1.0 INTRODUCTION
1.1 INTRODUCTION TO ALLERGIC RHINITIS

Allergic rhinitis is an inflammation disease and common medical condition mainly associated with nasal, throat and ocular irritation; nasal congestion sneezing and cough which occurs due to antibody Ig-E and which also responsible for direct or indirect cause of asthma. (Mandhare S. N., 2011). It is very serious allergic disease in all over the world affecting around the 35% of total populations. Those people who are suffering from allergic rhinitis may be due to certain type of food they eat and substance or any food which causes such allergic reaction are called as allergy triggering substances. It is substantially affects patient’s performance and quality of life. It is commonly co-exist with other respiratory disorder notably asthma this relationship is not totally comprehend, yet expert sympathize that every condition represent a single disease with inflammation and allergy retort could be have important role in every condition. About 40% of patient with allergic rhinitis have concomitant asthma. A stepwise therapy approach is required with oral second-generation antihistamines as first line pharmacotherapy and decongestant or leukotriene’s receptors antagonist should be added when further multi-symptoms relief is needed. (Prenner B. M., 2006). Rhinitis could be differentiate based on allergic response produced in the body and categorized into two types i.e. allergic rhinitis and non-allergic rhinitis further allergic rhinitis again divided into perennial (all year around) and seasonal (also called “hay fever”) depending upon the condition and symptom associated with it and alternative types of allergic condition depends on symptoms seriousness & incidence could be proposed and it helpful in decision for selection of pharmacotherapy. (Kemp A. S., 2009).

Rhinitis occurs at different age and occurrences of sensitivity towards allergens would be inserted particularly as inhalants and which shows progress throughout childhood and transfers as age passed up to adult life. (Kulig M., 2000). Seasonal Allergic rhinitis means hay fever is disease mainly appears in small children and particularly in young & would be appears to be very less common in early age. In this early age of young child perennial rhinitis with symptoms of nasal pathway blockage would be very common. The role of allergens in causing rhinitis is very complex and sensitization to inhaled allergens does not mean that allergens are the cause of allergic rhinitis. In some case hay fever associated with sensitization due to
pollen grains and link between allergens exposure & symptoms of allergic rhinitis occurs due to it could be clear but in case of perennial mainly occurs due to house dust mites. The link between inhalation of allergens & manifestation associated with rhinitis could be not quite clear. The related allergens would be change as per situation and geographic location. Various tests were performed in order to identify allergic condition based on symptoms, one of the important test among i.e Skin prick test.

**Table no. 1.1.1: Types of rhinitis and their characteristic features**

<table>
<thead>
<tr>
<th>Rhinitis type</th>
<th>Characteristic features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Allergic rhinitis</td>
<td></td>
</tr>
<tr>
<td>1.1 Seasonal allergic rhinitis</td>
<td>Occurs in spring and early summer due to pollens, timing dependent on plant species and geographical location.</td>
</tr>
<tr>
<td>1.2 Perennial allergic rhinitis</td>
<td>Symptoms occur year around due to house dust mite, pollens and pet animals.</td>
</tr>
<tr>
<td>2. Non allergic rhinitis</td>
<td></td>
</tr>
<tr>
<td>2.1 Idiopathic</td>
<td>Occurs due to strong smells and changes in temperature.</td>
</tr>
<tr>
<td>2.2 Infective</td>
<td>Most commonly repeated in viral infections due to nasal discharge.</td>
</tr>
<tr>
<td>2.3 Vasomotor</td>
<td>Sudden on onset and offset of watery discharge.</td>
</tr>
<tr>
<td>2.4 Occurs due to eosinophilia</td>
<td>Negative allergy tests but greater than 20% eosinophils on nasal smears.</td>
</tr>
<tr>
<td>2.5 Drug induced</td>
<td>B-blockers, chlorpromazine, aspirins, oral contraceptives and rebound symptoms from topical decongestant.</td>
</tr>
</tbody>
</table>

