SECTION 1.0
GENERAL INTRODUCTION

Pharmaceutical analysis simply means analysis of a pharmaceutical(s). Webster’s dictionary defines a pharmaceutical as a medicinal drug. It is generally known that a pharmaceutical is a chemical entity of therapeutic interest. A more appropriate term for a pharmaceutical is active pharmaceutical ingredient (API) or active ingredient. To distinguish it from the formulated product or drug product, API is also called drug substance. The drug product is prepared by formulating a drug substance with inert ingredients (excipients) to prepare a drug product that is suitable for administration to patients. So it is the drug product that is more likely to be administered to a patient as a medicinal drug than a drug substance by itself.

The scope of drug analysis includes the analytical investigation of bulk-drug materials, the intermediates in their synthesis, products of drug research (potential pharmacons), drug formulations, impurities and degradation products of drugs, biological samples containing the drugs and their metabolites with the aim of obtaining data that can contribute to the maximal efficacy and maximal safety of drug therapy and the maximal economy of the production of drugs [1].

The efficacy, safety and economy of drug therapy are extremely important issues not only from the point of view of public health, but their financial aspects are also immense. As a consequence of this, pharmaceutical and biomedical analysis is among the most important branches of applied analytical chemistry. To fulfill the rapidly increasing demands as regards the number and the quality of analytical measurements, great efforts have been made and are being made to apply, moreover further develop in this field, the latest achievements of analytical chemistry. Furthermore, there is a need for quality assurance of pharmaceutical products throughout their shelf life. This requires that we study interactions of the drug substance with the excipients in the presence of residual solvents, as well as other potential degradation reactions that may occur in the formulated product over a period of time under various stress conditions (these include conditions they may be subjected to during storage or shipment in the final package configuration) [2].
Headaches are one of the most common and universal human ailments, described in the Bible as well as in medical writings from ancient Egypt, Babylonia, Greece, Rome, India, and China. Severe chronic headaches were once treated by the oldest known surgical procedure, known as trepanning or trephining, in which the surgeon drilled a hole as large as 1–2 in diameter in the patient’s skull without benefit of anesthesia. Evidence of trepanning has been found in skulls of Cro-Magnon people that are about 40,000 years old.

Headache is a pain in the head and neck region that may be either a disorder in its own right or a symptom of an underlying medical condition or disease. The medical term for headache is *cephalalgia*.

**Description**

Contemporary doctors divide headaches into two large categories, primary and secondary, according to guidelines established by the International Headache Society (IHS) in 1988 and revised for republication in 2004.

**Secondary headaches**

Secondary headaches, which are caused by diseases or disorders, are categorized as either traction or inflammatory headaches. Traction headaches result from the pulling, stretching, or displacing of structures that are sensitive to pain, as when a brain tumor presses on the outer layer of nerve tissue that covers the brain. Inflammatory headaches are caused by infectious diseases of the ears, teeth, sinuses, or other parts of the head. Major causes of secondary headaches include the following:

- **Brain tumors.** Headaches associated with brain tumors usually begin as episodic nighttime headaches that are accompanied by projectile vomiting. The headaches may become continuous over time, and usually get worse if the patient coughs, sneezes, bears down while using the toilet, or does something else that increases the pressure inside the head.

- **Meningitis.** Meningitis is an inflammation of the *meninges*, the three layers of membranes that cover the brain and spinal cord. Meningitis is usually caused by bacteria or viruses, and may produce chronic headaches.
• **Head trauma.** Patients may complain of headaches as well as memory problems, general irritability, and **fatigue** for months or even years after a head injury. These symptoms are sometimes grouped together as post-concussion syndrome. In some cases, a blow on the head may cause some blood vessels to rupture and produce a hematoma, or mass of blood that displaces brain tissue, and can cause **seizures** or weakness as well as headaches.

• **Temporal arteritis.** First described in 1890, temporal arteritis is an inflammation of the temporal artery that most commonly affects people over 50. In addition to headache, patients with temporal arteritis may have fever, loss of appetite, and blurring or loss of vision. Temporal arteritis is treated with steroid medications.

