validation proper

Among all of the international organisations, the ICH guidelines achieved a great deal in harmonising the definitions of the required validation characteristics and their basic requirements. The International Conference on the Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human Use has harmonised the requirements in two guidelines [125,126]. The first one summarises and defines the validation characteristics needed for various types of test procedures; the second one extends the previous text 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 of submission. In order to fulfil the validation responsibilities properly, the background of the validation parameters and their consequences must be understood. Normally evaluated validation characteristics and their minimum number of determinations (if applicable) are listed in Table 1.3 [126].

There has been controversy regarding the technical term for the validation characteristic, i.e. specificity vs. selectivity, and the current definition of ICH is not clear in this respect. A clear distinction must be made between the terms “specific” and “selective”. 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 many exhibit selectivity. WELAC refers the selectivity of a method 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
Table 1.3. Validation characteristics normally evaluated for the different types of procedures [125] and the minimum number of determinations required (if applicable) [126].

<table>
<thead>
<tr>
<th>Validation Parameters</th>
<th>Minimum number</th>
<th>Test procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Identity</td>
<td>Impurities</td>
</tr>
<tr>
<td></td>
<td>Quantitative</td>
<td>Limit</td>
</tr>
<tr>
<td>Specificityb</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Linearity</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Range</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Accuracy</td>
<td>9 determinations over 3 concentrations (e.g. 3x3)</td>
<td>No</td>
</tr>
<tr>
<td>Precision</td>
<td>6 determinations at 100% or 9 determinations over 3 concentration levels (e.g. 3x3).</td>
<td>No</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2 series</td>
<td>No</td>
</tr>
<tr>
<td>*Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>^Including dissolution, content potency.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cIntermediate precision sufficient for submission.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dMay be needed in some cases.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
is perfectly selective for an analyte or a group of analytes is said to be specific. Thus a specific test procedure measures quantitatively a chemical or a physical parameter of functional group of one or more different analytes in the sample matrix while a selective one detect qualitatively the analyte in the presence of components which may be expected to be present in the sample matrix. Fig. 1.3 gives a graphical presentation for the differentiation between selectivity and specificity.

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

**Fig. 1.3.** 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).

Calibration range is defined by the expected concentration in the samples, and will be usually in the linear range of the detector for that analyte, where the determined response is directly proportional to the concentration. The calibration range should neither be too wide nor too narrow [122]. Least square linear regression is normally be used to define the calibration line mathematically. The range of an assay method can also be defined as the concentration intervals over which an analyte can be measured with acceptable precision and accuracy, whereas the linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample [125]. It has been generally accepted that the coefficient of correlation of the line of regression should exceed 0.99 [127]. However, there are certain analytical procedures (TLC,
fluorescence and atomic absorption spectrometry) with non-linear responses, which raises the question about the suitability of the calibration mode to be used in the procedure. The requirements and relevant parameters for the various calibrations are given in Table 1.4.

Table 1.4. Requirements for the different calibration modes with relevant parameters.

<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>Linear Function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
</tr>
<tr>
<td>External standard</td>
<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 at 100% test concentration).</td>
</tr>
<tr>
<td></td>
<td>Homogeneity of variances^a</td>
<td>F-test of the variances at the lower and upper limits of the range.</td>
</tr>
<tr>
<td><strong>Multiple Point Calibration</strong></td>
<td>Linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
</tr>
<tr>
<td>Linear, Unweighted</td>
<td>Homogeneity of variances^a</td>
<td>F-test of the variances at the lower and upper limits of the range.</td>
</tr>
<tr>
<td>Linear, Weighted</td>
<td>Linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
</tr>
<tr>
<td>Non-linear</td>
<td>Continuous function</td>
<td>Appropriate equation</td>
</tr>
<tr>
<td>100%-method (area normalisation for impurities)</td>
<td>For main peak: Linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
</tr>
<tr>
<td></td>
<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 at 100% test concentration).</td>
</tr>
<tr>
<td></td>
<td>Homogeneity of variances^a</td>
<td>F-test of the variances at the lower and upper limits of the range.</td>
</tr>
<tr>
<td></td>
<td>For impurities: Linear function</td>
<td>Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).</td>
</tr>
</tbody>
</table>

^a May be presumed for a limited range (factor 10 – 20).
Bias is the difference between the expectation of the results and an accepted reference value. Bias is a systematic error (determinate error) contrast to the random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the accepted reference value is reflected by larger bias value. Calibration curves are accepted for each individual batch during validation when no single standard has a % bias greater than 20% [122]. When a new analytical method is to be tested in a laboratory it may be used on samples of suitable reference material, but more often it is compared with an existing method on a range of suitable materials whose concentration levels are not known at all, precisely. It is important that the samples chosen cover the range of concentrations expected in future use, as the bias of the new method can vary with concentration. Regression techniques are commonly used to estimate the bias of the new method [128].

Accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value, and the value found [125]. The ICH guideline recommends the demonstration of accuracy over the whole working range. However, if only a narrow range is required (e.g. assay or impurities with a low specification limit), a six fold determination at a 100% test concentration may also be used. A quantitative approach to demonstrate the accuracy according to ICH guidelines have been given in Table 1.5. The analytical test to be validated is compared with another procedure or applied to a reference substance, the probably different specificities must be taken into account. Therefore, statistical tests should be performed only if the systematic bias based on these differences can be quantified and thus are corrected or are negligible. Otherwise the comparison should be performed as a qualitative verification of plausibility or an acceptable
maximum difference should be defined. The accuracy can be established in different ways, using statistical analysis.

Table 1.5. Quantitative approaches to demonstrate accuracy according to ICH [126].

<table>
<thead>
<tr>
<th>Drug substance</th>
<th>Application of the analytical procedure to a reference material. Comparison of the results with those of a second well characterised procedure.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug product</td>
<td>Application of the analytical procedure to synthetic mixtures of drug product components Spiking of analyte to drug product. Comparison of the results with those of a second well characterised procedure.</td>
</tr>
<tr>
<td>Impurities (quantitative)</td>
<td>Spiking of the impurity to drug substance or product. Comparison of the results with those of a second well characterised procedure.</td>
</tr>
</tbody>
</table>

Precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed condition [125]. Precision should be measured using homogenous authentic samples. However, if it is not possible or if it is not practical to obtain a homogeneous sample, it may be measured using artificially prepared samples or sample solutions. Precision experiments study the effect of random variations on the performance of a method. Precision must be assessed in several ways:

Repeatability, also termed as intra-assay precision, refers to the precision obtained under the same operating conditions over a short interval of time by applying the whole
analytical procedure to the sample. Repeatability should be assessed using a minimum of 6 determinations at 100% of the test concentration or a minimum of 9 determinations over the range of the procedure (e.g. 3 replicates for each of 3 concentrations). This will provide an estimate of the random error of the method.

Intermediate precision refers to within-laboratory variations, preferably performing a method over an extended period of time. The extent of investigation will depend on the intended use of the procedure. A typical investigation of intermediate precision would study various parameters that might include analysts, days, equipment, reagents, columns, etc. It is not required that each effect be studied individually so, therefore, experimental design is encouraged.

Reproducibility is an estimate of the variation between different laboratories and is usually assessed by performing an inter-laboratory (collaborative) study. A study of reproducibility is not required as a part of the marketing authorisation dossier.

Detection limit (DL) is the lowest amount of analyte that can be detected, as it (the analyte) yields instrumental response greater than a blank, but can not be quantified. It is a parameter of "limit test" and expected to produce a response, which is significantly different from that of a blank. On the other hand, quantitation limit (QL) is a parameter of "determination test" and can be defined as the lowest concentration of analyte that can be measured and quantified with acceptable precision and accuracy. Hence, it is the lowest concentration included in the standard curve and is used to interpolate unknown sample concentration. One should be aware that the determined QL (or DL) is strongly related to the equipment actually used, as well as to the time of determination. They may represent more
system parameters than characteristics of the analytical procedure. In cases where a general QL is required, as in pharmaceutical analysis, it is essential to define a realistic QL (or DL) for the analytical procedure, independently from the equipment used, because this limit has important consequences (e.g. for the reporting threshold for impurities or for method transfer).

DL and QL can be calculated from the standard deviation of the blanks. This approach is usually performed with instrumental methods. Measuring a blank sample, the standard deviation is calculated from the numerical outputs. DL and QL are defined as the 3.3 and 10 fold, respectively, of the standard deviation of the blank. The values are converted to a concentration by the slope of a corresponding calibration line [126].