The role of allergens in allergic rhinitis is very complex. There would be the sensitization occurs due to an inhalants allergen that would be present in environment that does not mean that allergen could be the cause of the such allergic rhinitis conditions where in some other situation like seasonal rhinitis with relevant pollen
sensitivity there could be link between allergen exposure & symptoms of allergic rhinitis would be clear but however in other conditions for example perennial rhinitis & house dust mite allergen sensitization in such case situation would be less clear because there would not any linkage between allergen and symptoms of rhinitis and moreover allergens which responsible for said condition could be differs significantly according to geographic location associated with it. Generally skin prick tests are performed for finding out relevant causes and symptoms likely to be present due to allergic rhinitis. If result of skin prick test found to be positive that does not mean that patient suffering from allergic rhinitis may be due to sensitization of patient body with production of IgE antibodies. In this skin prick test it would be very important to analyze the limit of the allergens which likely to be present and with huge amount of ragweed could be escort to produce hesitation & meaningless strive at allergen restraint. The outcomes of skin prick procedure may be correlated with background of patient for exemplar manifestation could be found to be less in early spring seasons which gives dismissive skin prick tests to pollen grass & would give cooperative skin prick test to house dust mite which provide basis for identification and assessing symptoms associated with it and could said that condition were non-allergic in terms of house dust mite. (Kemp A. S., 2009) Epidemiologic studies have described the relationship between allergic rhinitis and asthma means any person suffering from asthma having the symptoms of allergic rhinitis. (Linneberg A., 2002) this type of characteristic features for observing asthma in patient and same patient suffering from alternative respiratory related consequences would produce proof that asthma could be a congenital illness. Various review and research article were reported for clinical study regarding allergic rhinitis patient may not suffer from asthma related illness but they would be suffering from eosinophilic obstruction in mucous membrane. The patient with allergic rhinitis may have bronchial hyper-responsiveness due to certain type of seasons in which pollens were released in such seasons. Due to such pollen grains, allergens may causes stimulation of mucosal membrane of nose and produced bronchial hyper-responsiveness. The concept of one airway, one disease was introduced because of disambiguation of cells, inflammatory mediators & cytokines which are available between respiratory tracts (Holgate S. T., 2006). As per above justification allergic rhinitis and asthma were not different disease condition but simultaneously asthma in allergic rhinitis persons & concurrent allergic rhinitis in asthmatic patients should be examined and treated one by one for respiratory tract.
In the past, allergic rhinitis was subdivided into two types: seasonal or perennial allergic rhinitis. Such types of allergic rhinitis occur due to internal irritants like home dust and fungi and outdoor allergens happen due to seasonal pollens. However, in some specific areas, such pollens cause perennial allergic rhinitis when patients come in contact with allergens like pollens which are likely to be present in carpet and home furniture after pollinating seasons. While in case of perennial allergic rhinitis, symptoms associated with it would not continue for all-around the year, and in seasonal allergic patient, may sensitive to various allergens causing allergic rhinitis condition in every time of the year. Moreover, there may be seasonal worsening in perennial allergic patients when they were unprotected to pollen grains. Due to these reasons, ARIA (allergic rhinitis in impact on asthma) in 2001 had proposed the word intermittent and persistent instead of seasonal and perennial and severity of illness was categorized based on it as a mild and moderate by taking into mind their effect on work, daily activities, and sleeping conditions as shown in figure (Bousquet J., 2001).

![ARIA Classification of Severity of Allergic Rhinitis](image)

**Fig. no. 1.1.1:** ARIA classification of severity of allergic rhinitis.
1.1.1 Pathophysiology of Allergic rhinitis

The **pathophysiology** of allergic rhinitis and inflammatory reaction associated with it mainly take place by 2 stage: an early phase retaliation & late phase retaliation, early phase retaliation mainly associated with degranulation of mast cells, which are concentrates near the area of nasal mucosal epithelium in patient with allergic rhinitis, is a critical part of the early phase retaliation, which are occurs within minutes of allergens challenge while late-phase response occurs due to chemotactic factors which is released from mast cells perpetuate the inflammatory response by stimulating the migration of neutrophils, eosinophil, basophils & T-lymphocytes to the site of the allergens unprotected. These cells were fundamentally authoritative for sustaining late phase response, which happened hours after an allergens challenge. (Robert A., 2003). Normally two to eight hours’ time period after exposedness to allergic antigens causes released of inflammatory mediators & would affect tissue destruction and produced severe destruction of mucosal epithelial cell damage. This type of delayed response produced due to mediators which increased sensitivity toward the repeated exposure allergic conditions to certain pharmacological agents. Mast cells which are released due to chemical mediators are present in human body and high proportion of these types of cells was found in blood vessels of respiratory airways and conjunctiva. After degranulation of mast cell, histamine released form mast cell which was known for causing various allergic conditions by binding with the different histamine receptors and developed vasodilation & undisturbed muscularity elision leads to expeditious fluid ejaculation and passed into nasal tract. (Evans R., 1993).

**Sensitization and early phase reaction**

During periods of continuous exposure of allergens which increased the number of IgE- coated mast cells resulting into traverse of epithelium and make it granulate. (Naclerio R. M., 1991). Due to degranulation various mediators such as histamine, tryptase, chymase, heparin and other enzymes where released from it and in addition to this mast cell also secrets several inflammatory mediators including prostaglandins D$_2$ and leukotrienes. These arbitrator provocation leakages in blood vessels and produce mucosal edema together with watery rhinorrhea, main characteristics of allergic rhinitis. These mediators also stimulate sensory nerves, which transfer the sensation of nasal itch and congestion and produce fundamental...
involuntary respond like sneezing. The above response developed intervening in the few seconds of allergens divulgence; hence it is termed as early phase or sensitization phase (Mygind N., 1994). The human airway mucosa of upper side consist of antigens presenting cells which are in dense network of heterogeneous group of cells which were closely related to dendritic cells & monocytes. Dendritic cells present inside inter most cellular channels which are surrounded by lowest epithelial corpuscles & they were frequently efficacious corpuscles which are responsible for persuade & improving unaffected rejoinders. Due to such type of primary immune response lead to sensitizing of air passage of mucosal membrane takes place when an inhaled allergen come in contact with antigens presenting cells in human airway mucosal walls. Antigens presenting cells distinguish insolence & process antigen into small peptides which could be associated with major histocompatibility complex (MHC) Class II molecules. Antigens presenting cells also transform to T helper corpuscle to the cells by means of cytokines. These antigens presenting cells further produce different cytokines which done several function for breakthrough of antigen specific IgE prolongation. In a process termed as isotype switching, specific B cell subsets transform into plasma cells, which switch from IgM to IgE prolongation. (Skoner D. P., 2001).