• **Stroke.** Headaches may be associated with several conditions that may lead to stroke, including high blood pressure and heart disease. Headaches may also result from completed stroke or from the mini-strokes known as transient ischemic attacks, or TIAs.

• **Lumbar puncture.** About 25% of patients who undergo a lumbar puncture (spinal tap) develop a headache from the lowered cerebrospinal fluid pressure around the brain and spinal cord. Lumbar puncture headaches usually go away on their own after a few hours.

• **Sinus infections.** Acute sinusitis is characterized by fluid buildup inside sinus cavities inflamed by a bacterial or viral infection. Chronic sinusitis usually results from an allergic reaction to smoke, dust, animal fur, or similar irritants.

• **Referred pain.** This type of pain is felt in a part of the body at a distance from the injured or diseased area. Headache pain may be referred from diseased teeth; disks in the cervical spine that have been damaged by spondylosis (degeneration of the spinal vertebrae caused by osteoarthritis); or the temporomandibular joint, the small joint in front of the ear where the lower jaw is attached to the skull.

• **Idiopathic intracranial hypertension.** Also known as **pseudo tumor cerebri**, this disorder is caused by increased pressure inside the skull in the absence of any abnormality of the **central nervous system** or blockage in the flow of the cerebrospinal fluid. In addition to headache, patients with this disorder experience diplopia (seeing double) and other visual symptoms.
**Primary headaches**

**Migraine headaches.** Migraine headaches are characterized by throbbing or pulsating pain of moderate or severe intensity lasting from four hours to as long as three days. The pain is typically felt on one side of the head; in fact, the English word “migraine” is a combination of two Greek words that mean “half” and “head”. Migraine headaches become worse with physical activity and are often accompanied by nausea and vomiting. In addition, patients with migraine headaches are hypersensitive to lights, sounds, and odors.

**Cluster headaches.** Cluster headaches are recurrent brief attacks of sudden and severe pain on one side of the head, usually most intense in the area around the eye. Other names for these headaches include histamine cephalalgia, Horton neuralgia, or erythromelalgia. Cluster headaches may last between five minutes and three hours; they may occur once every other day or as often as eight times per day. The IHS classifies cluster headaches as either episodic or chronic. Episodic cluster headaches occur over periods lasting from seven days to one year, with the clusters separated by headache-free intervals of at least two weeks. The average length of a cluster ranges between two weeks and three months. Chronic cluster headaches occur over a period longer than a year without a headache-free interval, or with pain-free intervals that are shorter than two weeks.

**Tension headaches** Tension headaches are the most common headaches in the general population; other names for them include muscle contraction headache, ordinary headache, psychomyogenic headache, and stress headache. The IHS classifies tension headaches as either episodic or chronic; episodic tension headaches occur 15 or fewer times per month, whereas chronic tension headaches occur on 15 or more days per month over a period of six months or longer.
Rebound headaches. Rebound headaches, which are also known as analgesic-abuse headaches, are a subtype of primary headache caused by overuse of headache drugs. They may be associated with medications taken for tension and migraine headaches.

“More than 90 % of all headaches are primary headaches. Secondary headaches are caused by disease or medical condition; they account for fewer than 10 % of all headaches” [3].

MIGRAINE
A throbbing headache that usually affects only one side of the head. Nausea, vomiting, increased sensitivity to light, and other symptoms often accompany a migraine. Epidemiological studies have documented its high prevalence and high socio-economic and personal impacts [4].

Demographics
Over a lifetime, only 1 % of us escape headaches altogether. Over a year, it is estimated that 90 % of the population get at least one headache. About 16-17 % of the population gets a migraine headache sometime in their life – that means over a billion people worldwide at some point get migraine. The World Health Organization estimated in 2003 that 303 million people worldwide were migraineurs. A 2004 article suggested that there are almost 20 million migraine attacks happening every day [5].