Robustness/ruggedness of an analytical method can be defined as “a measure of the capacity of analytical procedure to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage” [129]. It can also be described as the ability to reproduce the (analytical) method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method. Though these two terms have been frequently used as the synonyms [130] but sometimes a mutual differentiation have also been made [131], according to which the ruggedness is defined as the degree of reproducibility of the test results obtained under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days, etc. The ICH guidelines also recommend that “one consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g.
resolution tests) is established to ensure that the validity of the analytical procedure is
maintained whenever used” [129]. Deliberate variations in the parameters of a procedure will
provide an estimate of its reliability in routine use. The extent of robustness studies will depend
upon the type of method but this evaluation should be considered during the development
phase. Typical variations might include extraction time, flow rate through a testing device,
stability of test solutions, etc.

**Study Proper and Statistical Analysis**

Daily standard curves are generated to determine the sample concentrations. The
quality control sample sequence is carefully monitored for the systematic errors. For each
standard curve, the slope, the intercept, variance and correlation coefficient should be
reported. From the data generated, specific analytical parameters should be 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.

**Statistical calculations of the validation parameters and certain others of
common interest**

*Linear regression by least square method; Normalisation factor (unweighted linear regression:*

\[ w_i = 1, \ k_w = 1 \]:

\[ k_w = \frac{\sum (y_i)}{n} \]

\[ w_i = 1, \ k_w = 1 \]:

\[ \bar{x} = \frac{\sum (x_i \cdot k_w \cdot w_i)}{n} \]

\[ \bar{y} = \frac{\sum (y_i \cdot k_w \cdot w_i)}{n} \]
Sum of squares:

\[ Q_{xx} = \sum (k_i \cdot w_i \cdot (x_i - \bar{x})^2) \]
\[ Q_{yy} = \sum (k_i \cdot w_i \cdot (y_i - \bar{y})^2) \]
\[ Q_{xy} = \sum (k_i \cdot w_i \cdot (x_i - \bar{x}) \cdot (y_i - \bar{y})) \]

Residual sum of squares (\(Q_{yy}\)) and Residual standard deviation (\(S_y\)):

\[ Q_{yy} - \frac{Q_{xy}^2}{Q_{xx}} \quad S_y = \sqrt{\frac{Q_{yy} - Q_{xy}^2}{n - 2}} \]

Slope (\(b\)), standard deviation of slope (\(S_b\)) and Relative confidence interval of the slope (\(C_{lb}\)):

\[ b = \frac{Q_{xy}}{Q_{xx}} \quad S_b = \sqrt{\frac{S_y^2}{Q_{xx}}} \quad C_{lb} = \frac{100 \cdot (P, n - 2) \cdot \sqrt{S_b^2}}{b} \]

Intercept (\(a\)), standard deviation of intercept (\(S_a\)) and Confidence interval of the intercept (\(C_{la}\)):

\[ a = \bar{y} - b \cdot \bar{x} \quad S_a = \sqrt{S_y^2 \cdot \left( \frac{1}{n} + \frac{\bar{x}^2}{Q_{xx}} \right)} \quad C_{la} = a \pm (P, n - 2) \cdot \sqrt{S_a^2} \]

Standard error (\(s_{\alpha}\)) and Relative standard error of slope (\(V_{\alpha}\)):

\[ s_{\alpha} = \sqrt{S_y^2 \cdot \left( \frac{1}{n} + \frac{\bar{x}^2}{Q_{xx}} \right)} \quad V_{\alpha} = 100 \cdot \frac{s_{\alpha}}{\bar{x}} \]

Coefficient of correlation (\(r\)):

\[ r = \frac{Q_{xy}}{\sqrt{Q_{xx} \cdot Q_{yy}}} \]

Residuals and %-Difference:

\[ y_i - \hat{y}(x_i) \quad \frac{(y_i - \hat{y}(x_i))}{\hat{y}(x_i)} \times 100\% \]

Where \(y_i = \) experimental \(y\)-value and \(\hat{y}(x_i) = \) calculated \(y\)-value for the given \(x\)-value.
Calculation of concentrations with uncertainties:

Input of \( y \)-values: Calculation of concentration
\[
X_i (y_i) = \frac{(y_i - a)}{b}
\]

Input of \( x \)-values: Calculation of signal
\[
y_i = a + b \cdot x_i
\]

Uncertainty and Relative uncertainty:
\[
\pm \frac{t(P\%, n-2) \cdot s_y}{b} \cdot \sqrt{\frac{1}{n} + \frac{1}{m} \cdot \frac{(y_i - \bar{y})^2}{b^2 \cdot Q_{xx}}} 
\]
\[
\pm \frac{100\% \cdot t(P\%, n-2) \cdot s_y}{b \cdot X_i(y_i)} \cdot \sqrt{\frac{1}{n} + \frac{1}{m} \cdot \frac{(y_i - \bar{y})^2}{b^2 \cdot Q_{xx}}} 
\]

Accuracy

(1) by comparison and validation procedure:

Mean \( (\bar{x}) \),
\[
\bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i
\]

Confidence interval \((CL(\bar{x}))\) upper and lower limit,
\[
CL(\bar{x}) = \bar{x} \pm t(P, n-1) \cdot \frac{s}{\sqrt{n}}
\]

Variance \((s^2)\), Standard deviation \((s)\) and Coefficient of variation \((CV \text{ or } RSD)\),
\[
s^2 = \frac{\sum(X_i - \bar{x})^2}{n-1}
\]
\[
s = \sqrt{s^2}
\]
\[
CV = \frac{s}{\bar{x}} \cdot 100
\]

Range \([R\%]\),
\[
R = \frac{x_{\text{max}} - x_{\text{min}}}{\bar{x}} \cdot 100
\]

Precision (Analysis of variance study):

(1) Test for the homogeneity of variances according to Cochran.
\[
C' = \frac{s^2_{\text{max}}}{\sum_{j=1}^{k} s^2_j}
\]

If \( > Cr(P, n-1) \); variances are inhomogeneous. \( k_{\text{max}} = 10 \), \( n_{\text{max}} = 10 \)

Statistical linearity test

As statistically based linearity test, the significance of the quadratic coefficient can be checked. If the confidence interval of the quadratic coefficient \( c \) \((y = a + b \cdot x + c \cdot x^2)\)
includes zero, the quadratic term becomes neglectable and the equation is reduced to a linear function (equations).

Significance of the quadratic coefficient: \[ CI_c = t(P, n - 3) \cdot \sqrt{s^2_c} \]

\( CI_c \) = confidence interval of the quadratic coefficient.

If \( c \pm CI_c \) includes 0, quadratic coefficient is not significant, and hence, no significant better fit by quadratic regression.

The test according to Mandel is also based on a comparison of a linear and a quadratic response function. In this case, a test is performed to determine whether the quadratic function results in a significantly better fit by comparing the residual variances of both calibrations. If

\[ PG = \frac{(n - 2) \cdot s^2_y - (n - 3) \cdot s^2_{y2}}{s^2_{y2}} > F(P, F_1 = 1, F_2 = n - 3) \]

significant better fit by quadratic regression. Where \( s^2_y \) is residual variance linear regression and \( s^2_{y2} \) is residual variance linear regression.

**Linearity (overall analysis, unweighted linear regression)**

Comparison with the slope of the 1st series:

Limits of the equivalence interval:

\[ C_U = 100 \left\{ \left( \frac{b_j}{b_i} \right) \exp\left[ s \cdot t(P, n_1 + n_j - 4) \right] - 1 \right\} \]

\[ C_L = 100 \left\{ \left( \frac{b_j}{b_i} \right) \exp\left[ - s \cdot t(P, n_1 + n_j - 4) \right] - 1 \right\} \]

\( \delta\% \) = Acceptable difference (percentage).

\( t(P,f) \) = Student's \( t \) value for level of statistical confidence \( P \) and degrees of freedom, \( f = n + n - 4 \).

Where \( b_1, s_b_1, n_1 \) = slope, standard deviation of the slope, and number of values of series 1 and 2, respectively.

The slopes of series 1 and 2 are equivalent, if \( - \delta\% \leq C_L \leq C_U \leq \delta\% \).
Accuracy (recovery from linearity): Comparison of the slope with the theoretical value of $t$.