Late phase reaction

The mast cell-derived mediators released during sensitization stages are hypothesized to execute on post capillary endothelial corpuscle to enhance the articulation of vascular endothelial corpuscle & E-selection, which helps the attachment of circulating leukocytes to endothelial cells. Late phase reaction could be associated with increasing the amount of lymphocytes, eosinophils, basophils & neutrophils would be discharge different types of cytokines & other chemical troubleshooter. In this late phase reaction eosinophils play major role in producing allergic reaction by producing free radicals of oxygen like superoxide anion & H$_2$O$_2$ could be severely affects the epithelium layer & causes the harmful inflammatory consequences. (Bascom R., 1988). This protein are capacity to produce nasal hyper-reactivity and affecting harmful damage to air passage layer of epithelium and exposes the local nerve fibers. The late phase response could be primarily occurs due to nasal obstruction as well as sneezing and rhinorrhea that persist from the early phase response.
This action additionally accounts interference in the elongation of natural endopeptidase & thus neuropeptides secreted by the nerve fibers remain ungraded prolonging the inflammatory process indefinitely. (Cameron L., 2000).

Fig. no. 1.1.2: Allergen-induced sensitization and inflammation.

**Neurogenic inflammation**

When epithelial membrane of respiratory were demolished and nerve endings were expose to proteins present in the eosinophilic cells and get excited due to stimulations and called retrograde axonal reflex. Due to this sensation producing nerve fibers would disguise neuropeptide and produce shrinkages of unruffled tendons, mucosal seepage of goblet corpuscles and plasma exudation from capillaries. These whole procedures occur due to respiratory epithelium exposure is called neurogenic inflammation.
Nonspecific hyper-responsiveness

In certain conditions nonspecific hyper responses also happen which would be one of the clinical features & characteristics of allergic inflammation which occurs because of infiltration of eosinophilia & damage of mucosal membrane of nasal tract. The mucosal membrane of nasal tract very hyper reactive towards above said conditions and causes the various symptoms related to allergic rhinitis. This type of nonspecific reaction may not be related to IgE because it is non immune response and hypersensitivity towards these responses may increases in allergic symptoms in allergic rhinitis patients (Togias A., 2000).

1.1.2 Diagnosis of Allergic rhinitis

The prevention and treatment of allergic rhinitis would be depended on a diagnostic tests and allergic symptoms history associated with the patient. When two or more two symptoms of allergic rhinitis were found out of various symptoms like watery rhinorrhea, sneezing, nasal obstruction and nasal pruritus happens for more than one hour on most of the days than allergic rhinitis would be strongly present. In such type of condition, severity of disease condition were classified as per ARIA guidelines and diagnosis test could be confirms by carrying and performing skin prick test & serum-specific IgE level where as mucoid postnasal drip, unilateral nasal stuffiness, pain, anosmia were normally not associated with allergic rhinitis.

Skin testing

The one of the important test to identifying allergic rhinitis i.e. skin testing which gives idea about presence of allergens which are responsible for causing the allergic rhinitis. There are various tests available in order to assessing the allergic conditions like intradermal test, skin prick test, patch test and scratch test. Among all above tests mainly skin prick procedure would be usually helpful in clinical suiation. In above said skin prick test, various false negative & positive reactions are performed. In false positive reaction specific allergens does not consistently have direct relation to exact allergic consequences in the nasal cavity. But it would be squabble respecting the perceptions of the outcomes of test and exemplars for positivity are different among allergy clinics. Furthermore, skin testing procedure could have certain disadvantage due to some factors like presence of drugs and
patients' age would influence the result of test. This test could not work if patient suffering from dermatologic disease and hence it would be difficult to perform. (Guerra S., 2002).

**Serum specific IgE level**

The radio allegro-sorbent test was the prime & first strategies for identification serum IgE level and due to requirement of costly instrument and radio-isotope these tests were not widely used. Another drawback of said test could not able to detect multiple antibodies simultaneously. Because of limitations with radio allegro-sorbent test new method i.e. multiple allergen simultaneous test (MAST) was used as like above said allergic conditions. Multiple allergen simultaneous tests have certain advantage over radio allegro-sorbent test due to cheapness of reagent. In the multiple allergen simultaneous tests uses of photo reagent in place of radio isotope which does not required costly equipment and could detect multiple allergens simultaneously. Another most important advantage of multiple allergens simultaneous tests i.e. does not influence by presence of other drug substance or any interfering agent. Moreover multiple allergens simultaneous test have low sensitivity as compared to other diagnostic test. One of another important test also carried out for identifying symptoms of allergic rhinitis based on hydrophilic carrier polymer i.e. capsulated hydrophilic carrier polymer were gives better result with high accuracy and detects allergens more quantitatively than the multiple allergens simultaneous tests. Capsulated hydrophilic carrier polymer performed using cellulose polymer that have high affinity towards the antigens by binding on it. (Finnerty J. P., 1989)

**Clinical parameters related to asthma**

The seriousness of allergic rhinitis have a linkage related to effect of asthma means any person suffering from allergic rhinitis condition always have higher risk of asthma due to increasing level of serum antibodies and eosinophilic count number of nasal tract were also high and these types of conditions leads to causes the bronchial hyper-responsiveness and due to the bronchial hyper-responsiveness there were change in the number and distribution of eosinophils which were present in the nasal cavity may also be altered. (Silvestri M., 2005). By performing study on eighty three pediatric patients suffering from allergic rhinitis and thirty two normal pediatric
patients they have reported that if there were development of inflammation in nasal tract than there is a chance of bronchial hyper-responsiveness which leads to allergic conditions. Usually bronchial hyper-responsiveness was found higher than the normal pediatric patient and there are chances of developing asthma to the bronchial hyper-responsiveness patient. The probability and risk of developing asthma were higher because of internal allergens like dust mites and cat dander.