25 % of women and 8 % of men get migraines sometime in their lifetime. About half of these people get their first migraine before the age of 20, and 98% before the age of 50. 5 % get migraine before they are 15 years old and about a third of those get migraine before they are even 5 years old. Most migraines, however, occur between the ages of 25 and 50. According the Kids Health, Org., up to 10 % of children between 5 and 15 may experience migraine. Before puberty, girls and boys are almost equal in the migraines they suffer, possibly due to the estrogen changes that women go through at various stages in life. About 70 % have some other close (first degree) relative with migraine [6].
85% of migraineurs could report something that triggers the migraine. These include weather (up to 50%), missing a meal (40%), stress (about 50%), alcohol (50%) and various types of food (45%). About 50% of women report menses as a trigger. A recent study showed that over 50% considered crying to be a trigger. 38% of migraineurs suffer from 1-12 times each year, 38% get 1-3 times a month, 37% get once per week, and 11% get 2-6 times in a week [7].

Migraine remains undiagnosed and undertreated in at least 50% of patients, and less than 50% of migraine patients consult a physician [8].

Types of migraine

The two major categories of migraine are migraine without aura and migraine with aura.

Migraine without aura

Previously termed as hemicrania simplex or common migraine (because it is the most common type). It is a clinical syndrome characterized by headache with specific features and associated symptoms. Recurrent headache disorder manifesting in attacks lasting 4-72 hours. Typical characteristics of the headache are unilateral location, pulsating quality, moderate or severe intensity, aggravation by routine physical activity and association with nausea and photophobia and phonophobia.

Migraine with aura

Previously termed as classical migraine or ophthalmic. Aura is the complex of neurological symptoms that occurs just before or at the onset of migraine headache. Approximately 15 to 30% of patients with migraine experience the warning phenomenon of aura with some or all of their headaches. Headache that follows aura is often less intense and not as difficult to treat.

The five phase of migraine

- Prodrome: Occurs hours to days before migraine without headache
• Aura: Neurological phenomena such as disturbance of vision just before headache
• Pain phase: Headache on one side of head with nausea, photophobia and other classic migraine symptoms
• Resolution: Vomiting, deep sleep
• Postdrome: Exhaustion, irritability, diuresis (Figure 1.0.1)

![Figure 1.0.1 Five phase of migraine attack](image)

**Causes of migraine**

The tendency to develop migraine is inherited; up to 90% of people with migraine have a close relative who gets them, too. Some studies show that if one parent has migraine, each child has a 40% chance of developing it; if both parents have migraine, each child has a 75% chance.

Four main theories about the cause of migraine have been proposed. They are: (1) the brain (the central theory); (2) the blood vessels (the vascular theory); (3) inflammation (the neurogenic inflammation theory, which involves the trigeminovascular system); and (4) a combination of these factors (the unifying theory), which pulls the three interrelated theories together [9].
Where does the headache come from?

- When a headache occurs, serotonin and magnesium levels drop
  - Trigeminal nerve releases neuropeptides
  - Neuropeptides travel to outer covering of the brain and cause dilation and inflammation of meningeal blood vessels as show in Figure 1.0.2.
- Dura and dura vessels disrupted but brain structure remains intact. No increased risk of a brain tumor.

![Migraine process diagram](image)

**Figure 1.0.2** Migraine process.

Migraine triggers

Many migraine patients are unusually sensitive to internal (within the body) and external (outside the body) environmental changes.

**Environmental triggers**

- Changes in weather or barometric pressure
- Vapours and fumes
• Noises

**Physiological triggers**
- Hormonal fluctuations in women (menses)
- Oversleeping or lack of sleep

**Medicines**
- Oral contraceptives or estrogen replacement
- Vitamin A and retinoic acid derivative

**Dietary triggers**
- Foods containing tyramine such as aged cheese, pickled foods, fresh baked yeast products, marinated foods
- Nitrates in cured meats
- Chocolate
- Monosodium glutamate (MSG)
- Red wine
- Excessive caffeine or caffeine withdrawal

**Psychological triggers**
- Stress
- Emotions such as fear, anger, anxiety or depressed mood [10]

**Symptoms of migraine**

Headache on one side of the head, throbbing or pulsating pain, moderate to severe pain that makes it difficult or impossible to function, worsening of pain in response to routine physical activity such as bending over or climbing stairs, nausea, vomiting, sensitivity to light and sound etc. Other symptoms of a migraine attack may include dizziness, frequent urination, diarrhea, sweating, and cold hands.