Limits of the Equivalence interval:

$$C_L = b - t(P, n-2) \frac{s_y}{\sqrt{Q_{xx}}} \quad C_U = b + t(P, n-2) \frac{s_y}{\sqrt{Q_{xx}}}$$

$\delta$ = acceptable difference (absolute value).

$t(P,f)$ = Student's $t$-value for level of statistical confidence $P$ and degrees of freedom, $f = n-2$

$s_y, Q_{xx}$ = residual standard deviation, sum of squares.

The slope is equivalent to the theoretical value of $t$, if $-\delta \leq C_L \land C_U \leq \delta$

Accuracy (100% recovery, comparison to reference): Comparison of the mean with a nominal value.

Limits of the equivalence interval:

$$C_U = T - \bar{x} + t(P, n-1) \frac{s}{\sqrt{n}} \quad C_L = T - \bar{x} - t(P, n-1) \frac{s}{\sqrt{n}}$$

$T$ = nominal value (100% or reference value).

$\delta$ = Acceptable difference (absolute value).

$t(P,f)$ = Student's $t$-value for level of statistical confidence $P$ and degrees of freedom, $f = n-1$.

Where $\bar{x}, s, n$ = mean, standard deviation and number of values, respectively.

$T$ are equivalent, if $-\delta \leq C_L \land C_U \leq \delta$

Accuracy (Comparison to another procedure): Comparison of two means

$$C_L = 100 \left\{ \frac{\bar{x}_1}{\bar{x}_2} \exp \left[ -t(P, n_1 + n_2 - 2)s \right] - 1 \right\} \quad C_U = 100 \left\{ \frac{\bar{x}_1}{\bar{x}_2} \exp \left[ t(P, n_1 + n_2 - 2)s \right] - 1 \right\}$$

$$s = \sqrt{\frac{s_{\bar{x}^2 \times x^2}}{\frac{1}{n_1 \frac{x_1^2}{2}} + \frac{1}{n_2 \frac{x_2^2}{2}}} \quad s_{\bar{x}^2}^2 = \frac{(n_1 - 1)*s_1^2 + (n_2 - 1)*s_2^2}{n_1 + n_2 - 2}$$

$\delta\%$ = Acceptable difference (percentage).

$t(P,f)$ = Student's $t$-value for level of statistical confidence $P$ and degrees of freedom, $f = n_1 + n_2 - 2$. 
Where $\bar{x}_{1,2}, s_{1,2}, n_{1,2}$ = mean, variance, and number of values of series 1 and 2, respectively.

The two means are equivalent, if $-\beta \% \leq C_L \cap C_U \leq \beta \%$

**Precision (individual series):** Comparison of the standard deviation with a nominal value.

Limits for the equivalence interval:

$$C_L = 100 \left\{ \frac{s}{T} \sqrt{\frac{n - 1}{\chi^2 (P, n - 1)}} - 1 \right\}, \quad C_U = 100 \left\{ \frac{s}{T} \sqrt{\frac{n - 1}{\chi^2 (1 - P, n - 1)}} - 1 \right\}$$

$\beta \%$ = Acceptable difference (percentage of the standard deviation).

$\chi^2 (P, f)$ = Chi-squared distribution for level of statistical confidence $P$ or $1 - P$ and degrees of freedom $f = n - 1$.

$\bar{x}, s^2, n$ = mean, variance, and number of values.

Experimental and target standard deviation are equivalent, if $-\beta \% \leq C_L \cap C_U \leq \beta \%$