**Treatment of allergic rhinitis**

Remedial treatment for allergy causing illness could be prevented without influencing the day to day activities in order to intercept squeal such as asthma exacerbations or sinusitis. The restraint of feature and preventing various events associated with it and enhancement of forbearing status of life are prime objective related to any remedy related to allergic rhinitis. The almost all related prodromal corresponding to allergic rhinitis were congestion of nasal mucosa, rhinorrhea, itchy & eye with lots of water like fluid and finally repetitive sneezing particularly found in perennial allergic rhinitis. From recent online survey of more than 2000 people with allergic rhinitis, it was found that 75% among those are suffering from severe nasal decongestion and 40% of people with allergic rhinitis have concomitant asthma. The relationship between allergic rhinitis and asthma is not entirely comprehend but few specialist sympathize that both situation leading to one illness together with inflammation and allergic response playing important role in both the conditions. (Kay G. G., 1997) A large body of data establishes intranasal corticosteroids and antihistamines as the mainstays of therapy for allergic rhinitis. Numerous placebo-controlled studies of intranasal corticosteroids & oral non-sedating antihistamines have demonstrated them to be effective versus placebo at alleviating the range of features of hypersensitive & at upgraded sufferer well-being related quality of life.

In order to treat symptoms of allergic rhinitis together with asthma there are many drugs therapy among that cortisone steroid via inhalation route of administration, long acting bronchodilators and leukotriene receptor antagonists were prime treatment for asthma & certain second generation antihistamines like cetirizine, fexofenadine, loratadine & desloratadine have been signifies the capabilities in relieving asthma manifestations.
In illness person with sensitive condition of inflammation due to rhinitis & accompanying asthma in which drug called cetirizine play significant role in reducing the upper and lower respiratory tract features. According to ARIA guidelines second generation antihistaminic proves better welfare & capabilities in prevention of condition called of allergic rhinitis because there are various severe side effects like sedation and memory impairment occurs with first generation antihistaminic drugs there for second generation antihistaminic drugs were used in treatment of allergic rhinitis. Oral route of administration of above said second generation were very effective in diagnosis in various symptoms of allergic rhinitis but these drugs have low safety and efficacy profile when they are used through nasal cavity. The second generation antihistaminic drugs like terfenadine & astemizole when given along with antifungal drugs or any macrolide antibiotic then there are severe risk of cardiac toxicity occurs because these drugs effect the activity of isoenzyme, there for drugs like terfenadine & astemizole were not prescribed in most of country. The second generation drug like ebastine prescribed along with other drug because ebastine easily crossed the blood brain barrier and metabolized by isoenzyme but high dose of ebastine given with proper care because ebastine when given with other antifungal drug like ketoconazole and macrolide antibiotic like erythromycin produces prolongation of QT in electrocardiography with no clinical significance. Desloratadine remedies also recovered hay fever situation and various prodromal associated with asthma and reducing the requirement of β-agonists (Simons F. E., 2004). In many research & review article reported that topical application of antihistaminic drugs were reduces the various symptoms of allergic rhinitis but these drugs less effective than the intranasal corticosteroids. Use of this intranasal corticosteroid two times a day relieves the symptoms than the oral route antihistamines. (Berger W. E., 2003).

Moreover desloratadine would be very preventive as compared to montelukast in relieving in the features occurs together with asthma & unfavourable implementation produced because of above drugs were similar in nature in both preventive categories. There for patient suffering from said allergic condition combination therapy prove better symptoms relief than the single alone. The combined therapy of antihistamine and leukotrienes antagonist by oral route of administration proves better than the other drugs in consideration of allergic situations.
Very large numbers of impersonal trials & boundless long term clinical use have been demonstrated because of their well abilities and overshadow tolerability and safety profiles as compared to first generation chemical entities & second generation antihistamines could also be help in safely use of combination with most other classes of anti-allergic agents. Intranasal route for administering the steroids could be the good treatment & curing the ill person having more manifestations and by administering the steroids could be help in additional prodromal reassurance. A leukotriene receptor antagonist appears a novel categories agent for the prevention and curing of any type of allergic rhinitis conditions.

To establish the role of the leukotriene receptor antagonists in managing of hyposensitized rhinitis & their unsympathetic capabilities requires to have correlated to that of non-sedating anti-histamines. Leukotriene receptor antagonists have been evaluated for efficacy alone and in combination with oral non-sedating antihistamines, and they have been compared in head-to-head studies with antihistamines. According to recent studies the efficacy of leukotriene receptor antagonists compared with placebo has been evaluated in two small studies. The first investigation, an antigen-challenge study, showed the leukotriene receptor antagonist montelukast could not appropriate effectiveness as compared to placebo for prevention of various symptoms of allergic rhinitis. (Philip G., 2002). In the second study, a single daily dose of the leukotriene receptor antagonist zafirlukast improved nasal congestion and, less consistently, rhinorrhea and sneezing versus placebo over a 2-day period in forbearing person with seasonal hay fever situation. The preliminary research manage to date, then, shows only equivocal benefit of leukotriene receptor antagonists compared with placebo in allergic rhinitis.

A leukotriene receptor antagonist was compared with the antihistamine loratadine in two randomized, doubleblind, placebo-controlled studies. In the first study the leukotriene receptor antagonist montelukast & loratidine taking dose of 10 mg daily for period of 2 weeks & were each very potential than placebo in terms of alleviating nasal related manifestation being measured. The observation of these studied were surprising in that both montelukast and loratadine demonstrated comparable efficacy at relieving nasal congestion. As an antihistamine, loratadine is not expected to be effective for nasal congestion, whereas montelukast is expected to relieve nasal congestion of nasal mucosa were related to its mechanism of action. (Donnelly A. L., 1995). So according to details study of various literature of allergic rhinitis

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combination of therapy of oral antihistamines & leukotriene antagonists were considered first line preventive measures for prevention & diagnosis of different situation related to hay fever.