**Anti-migraine drugs (drugs selected for this study)**

Anti-migraine medications are drugs that are given to lower the risk of a severe migraine attack or to reduce the severity of the headache once an attack begins. Preventive treatment for migraine headaches is called migraine prophylaxis or prophylactic therapy. Migraine therapy can be divided into three general categories of treatment: acute pharmacologic therapy, prophylactic pharmacologic therapy, and non
pharmacologic approaches. Acute pharmacologic therapy (also referred to as abortive therapy) can be further subdivided into nonspecific and specific migraine therapy. Nonspecific therapy relies on several classes of analgesics, anxiolytics and barbiturates. Specific therapy refers to drugs targeted against the pathophysiology of the migraine itself, and includes serotonin receptor agonists, both selective and nonselective.

<table>
<thead>
<tr>
<th>Nonspecific</th>
<th>Specific</th>
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<tr>
<td>Non steroidal anti-inflammatory drugs, or NSAIDs (Analgesics), Anxiolytics, Barbiturates, β-blockers, calcium channel blockers, etc. e.g., aspirin, naproxen, Diclofenac, ibuprofen, flurbiprofen, ketorolac, ketoprofen, atenolol, propranolol, flunarizine, etc.</td>
<td>5-hydroxytryptamine (5-HT1) agonists (selective serotonin receptor) e.g., triptans (non selective serotonin receptor) e.g., ergot alkaloids</td>
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Migraine pain is believed to come from activation of the trigeminovascular system. Activity in the trigeminovascular system is regulated by serotonin. The 5HT₁ presynaptic autoreceptors (5HT₁D) modulate neurotransmitter release and release of vasoactive neuropeptides. The post-synaptic receptors (5HT₁B) constrict blood vessel walls. The serotonin agonists (triptans) are specific for the 5HT₁B and 5HT₁D autoreceptors. It seems that levels of serotonin in the blood fall at the onset of headache but are normal between attacks.

**Migraine-specific treatment**

**Selective serotonin receptor (5-HT1) agonists**

Selective serotonin receptor agonists have been used to treat migraines since 1991. They work by activating serotonin receptors in the brain, which block an inflammatory process that affects the blood vessels in the head and leads to a leakage of blood plasma through the vessel walls. These drugs, which are also known as triptans or
5-hydroxytryptamine 1B agonists, are effective in treating about 70% of migraine patients. Sumatriptan (Imitrex) is the prototype of this class of medications. Second-generation triptans include such drugs as eletriptan (Relpax), naratriptan (Amerge), rizatriptan (Maxalt), almotriptan (Axert), frovatriptan (Frova), and zolmitriptan (Zomig). The second-generation triptans were developed to increase the speed of the drug’s absorption through the digestive tract and thus relieve the patient’s pain more rapidly. All the triptans are prescribed for moderately severe or severe migraines; one, sumatriptan, is available as a nasal spray or injection for patients with severe vomiting. One major drawback of the triptans, however, is that moderate-to-severe headache pain tends to recur within 24 hours of the first dose.

As a class, the triptans constrict blood vessels in the head and reverse inflammation around blood vessels in the meninges (the brain covering). They may, however, constrict other blood vessels on occasion and thus should not be given to patients with coronary artery disease, stroke, other blood vessel or vascular disease, uncontrolled high blood pressure.