**Test for Outliers**

With regard to linearity, an outlier is characterised by a significant deviation from the regression curve. The 95% prediction interval can be used as a criterion [132]. This interval describes the range around the regression function in which a repeated analytical value can be expected in 19 out of 20 cases. If the calculation of the 95% prediction interval is performed with all values, a possible outlier might have a considerable influence on the calculation (by broadening the interval). The suspected value or values can be inactivated manually and a second regression is performed. If the deactivated value or values are now outside the 95% prediction interval, they can be suspected as being outliers. In the same manner, deviations from a linear response performing a linear regression can be detected. However, it must be taken into consideration that the prediction interval will be less stringent (broader) as the
extrapolation increases ("trumpet-shaped"). Most statistical tests and calculations are based on the assumption that the experimental values are only influenced by random variability (i.e. that they are normally distributed). Data that do not fulfill these assumptions (e.g. due to so-called "gross errors", weighing, dilution, or by problems with the instrument etc.) will affect the results. It is the aim of outlier tests to identify such values in order to eliminate them before performing further calculations. However, the problem is especially with a small number of data where groupings could easily occur avoiding the incorrect rejection of values belonging to the same distribution. Therefore, outlier tests should be applied carefully and only obviously deviating values should be eliminated. When an outlier is identified, the absolute magnitude of the coefficient of variation (relative standard deviation) must also be considered for evaluation. If this parameter (calculated including the suspected "outlier") lies in a normally expected range, preferably all values should be retained. The question of systematic effects influencing the values (e.g. degradation or adsorption of the analyte, shifts in the equipment settings, etc.) is of more importance. This can be investigated by applying a trend test in which an unweighted linear regression is performed using the values in the order of the input (equidistant spacing with respect to the \( x \) dimension). The 95% confidence interval of the slope is then tested against the slope itself. If a slope can not be detected statistically, the hypothesis of a systematical trend can be rejected.

Unweighted linear regression of \( i = 1 \) to \( n \) vs. \( x \). If \( C_l > \text{abs}(b) \) then no trend.

Where \( C_l = \text{confidence interval of the slope} \ t(P, n - 2) = \text{Student's } t\text{-value for the level of statistical confidence } P \text{ and degrees of freedom } n - 2. \)
The outlier test according to Grubbs calculates the ratio of the difference between the largest/smallest value and the mean to the standard deviation. This parameter is then compared with the tabulated critical values. The test for double outliers searches for the occurrence of two outlying results.

\[ n_{\text{max}} = 50. \] Range of the values \( x_i \) from 1 to \( n \).

\[ PG_{\text{max}} = \frac{(x_n - \bar{x})}{s} \quad PG_{\text{min}} = \frac{(\bar{x} - x_1)}{s} \]

**Test for single outliers:**

\[ \bar{x} = \text{mean}, \ s = \text{standard deviation}. \]

An outlier may be suspected if \( PG \) is larger than the tabulated value [133].

**Test for double outliers:**

\[ d_0^2 = \sum_{i=1}^{n} (x_i - \bar{x})^2 \]

**Test of the two smallest values:**

\[ PG = \frac{d_{12}^2}{d_0^2} \quad d_{12}^2 = \sum_{i=3}^{n} (x_i - \bar{x}_{12})^2 \quad \bar{x}_{12} = \frac{1}{n-2} \sum_{i=3}^{n} (x_i) \]

**Test of the two smallest values**

\[ PG = \frac{d_{12}^2}{d_0^2} \quad d_{12}^2 = \sum_{i=3}^{n} (x_i - \bar{x}_{12})^2 \quad \bar{x}_{12} = \frac{1}{n-2} \sum_{i=3}^{n} (x_i) \]

An outlier may be suspected if \( PG \) is smaller than the tabulated value [133].

For the outlier test according to Dixon [134], differences between ordered values at the upper and lower extremes are divided by the whole range. This parameter is then compared with the critical tabulated values.

\[ n_{\text{max}} = 40. \] Range of the value \( x_i \) from 1 to \( n \).

For \( n = 3 - 7 \)

\[ PG = \frac{x_2 - x_1}{x_n - x_1} \quad PG = \frac{x_n - x_{n-1}}{x_n - x_1} \]
or

For $n = 8 - 12$: \( P_G > \)

\[
P_G = \frac{x_2 - x_1}{x_{n-1} - x_1}
\]

or

For $n = 13 - 40$: \( P_G > \)

\[
P_G = \frac{x_3 - x_1}{x_{n-2} - x_1}
\]

An outlier may be suspected if $P_G$ is larger than the tabulated value [135].