**Table no. 1.1.2: Drug used in diagnosis of allergic rhinitis conditions.**

<table>
<thead>
<tr>
<th>Class</th>
<th>Mechanism of action</th>
<th>Generic name</th>
<th>Major side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-histamines</td>
<td>Histamines H₁ receptor antagonist</td>
<td>Desloratidines</td>
<td>Bitter taste, epistaxis, headache</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Olopatadine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rupatadine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fexofenadine</td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>Glucocorticoid receptor agonist, anti-inflammator action because of different consequences</td>
<td>Prednisolone</td>
<td>Transiently irritation in nasal mucosa, pharyngitis, taste, voice changes epistaxis.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dexamethasone</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Budesonide</td>
<td></td>
</tr>
<tr>
<td>Leukotriene inhibitors</td>
<td>LTD₄ receptor antagonism</td>
<td>Montelukast</td>
<td>There may be possibility of enhancing the risk of suicide with Montelukast Na.</td>
</tr>
<tr>
<td>Decongestants</td>
<td>α-Adrenergic receptor agonism</td>
<td>Phenylephrine HCl</td>
<td>Nervousness, insomnia, irritation, tachycardia, hypertension &amp; aggravation of urinary obstruction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pseudoephedrine HCl</td>
<td></td>
</tr>
<tr>
<td>Mast cell stabilizers</td>
<td>Mast cell stabilization</td>
<td>Cromolyn sodium</td>
<td>Nasal burning and sneezing or sneezing in 10% patient.</td>
</tr>
</tbody>
</table>
1.2 INTRODUCTION TO METHOD VALIDATION

Pharmaceutical analysis is the branch of science which deals with identification of substances and determination of amount present in particular sample. Pharmaceutical analysis covers the bulk materials, dosage forms and more recently, biological samples in support of bio-pharmaceutical and pharmacokinetic studies. Analysis can be divided into areas called qualitative and quantitative analysis. Pharmaceutical products synthesized and identified using instrumental techniques (Sethi P. D., 2001). Developing analytical methods are necessary because it could be used for product development and very important in process control & analyzing quality control aspect of chemicals. One can developed analytical method by using techniques mainly spectroscopic or chromatographic which have their own special features which must be considered while carried out these techniques. Each step in analytical method are equally important there for it must be investigated in order to determine active content of any drug product in presence environmental condition & matrix to know the effect procedural variables which could be affect the estimation of active content of drug in presence of matrix. (Bressolle F., 1996).

Need for pharmaceutical Analysis

- New Drug Development.
- Method Validation as for ICH Guidelines
- Research in Pharmaceutical Sciences.
- Clinical Pharmacokinetic Studies.

When encouraging outcomes were resulted because of explorative validation practice during the strategies expansion stage, then only full validation should be stared. Pharmaceutical analysis require very precise and accurate assay methods to identifying active drug moieties in Pharmaceutical or microbial preparations. The assay procedures could be delicate, selective, rugged and reproducible. The Laboratory controls shall include the establishment of sound and appropriate specification standards and test procedure to assure that the final drug substances conforms to the required standards of identity, strength, quality and purity. Modern physical methods of analysis are extremely sensitive, providing precise and accurate information about the standards of chemicals or drugs up to nanogram levels like HPLC (Glajch L., 1999). These methods are used extensively in the quality assurance.
of raw materials, in process quality assessment, stability of the drugs on storage and monitoring drugs concentrations in various body fluids or tissues.

Today analysis of drugs and pharmaceutical products are performed by analytical chemist specializing in chromatographic, spectroscopic and wet chemical analysis (Remington 2007). A drug could be explain as chemical entity which could use in relieving manifestation and used for diagnosis, cure, mitigation, prevention & treatment of illness which would likely to appear in patient or in animals and which would be affect the various normal function and structure of patient & animals (Mithal B. M. 2003). Medicinal chemistry is a branch of chemistry mainly concerned with determining the influence of chemical structure on biological activity (Foye W. O., 1989). Pharmaceutical chemistry could be explained as terminology of science that deals with use of general principles of chemical science in order to study the various chemical entities for manufacturing, nature, constitution, framework which could be alter function of an organism and which could also include methods of quality control & their specification for storage and they were broadly categories according to

1. Pharmacodynamic agents

2. Chemotherapeutic agents

Pharmacodynamic agents are agent or group of medicament which were responsible for stimulating or depressing different physiological functions of human body so as to provide some relief to the body in case of body abnormalities, without curing the disease. They are mainly used in case of non-infectious diseases; so as to correct the abnormal body functions. Non-selective central nervous system modifiers (depressants or stimulants), adrenergic stimulants and blocking agents, cholinergic and cholinergic blocking agents, cardiovascular agents, diuretics, antihistaminic agents and anti-coagulating agents were few demonstrations of these categories & These agents could not have any specific effect on transmittable organisms, which cause various diseases. Chemotherapeutic agents are agents who could be preferably selective in nature and very poisonous to illness producing organisms without harmful effect to the host. Some of the examples of this group are organometallic agents, anti-malarials, anti-bacterials, anti-protozoals, anti-fungal agents, anthelmintics, anti-septics, anti-tubercular agents, anti-neoplastics, etc. Every country has legislation on
bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called “Pharmacopoeia” (e.g., IP, USP, BP: and Martindale Extra Pharmacopoeia, MEP). Pharmaceutical analysis could not only relate to medicaments (chemical entity and its composition) but also related to their starting materials i.e., with the raw material on which degree of pureness & confines of medicament depends & quality of medicated could be deduced only after determining their final profanes by performing various testing methodologies in order to assess their purity & confines quality of actual entity in appropriate substance in their constitution of formulations (Beckett A. H., 1986).