**Ergot alkaloids**

Ergot alkaloids are an older group of drugs that include such compounds as ergotamine tartrate (Ergostat) and dihydroergotamine (DHE-45, Migranal). These drugs are derived from ergot, a compound produced by a fungus (Claviceps purpurea) that grows on rye plants. The medications work by causing the blood vessels in the head to constrict or narrow, which counteracts the dilation of the blood vessels that causes pain. Some medications in this group are combinations of ergotamine tartrate and caffeine (Cafergot, Ercaf); the caffeine intensifies the vasoconstrictive effect of the alkaloid. Like the triptans, the ergot alkaloids are used to treat moderate- to-severe migraines. They are not prescribed as frequently as they once were, however, because of the severity of their side effects and because they cannot be given to patients with coronary artery disease or other vascular disorders.
Migraine non-specific treatment

Analgesics

Analgesics in general are medications given to relieve pain. These drugs are used to treat patients who have infrequent migraine headaches, or who cannot be treated with triptans. There are two main types of analgesics used as acute treatment for migraines, nonsteroidal anti-inflammatory drugs, or NSAIDs, and combination analgesics. NSAIDs include aspirin, naproxen (Naprosyn), diclofenac (Voltaren, Cataflam), ibuprofen (Advil, Motrin), flurbiprofen (Ansaid), ketorolac (Toradol), and ketoprofen (Orudis). Combination analgesics include butalbital plus acetaminophen (Fioricet), butalbital plus aspirin (Fiorinal), and isometheptene plus acetaminophen and dichloralphenazone (Midrin). As of 2004, doctors disagree about the use of opioid (drugs that are or act like narcotics) analgesics to treat migraine pain [3].

β-blockers

β-blockers may work by stabilizing arteries or preventing the central generator of migraine in the brainstem from firing. Of the many β-blockers, propranolol (Inderal), atenolol (Tenormin), metoprolol (Lopressor, Toprol-XL), nadolol (Corgard), and timolol (Blocadren) are the most effective for prevention of migraine.

Calcium channel blockers

Calcium channel blockers prevent calcium from entering certain cells in the brain and muscles. This, in turn, prevents blood vessels from dilating, which blocks part of the migraine process. The most widely used calcium blocker for migraine in the United States is verapamil (Calan, Isoptin), followed by amlodipine (Norvasc) and diltiazem (Cardizem). Others such as nisoldipine (Sular), nicardipine (Cardene), and flunarizine (Sibelium) may also be used [9].
SECTION 1.1 SCOPE OF THE PRESENT WORK

The main objective of the work is to develop new methods for the analysis of pure active ingredients (active ingredient analysis) and analysis of medicinal preparations used in the treatment of migraine. The latter can exist in different forms (tablets and/or capsules). The medical preparations consist of the pharmaceutically active substance and at least one pharmaceutical excipient that is suitable for administration to patients. Impurities usually stem from the synthesis of the active ingredient; they are usually monitored according to the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [11] and the pharmacopoeias [12]. Many precise and accurate analytical methods for the commonly used active ingredients and excipients are monographed in detail in pharmacopoeias like United State pharmacopoeia, British pharmacopoeia, European pharmacopoeia, Indian pharmacopoeia etc. Pharmacopoeia (literally, 'drug-making'), in its modern technical sense, is a book containing directions for the identification of samples, preparation of compound medicines, contain the statutory requirements regarding the identity, content, quality, purity, packaging, storage and labeling for active pharmaceutical ingredients and other products used for therapeutic purposes [13]. In the pharmaceutical industry, pharmaceutical analysts working in research and development (R&D) play a very comprehensive role to develop and refine the methods that eventually will be utilized to test the identity, quality, purity, potency, and composition of marketed products. Following approval, quality control (QC) personnel provide the data to assure consistent quality and stability for the marketed product and to support the inevitable changes that occur in every product’s life cycle [1].

Assay or analysis of active pharmaceutical ingredients (API) and medicinal preparations has three classes [14] as shown below:

1. Chemical assay: The determination of potency of the active component present in drug preparation by chemical methods is known as chemical assay.

2. Bio assay: It is defined as a method of determination of the potency, chemical or biological agent by means of a biological indicator.
3. Immunological assay: It is defined on the principle that a hormone antigen reacts with its specific antibody.