**Test for normal distribution (David's test)**

For all statistical tests used here, it is assumed that the experimental values are only influenced by random variability (i.e. that they are normally distributed). This can be checked with a statistical test according to David [136]. For this test, the ratio of the range (maximum to minimum value) to the standard deviation is compared with the tabulated critical limits. Relying on only a small number of data, the test for normal distribution is restricted to similar problems as discussed for the outlier tests. For a large number of analytical procedures, a normal distribution of the whole population can be generally assumed. The question of systematic effects influencing the values (e.g. degradation or adsorption of the analyte, shifts in the equipment settings, etc.), is of more importance. This can be investigated by applying a trend test.

\[ R = \text{range (maximum value} - \text{minimum value)} \]

\[ s = \text{standard deviation} \]

The values are (probably) normally distributed if $G_l < R/s < G_u$

$G_l$, $G_u$: lower and upper test limit. Tabulated value [136].
F- and t-tests:

(i) Mean t-tests:

\[ F - \text{test: If } \frac{s_1^2}{s_2^2} > F(P, f_1, f_2) \text{ with } s_1^2 > s_2^2 \text{ then significant difference of variances exists. } F(P, f_1, f_2) = \text{Fisher's } F \text{-value for level of statistical confidence } P \text{ and degrees of freedom } f_1 \text{ and } f_2. \]

If \( \frac{D_j}{S_d} \cdot \sqrt{\frac{n_j}{n_{\text{comparison}}} > t(P, f)} \) significant difference of mean exists.

Where \( S_d = \sqrt{\frac{n_j - 1}{n_j} s_j^2 + \frac{n_{\text{comparison}} - 1}{n_{\text{comparison}}} s_{\text{comparison}}^2} \) \( D_j = |\bar{x}_j - \bar{x}_{\text{comparison}}| \)

(ii) Nominal value t-test:

If \( \frac{D_j}{S_j} * \sqrt{n_j} > t(P, f) \) significant difference to the reference exits.

Where \( f = n_j - 1 \) and \( D_j = |\bar{x}_j - \bar{x}_{\text{reference}}| \)

\( t(P, f) = \text{Student's } t \text{-value for level of statistical confidence } P \text{ and degrees of freedom } f. \)

and \( \bar{x}, s, n_j = \text{mean, standard deviation, number of values in validation series.} \)

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 the natural sources/products. All the drugs having medicinal importance can be broadly divided into two classes.

**Chemical classification:** Here the drugs are classified according to their chemical structure and properties without taking the pharmacological actions 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/organ (viz. heart, brain, lymphatic system, respiratory system,
endocrine system, central nervous system etc.). Hence these drugs are named like antianginal,
narcotics, soporifics, analgesics, diuretics and anaesthetics etc. Further classification of each
group is done according to the therapeutic/pharmacological specificity with the relevant organ.
A detailed classification of drugs with respect to their therapeutic importance for the
cardiocascular system has been given in Table 1.6 [137].

**ANTIANGINALS**

*Angina pectoris* is a heart ailment characterised by pressing chest pain that often
radiates to the neck area and arms and shoulders (often towards left side). As a clinical entity
it was first characterised under the name *Pectoris Dolor* by William Heberden in 1768, although
its symptoms had been noted as early as 1632 [138–140]. In the recent medical terminology
it is referred as the symptom of ischaemic heart disease. For nearly a century following its first
description, there was not much that could be done to relieve an angina patient of his agony;
brandy, ether, chloroform, ammonia and other stimulants and depressants had been tried, but
nothing seem to bring comfort. The breakthrough came in 1867 when T. Lauder Brunton, a
British physician, reported his success with amyl nitrite. In the authoritative but lively journal of
medicine, the *Lancet*, he wrote, “*On pouring from five to ten drops of (amyl) nitrite on a cloth
and giving it to the patient to inhale, the pain completely disappeared and, generally did
not return till its wonted time next night*” [141]. And finally the continuous effort of the medical
Table 1.6. Classification of drugs used in the treatment of cardiovascular disorders.

Classification of drugs for cardiovascular system

- Antiarrhythmics
- Antihypertensives
- Antianginals
- Cardiac Failure & Shocks
- Vasodilators
- Coagulants
- Anticoagulants, Thrombolytics & Antiplatelet drugs

- Antiadrenergic agents-
  - Centrally acting
  - Peripherally acting
- Antiadrenergic agents-
  - Blockers
- Antiadrenergic agents-
  - $\alpha / \beta$ blockers
- Drugs acting on vascular smooth muscle
- Calcium channel blockers
- Ace inhibitors
- Angiotension II receptor antagonists
- Diuretics
- Certain others like
  - Eicosapentaenoic acid

- Nitrates
- Beta adrenergic blockers
- Calcium channel blockers
- Potassium channel activators
- Antiplatelet drugs
- Other drugs like oxyfedrine, dilazep, lidoflazone etc

- Positive ionotropic drugs
- Vasopressors used in shocks
- Ace inhibitors
- Plasma expanders
- Parenteral anticoagulants
- Oral anticoagulants
- Thrombolytics (Fibrinolytics)
- Antiplatelet drugs
- Others
researchers, they got succeeded in knowing the different reasons with this life-threatening ailment and its therapeutic solutions.