Quality is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods there can be no “second quality” in drugs. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. As a matter of fact, it is built in from the time of inception of the thought to make a product, to the time; it is finally made and sent out with an okay quality report. In popular practice, the quality of medicines or pharmaceutical products is assured through quality control. It is therefore, essential that quality assurance department must adopt “good laboratory practice” to ensure reliability and accuracy of results given out by them. The assurance of the quality and the reliability of pharmaceuticals, together with their careful control are our moral obligations towards the sick human beings. Consequently the manufacture and the control of drugs are very responsible task and they need substantial knowledge of the science. The decision to release or reject a product is based on their one or two categories of dominance & combination of both. If the product is a single entity of high purity, the analytical data is the basis for decision but most of time; the formulation is a physical mixture of different potential medicament & with expansion of pharma related companies throughout last several years, there has been a rapid progress in the field of pharmaceutical analysis involving complex instrumentation, providing simple analytical procedures for complex formulations is a matter of foremost importance.

Some of the medicinal products are still being assayed by the time-tested procedures of gravimetric and titrimetric techniques, though use of electronic balances and recording titrators have improved these classical procedures considerably. A wide diversity in the type of analytical technique has been
characteristic of assay method for pharmaceuticals. Simple distillation is useful for determining alcohol contents of the galanicals, or other substances being volatile in current of steam such as menthol, thymol and even certain alkaloids such as ephedrine. Moisture contents have been determined by drying in a dessicator or in a heated oven. Use of moisture balance in which sample pan is directly heated by infrared lamp without removing the sample from the balance has been an innovation, though the most specific and convenient procedure being Karl-Fischer titration. Chromatographic techniques are separation techniques which are very important in analysis of drug or any chemical entity in pharmaceutical field of research but due to availability of modern spectrophotometer one could do analysis with the help of advance features of which were incorporates into it have becomes essential tool for analysis of active pharmaceuticals in field of pharmacy. With the help of such modern spectrophotometer analysis of few colorless substances could be possible by converting them into specific derivative which can able to produces the color and intensity of these color derivatives could be measured by using suitable wavelength & compared with known color derivative of reference material having standard purity. The fluorimeter measures fluorescence that may be present in the sample such as riboflavin or may be developed into the sample such as thamine hydrochloride. Solvents which are used in spectrophotometric analysis for dilution purpose must have high purity and capable of producing desired strength of standard drug solution and sometimes it could be prefer to use blank method of analysis with the help of solvent and reagent in order to obtained correction in measurement due to presence of inherent substance so that we could easily get required absorbance due to presence of inherent chemical entity which likely to be present in the dosage form. (Sethi P. D., 1986).

The methods of determination of drug content could be differentiating according to nature and physico-chemical & biological properties but physico-chemical very frequently used in estimation and analysis of drug content. Amongst these Physical methods for determination of drug content carried out by studying physical properties of a drug substance which includes determination of solubility, moisture content, density, specific gravity for liquid samples and melting boiling points. While in case of physico-chemical method (Basett J., 1986) determination of drug content carried out by studying the fleshly behavior that could be occur due to outcomes of chemical rearrangement. Among the physico-chemical methods, the most important are...
optical (refractometry, polarimetry, emission and fluorescence methods of analysis, photometry including photocolorimetry and spectrophotometry covering UV, visible and IR regions, nephelometry or turbidimetry) and chromatographic (column, paper, thin-layer, gas liquid, HPLC) methods. Methods such as nuclear magnetic resonance (NMR) and paramagnetic resonance (PMR) are becoming more and more popular. The chemical system comprised of precipitation producing strategies & volume measurement estimation which could be depended on coordination producing, acid base, precipitation and redox reactions.

Modern pharmaceutical analysis of any drug substance must need the following requirements in order to assessing their quality aspects.

1. The analysis of drug content should performed in limited time period
2. The accuracy of developed method must be in range as per guidelines
3. The analysis of drug content should very economical in nature
4. The analytical method which were selected must be gives precise results
5. These requirements could be fulfills by the physico-chemical methods of analysis and advantage of use of analytical strategies in small pharma related companies due to following reasons (1) Biological and microbiological methods are expensive and time consuming (2) Modern analytical methods of choice such as HPLC, GC, NMR, Mass etc. depend on sophisticated equipment which are costly. So they are not within the reach of most laboratories. Visible spectrophotometric methods are very simple, economical and maintenance problems of the instruments are minimal.

The types of analysis can be distinguished in two ways:

**Qualitative Analysis**: To refer identity of product, i.e., it yields helpful ideas from molecule constitution and composition of atoms and their frame work and element present in sample under investigation could be identified.

**Quantitative Analysis**: To refer the purity of the product, i.e., the results are in the form of numerical data corresponding to the concentration of analytes.

**Types of Analytical Methods**:

The various methods of analysis can be grouped into two categories. They are:
1. Chemical methods.

2. Instrumental methods.

**Chemical Methods:**

In these methods, volume and mass are used as means of detection.

1. Titrimetrical methods like acid-base, oxidation-reduction, non-aqueous, complexometric and precipitation titrations.
2. Gravimetric and thermo gravimetric methods.
3. Volumetric methods.

**Instrumental Methods:**

Based on principles different Instrumental methods are available and these analytical process were related to identifying & deducing the active and non-active fleshly parameters of a substance (*Ashutoshkar S., 2005*).