Generally, chemical assay is more reliable and precise compared to biological and immunoassay. Chemical methods of analysis include gravimetric [15] and volumetric [16] procedures (which are based on complex formation, precipitation, acid-base and redox reactions). Physico-chemical methods are also commonly employed for the analysis and depend on the physical phenomena that occur as a result of chemical reactions.

The pharmaceutical industries have employed several analytical techniques to penetrate chemical determinations not only of the active ingredient but also the quantification of related compounds or impurities in incoming chemicals, drug materials and formulations. An important feature of modern pharmaceutical chemistry is the introduction of more refined and sensitive methods of physico-chemical analyses [17] such as optical (atomic absorption spectrophotometry [18], emission and fluorescence methods [19], polarimetry [20], photometry [21] including spectrophotometry covering UV [22], visible [23-26] and IR regions [27] or turbidimetry [28], electro-chemical coulometry [29], conductometry [30], polarography [31], voltammetry [32], potentiometry [33] and amperometry [34] and chromatography (thin layer chromatography (TLC) [35], high performance liquid chromatography (HPLC) [36, 37], liquid chromatography (LQ) [38], high performance thin layer chromatography (HPTLC) [39], gas chromatography (GC) [40] and capillary electrophoresis (CE) [41,42] for the assay of individual components of a mixture. Other analytical techniques including kinetic-spectrophotometry [43], thermal [44] and flow injection chemiluminescence [45] methods are also being used for the assay of drugs. In addition, NMR technique [46] is also used for the assay of individual components of a mixture. The combination of mass spectroscopy with gas/liquid chromatography [47,48] is one of the most powerful tools employed in identification and quantification of analyte in bulk or dosage forms.

Modern methods of analysis (HPLC, GC, NMR and Mass) involve sophisticated equipments which are costly and pose problems of maintenance. Hence, they may not be in the reach of most of the laboratories and small scale industries, which produce bulk drugs and pharmaceutical formulations. Among various techniques,
spectrophotometry still enjoys significant role in the assay of several class of drugs at micro or nano gram levels. It is simple, economically viable and easy to carry out. The importance of a spectrophotometric method lies in the chemical reaction(s) upon which the procedures are based, rather than upon the sophistication of the instrument. Many reactions which yield colored species for a particular drug are found to be selective or can be rendered selective through the introduction of masking agents, control of pH, use of solvent extraction techniques, by prior removal of interfering substances and so on. UV-spectrophotometry is the simplest of the spectroscopic techniques and it is in wide use in the quantitative analysis of the active substances. UV-spectrophotometric procedure is also recommended in Pharmacopoeial monographs such as Indian Pharmacopoeia [49], British Pharmacopoeia [50] etc. Hence, spectrophotometry is generally preferred in small-scale industries and most of the laboratories for routine quality assurance. Titration is also a simple technique giving accurate and precise results. The non-aqueous titration with visual or potentiometric end point detection has maintained its considerable importance in pharmaceutical analysis and has been accepted by the majority of modern pharmacopoeias [51-54] as an official analytical method.

Adopting these techniques like UV/visible spectrophotometry, titrimetry (including aqueous and non-aqueous) with visual or potentiometric end point detection, the author has developed many simple, selective, sensitive and cost-effective analytical methods for the determination of following anti-migraine drugs:

1. Atenolol, a β-blocker
2. Rizatriptan benzoate, selective serotonin receptor
3. Propranolol hydrochloride, a β-blocker
4. Sumatriptan succinate, selective serotonin receptor
5. Flunarizine dihydrochloride, calcium channel blocker
6. Zolmitriptan, selective serotonin receptor

The developed methods have been applied to different pharmaceutical formulations like tablet and capsule, and also to spiked human urine wherever possible. Targeting the analytically useful functional groups on the molecule, several visible spectrophotometric methods based on redox reaction, charge-transfer complexation, ion-
pair complexation, bromination, nucleophilic addition followed by elimination, etc., have been developed.