Drugs used in angina pectoris are those that reduce cardiac work and myocardial 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. The drugs that either improve myocardial perfusion or reduce the metabolic demand, or both can counteract it. Several antianginal drugs are available for the treatment of angina. These include (a) organic nitrates, (b) beta-adrenergic blockers, (c) calcium channel blockers, (d) potassium channel activators, (e) antiplatelet drugs and (f) certain others like diazepam, oxyfedrine etc.

This thesis deals with the quantitative analysis of certain antianginal drugs namely, nifedipine, verapamil hydrochloride, amlodipine besylate and diltiazem hydrochloride in pharmaceutical formulations.

**Nifedipine** \((\text{C}_{17}\text{H}_{18}\text{N}_{2}\text{O}_{6})\)

Nifedipine is chemically dimethyl–1,4-dihydro–2,6-dimethyl–4–(2–nitrophenyl)pyridine–3,5–dicarboxylate promising for the following structure (Structure I). It is referred as the prototype compound of dihydropyridine class of calcium channel blockers having properties of peripheral and coronary vasodilation. It is orally administered for the management of hypertension and angina pectoris and functions primarily through its vasodilating properties as an afterload reducer.

Since nifedipine is extremely sensitive to photo-oxidation, manufacturers take great care in the storage and packaging of nifedipine to protect it from photodegradation due to
inadvertent light exposure and thus to maintain its therapeutic potency. But due to the same reason, official United States Pharmacopoeia recommend that the measured amount of nifedipine in commercial preparations may vary in the range 90—110% of the labelled quantity.

**Verapamil hydrochloride (C$_{27}$H$_{38}$N$_2$O$_4$. HCl)**

Verapamil hydrochloride, chemically designated as 5—[N—(3,4—dimethoxyphenylethyl) methylamino]—2—(3,4—dimethoxyphenyl)—2—isopropyl valeronitrile monohydrochloride is an antiarrhythmic as well as a calcium channel blocker belonging to the phenylalkylamine class of compound. The following structure (Structure II) has been suggested for verapamil hydrochloride.

Verapamil is administered orally as the racemic mixture and well absorbed rapidly.

Verapamil is metabolised stereoselectively, the more active enantiomer being more rapidly
metabolised. According to pharmacopoeial definitions, it contains not less than 99.0% and not more than 101.0% of C_{27}H_{38}N_{2}O_{4}. HCl, calculated with respect to the dried substance.

**Amlodipine besylate (C_{20}H_{25}ClN_{2}O_{5}. C_{6}H_{6}O_{3}S)**

Amlodipine besylate is comparatively a new calcium channel blocker, and is chemically defined as 3-ethyl 5-methyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl pyridine-3,5-dicarboxylate monobenzene sulphonate (Structure III).

![Structure III](image)

**It also belongs to dihydropyridine group of calcium channel blockers, undergoing photodegradation and showing almost similar therapeutic behaviour.**

**Diltiazem hydrochloride (C_{22}H_{26}N_{2}O_{4}S. HCl)**

*Diltiazem hydrochloride has been defined in the pharmacopoeias as (+)−5−[2−(dimethylamino)ethyl]−cis−2, 3−dihydro−3−hydroxy−2−(p−methoxyphenyl)−1, 5−benzothiazepin−4 (5H)−one acetate, containing not less than 98.5% and not more than 101.5% of C_{22}H_{26}N_{2}O_{4}S. HCl (Structure IV), calculated on the dried basis.*
Diltiazem bears the peripheral and coronary vasodilation therapeutic properties. It lowers blood pressure and has some effect on cardiac conduction. It is orally administered for the management of angina pectoris and hypertension, and may also be given intravenously in the treatment of atrial fibrillation or flutter and paroxysmal supraventricular tachycardia.
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