**Table no. 1.2.1: List of instrumental method**

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Principle</th>
<th>Instrumental Method</th>
</tr>
</thead>
</table>
Analytical method development and validation is a good research in the field of pharmaceutical analysis utilized to determine the drug content from hugeness, pharma related dosage regimen and also form bio materials like blood serum, urine etc in view of the industrial scenario and literature, by using spectral and chromatographic techniques, revealed the utility of analytical instruments like high pressure chromatography’s techniques & hyphenated techniques for the quantification of API’s at greater accuracy and precision in quantification of drugs in formulation and in biological fluids even at low concentration. Once the ideal technique without interferences is selected for the analysis of medicinal formulations, which is most suitable one, the analysis should be performed at least in duplicate but preferably in triplicate. A simple calculation is converted into information through experimental data obtained which is the reflection of analytical sample to be determined. The results obtained through practical experiments will be always associated with a level of some error and could be right for any physic-chemical divination. Because of this reason, it is always obligatory to establish the magnitude of this uncertainty to turn the data into accurate & precise result of the analysis. For this reason it becomes very necessary to validate the developed method and prove the ability of the particular technique for the correct analysis of sample under investigation (Jeffery G. H., 1994a).

Determination of Active medicament in terms of purity is essential in the department of quality control and quality assurance for the pharmaceutical dosage form, prior to its entry into market. Now days, potency of the drugs and efficacy are playing major role in patient compliance during therapy. Hence, No matter the therapeutic agent present as individual or in the combination with other drugs, the amount of active ingredient at individual dose should be complied as per posology direction in order to achieve efficacy. However, in recent decades there are lots of mechanism were outlined and presented for their divination of medicament in quality control & quality assurance aspects. There is a rapid advancement and developments in the field of pharmaceutical analysis where sensitive chromatographic and spectral pattern could be evoked for their ascertainment of drugs in various pharma related products in pharmaceutical industry, the analyst plays a vital role in FDA approval of newer potent drugs with respect to method development, validation and determination of drugs (Smith R. V., 1981).
**Need for method validation**

A method of analysis is supposed to be tested for validation when it is crucial to confirm that; the working characteristics are satisfactory to be used for a definite analytical difficulty, for example

- When the established technique needs perfection.
- Development of new method for particular problem.
- Established scheme used in another laboratory or with separate analysis or dissimilar
- Instruments.
- To show the uniformity between two dissimilar techniques for example, a novel method and a reference Pharmacopoeial method.

Analytical method validation includes all procedures and checks required to prove the reliability of a process for quantification estimation in a sample containing a component or a known series of components.

For analytical method validation, US-FDA has given some guidelines in USP and is referred as “Eight steps of analytical method validation” ([Sethi P. D., 1993](#)).

Patterns framing includes identifying, characterization & optimization of different phases of analyte arrangement, high pressure chromatograph quantification, identification & determinations.

Prior to method development of selected drug it is important for extensive literature survey regarding ([Hubert P. H., 1999](#)):

1. Choice of the instrument which is suitable for the analyte such as
   - Gas Chromatography (GC)
   - UV Visible spectroscopy
   - High Pressure Liquid Chromatography (HPLC)
   - Combined GC and LC Mass Spectrometry (GCMS)
   - HPLC-MS
   - HPTLC
   - LC-MS-MS
     - Choice of the mass parameters such as parent ion, product ion.
Choice of the ionization mode such as positive mode or negative.
Choice of the gas parameters such as curtain gas, nebulizer gas, heater gas and CAD gas

2. Choice of the chromatographic conditions such as Mobile Phase
   - Column
   - Autosampler conditions
   - Flowrate, injection volume

3. Choice of the internal standard.
4. Choice of extraction method.
5. Choice of regression methods.

The procedure expansion & initiation for any analytical strategy could be incorporated estimation of selectivity, accuracies, preciseness, recoveries, calibration curve & whether analyte could be stable in added or spiked preparation (Breda C. A., 2004).

It is not necessary at all times to evaluate analytical working parameters, as distinct assessment methods need distinct validation schemes. Nearly all general class of assays for which data for validation must be essential is as follows:

- Quantification of major constituents or active pharmaceutical ingredients.
- Impurity detection or degraded constituents.
- Determination of working parameters.

There are three different categories of drugs which should cover different parameters of validation. Some of the methods require all the parameters to be covered, where as some methods need not cover all the parameters. The present work consist the techniques falling under category I of validation parameters. The applications of the proposed method fall under category III of validation parameters.

**Category –I:** It includes methods of analysis for quantification of chief constituents of drugs in bulk or active pharmaceutical constituent (together with stabilizers) in accomplished medicinal preparations.

**Category –II:** Includes methods of analysis for the impurity detection in drugs in bulk or degraded components in accomplished pharmaceutical preparations which include assays for quantification and limit tests.
**Category –III:** is for the methods of analysis for evaluation of working characteristics (example dissolutions, drug releases).

The following table is the chart for data essentials which are necessary to carry out assay validation. The kind of process and its proposed use decides what parameters are compulsory to be inspected for category I, II and III are illustrated as shown in following Table.