1.2 METHOD DEVELOPMENT

A feature of organic drugs is the presence of functional groups in their molecules, i.e., reactive atoms or groups of atoms which can be determined by chemical reactions. Functional groups pave the way of analyzing organic drugs because they are responsible for the properties of substances and determine the identification reactions and the methods of quantitative determination. Knowing the reactions for detecting functional groups present in a certain drug, one can develop new analytical methods for the analyzing of that drug. The variety of functional groups such as aliphatic and aromatic amines, allylic moiety, electrophilic ring, chloride of the hydrochloride salt and oxidizing or reducing positions which exist in the selected anti-migraine drugs was exploited to develop new analytical methods through some important reactions.

As mentioned above under Section 1.1, the author’s studies in this thesis are focused on use of titrimetric and spectrophotometric techniques. In titrimetric procedures, the end point was detected either visually using suitable indicators or potentiometrically which depends on the principle involved in the method and the calculations are based on the reaction stoichiometric relationship between the drug and the titrant (direct titration)/reactant (indirect titration). The reactions used in the titrimetric procedures include reduction-oxidation, bromination substitution, and titration in non-aqueous medium (visual and potentiometry). In spectrophotometric methods, the measured property is the absorbance which is directly proportional or inversely proportional to the concentration of the analyte based on the basis of the method. Reactions based on charge-transfer complexation, ion-pair complexation (extractive and extraction-free), reduction-oxidation, bromination, and nucleophilic addition were performed with the spectrophotometric technique.

Since the reaction products of the drugs studied in this thesis (analyte) and the reagents used were not isolated, the precise reaction mechanism could not be given in some cases. Hence, based on the literature knowledge as well as the reactivity of the
functional groups present in the drugs and based on the experimentally found reaction stoichiometry, the probable reaction pathways of the proposed methods are presented.

In all developed methods, the effect of various experimental variables such as acid concentration, type and volume of the buffer, pH, reagents concentration, reaction time, organic solvent and color stability for the colorimetric methods was carefully studied and optimized.

### 1.3 METHOD VALIDATION

Analytical methods used in pharmaceutical analysis must be sufficiently accurate, specific, sensitive and precise to conform to the regulatory requirements as set out in the relevant guidelines of ICH [11], which are applied by the licensing authorities and by pharmacopoeias. So, validation is a basic requirement to ensure quality and reliability of the results for all analytical applications [13]. The ICH guideline is regarded as the basis and philosophical background to analytical validation. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose [13]. Following the ICH guideline [11], the developed methods have been validated for linearity, sensitivity, precision, accuracy, robustness, ruggedness and selectivity. Sensitivity parameters such as apparent molar absorptivity and Sandell’s sensitivity values, the limit of detection (LOD) and the limit of quantification (LOQ) were also calculated in spectrophotometric methods.

**Sensitivity of a spectrophotometric method**

Sensitivity refers to the least determinable concentration or amount of the species to be determined [11]. According to Beer’s law, $A = \varepsilon lc$, where ‘$\varepsilon$’ is the molar absorptivity, ‘$l$’ is the path length of the cell holder (in units of centimeters, cm) and “$c$” is the concentration of the solution (in units of molar, M). The ‘$\varepsilon$’ is a valuable index in knowing the relative sensitivity of different spectrophotometric methods. For sensitive spectrophotometric methods, ‘$\varepsilon$’ would be greater than $1 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ and the values of ‘$\varepsilon$’ below $1 \times 10^3$ L mol$^{-1}$ cm$^{-1}$ correspond to less sensitive methods.

The sensitivity is often expressed in terms of sensitivity index as given by Sandell [55], which represents the number of micro/nanogram of the analyte/determinant per ml
of a solution having the absorbance of 0.001 for a path length of 1 cm. Sandell’s sensitivity is expressed in \( \mu g \text{ cm}^{-2} \) or \( \text{ng cm}^{-2} \).

**Limit of detection (LOD) and limit of quantification (LOQ)**

LOD and LOQ are the smallest amount of an analyte which can be detected and quantified by a particular method. LOD and LOQ were calculated according to the ICH guidelines using the formulae:

\[
\text{LOD} = 3.3\sigma/b \quad \text{and} \quad \text{LOQ} = 10\sigma/b
\]

where \( \sigma \) is the standard deviation of five reagent blank determinations and \( b \) is the slope of the calibration curve.

In UPLC, the LOD and LOQ were obtained by signal to noise (S/N) ratio method.

**Robustness**

Robustness is one of the most important validation parameters. The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage. The proposed methods have been validated for robustness by variations in volume of reagents, volume of acid, wavelength and reaction time.

**Ruggedness**

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions. The ruggedness of the methods was validated by varying different analysts and different instruments.

**Selectivity**

Selectivity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Selectivity of the methods was performed by placebo and synthetic mixture analyses. Any interference found in the analysis was removed by suitable solvent extraction [13].

**Precision and accuracy**

Precision and accuracy of the methods are performed on the basis of intra-day and inter-day analyses. The term precision describes the reproducibility of a result. It can be defined as the agreement between the numerical values of two or more measurements that have been made under identical conditions. One of the most common statistical terms
employed is the standard deviation of a population of observations. The standard deviation (S) is the square root of the sum of squares of deviations of individual results \((x_i)\) from the mean \((x)\) divided by one less than the number of results in a set. It is calculated using the following equation:

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

Relative standard deviation is the standard deviation expressed as a function of the mean i.e. \(S/x\). It is sometimes multiplied by 100 and expressed as a percent relative standard deviation to make it a more reliable expression of precision.

\[
\% \text{ Relative standard deviation} = \frac{S}{x} \times 100
\]

Accuracy describes how close a measured value is to ‘true value’ and it is expressed in terms of error. According to IUPAC [11], accuracy relates to the difference between a result (or mean) and the true value. Accuracy of a method is checked by taking varying amounts of the analyte and proceeding according to the chosen procedure. The difference between the mean of an adequate number of results and the amount of analyte actually present, is usually expressed as parts per hundred (%) i.e. % relative error (% RE).

Precision and accuracy of the method are also confirmed by a comparative method.

**Comparative method**

In this method, the results of a proposed method with those of an official/reported method are compared by calculating Student’s \(t\)-test for accuracy and \(F\)-test for precision. **Student \(t\)-test:** This is used to compare the means of two related (paired) samples analyzed by reference and proposed methods. It gives answer to the correctness of the null hypothesis with a certain confidence at 90% or 95% or 99%. The calculated “\(t\)” values are compared with the tabulated value for a given number of replicates at the desired confidence level. If the calculated \(t\)-values are smaller than the tabulated \(t\)-values, one can conclude that the two methods are not significantly different at a given confidence level.
**F-test:** The significance of the difference in variances of reference and proposed methods can be tested by *F*-test. Suppose that one carries out ‘n₁’ replicate measurements using the proposed method and ‘n₂’ replicate measurements using the reference method. If null hypothesis is true, then the estimates $S_T^2$ (variance of proposed method) and $S_R^2$ (variance of reference method) do not differ very much and their ratio should not differ much from unity. In fact, one uses the ratio of the variance: $F = S_T^2 / S_R^2$ or $F = S_R^2 / S_T^2$.

It is conventional to calculate the *F*- ratio by dividing the large variance by the smallest in order to obtain a value equal or larger than unity. If the calculated *F*-values are smaller than the tabulated *F*-values, it could be concluded that the procedures are not significantly different in precision at a given confidence level.

**Recovery experiments (Standard addition method)**

Recovery studies were conducted by analyzing each pharmaceutical formulation in the first instance for the active ingredient by the proposed methods. A known amount of the pure drug to be determined was added to each one of the previously analyzed samples and the total amount of the drug was once again determined by the proposed methods. The amount of the added pure drug was determined by the difference. Satisfactory recovery values will enhance the accuracy of the proposed procedures.
REFERENCES
49. Indian Pharmacopoeia. Govt. of India, Ministry of Health and Family Welfare, Delhi.