**Table no. 1.2.2:** Data essentials necessary for assay validation

<table>
<thead>
<tr>
<th>Data treatment parameters</th>
<th>Assays category- I</th>
<th>Assays category- II</th>
<th>Assays category- III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quantitative</td>
<td>Limit tests</td>
<td></td>
</tr>
<tr>
<td>1. Accuracy</td>
<td>√</td>
<td>√</td>
<td>*</td>
</tr>
<tr>
<td>2. Precision</td>
<td>√</td>
<td>×</td>
<td>√</td>
</tr>
<tr>
<td>3. Specificity</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>4. LOD</td>
<td>×</td>
<td>×</td>
<td>√</td>
</tr>
<tr>
<td>5. LOQ</td>
<td>×</td>
<td>√</td>
<td>×</td>
</tr>
<tr>
<td>6. Linearity and Ranges</td>
<td>√</td>
<td>√</td>
<td>×</td>
</tr>
<tr>
<td>7. Ruggedness</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

* might be applicable based on the character of definite experiment

**Parameters to be considered for Method Validation:**

1. Accuracy
2. Precision
3. Specificity
4. Linearity and range
5. Ruggedness and Robustness
6. LOD
7. LOQ

**Accuracy:** It could be explained like concordance or nearness of results amongst true outcomes & observed (determined) investigative values obtain by the application of an experiment technique number of times. Accuracy of an analytical process is resolute by systematic or methodical error involved. The accuracy is satisfactory if the
distinction among the proper value and the obtained mean value is not greater than the Relative Standard Deviation values attained for repeatability of the technique. The parameters provide details about the drug recovered from analytical sample and influence of environment, as recoveries are likely to be excessive as well as deficient.

**Precision:** the precision of a technique of analysis is the concordance or degree of conformity between the various outcomes of same measurement of several samples under test & could be determined by assessment of adequate sequences of multiples samples to estimates the standard deviations & relative standard deviations. Reproducibility (precision on comparison): Precision beneath diverse conditions (dissimilar sample for analysis, another laboratory, separate days and chemicals from variety of sources) using the unchanged sample. Relative Standard Deviation $\geq 10\%$ within the laboratory reproducibility.

**Specificity:** It is the capacity of a technique of analysis to review clearly the component of significance in the existence of constituents that may be predictable to be present, such as impurity and degraded components including constituents of surrounding medium present in the matrix. In case of assays, demonstration of preciseness is required so that the process were unchanged by the existence of impurity & additives. In routine, this can be demonstrated by treatment of the drug substances or product with proper levels of impurity & additives and confirming that the assay were not affected by the presence of these impurity & additives. In case of the non-availability of reference degradation product impurities, specificity parameter may be confirmed by the comparison of results of experimental containing impurities with other established method. These comparisons must consist of samples kept under similar relevant stressed environments for example: UV radiations, sun light, heat, moisture, acid- base hydrolysis, oxidative degradation etc.

**Linearity and range:**
The linearity of a process of analysis is its capacity to produce experimental outcomes in a direct or distinct mathematic conversion, proportionally relative to the amount of analyte in a sample within a certain series. It must be conventional around the limits of the process of analysis. The range is usually articulated in the same unit as result experiments (example: $\mu g/ml$, percentage, parts per million, parts per billion etc.)
Ruggedness: The extent of repeatability of assessment results observed by the examination of the sample trials under an array of similar parameters such as separate labs, separate analyst, another instrument and dissimilar set of reagents, different times, different temperature, and different day.

Robustness: The gauge of its ability to stay unchanged by little but purposeful changes in process characteristics and indicates a sign of its steadiness during regular practice. Example of typical variation in First derivatization UVspectrophotometric estimation is the stability of analytical solutions.

Most of the validation parameters described by ICH guidelines and USFDA guidelines have common features with slight variations. The above parameters provide a newly developed method a touch of correctness, repeatability, reproducibility, sensitivity, stability and assure the quality of novel method is up to the international standards and universally acceptable. It is related to monitoring of different parts in a preparation procedure. The main goal of validation should be such that it could have acceptable range of result which could easily confirm the specification by checking the raw materials & finished products in order to control the extent of impurities inserted accidentally or deliberately in pharmaceutical products.

Limit of Detection:

The smallest possible concentration of samples under study that produces response which can be easily detected without any difficulty, preferably thrice the noise level of the system. It is the minimum concentration of analyte below which it cannot be detected while the instrument can only detect the concentrations above this limit.

LOD can be calculated by using equation:

$$\text{LOD} = \frac{3.3\sigma}{S}$$

Where,

$$\sigma = \text{Deviation of pure analyte of different measurements}$$

$$S = \text{Slope of standard graph}$$
Limit of Quantitation:

It could be explained as small strength of sample under investigation which could be precisely and accurately measured and quantified. To determined LOQ the analyte concentration is reduced to a level where the precision of the method is not bothered.

Limit of quantitation can be calculated using following formula:

\[ \text{LOQ} = \frac{10 \sigma}{S} \]

Where,

\( \sigma = \) Standard deviation of replicate measurements

\( S = \) Slope of standard graph.

System Suitability Parameters:

Before starting the analysis of unknown sample, the analyst must establish that the instrument and developed method are capable of providing acceptable results. It may be accomplished with system suitability parameters. This testing is compulsory for a newly developed analytical procedure. The basic concept behind the test is that sample to be analyzed, electronics, equipment, and analytical procedure acts as an integral system. The various validation parameters must be prepares & examined for newly developed analytical procedure are based upon the type of procedure that has to be validated. These are the verification tests used to check reproducibility and resolution of chromatographic system so that determination can be carried out perfectly. Various standard parameters like theoretical plate, resolution, tailing factor and reproducibility of the method (%RSD for area of six replicate injections) are determined and compared with the standard recommended values (Sethi P. D., 1993 &ICH Product guidelines).
Table no. 1.2.3: System Suitability Parameters and Standard Specifications

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity factor</td>
<td>The peak obtained for the analyte should be properlyresolved from peaks of other compounds and void volume, generally value of ( k'' ) should be greater than 2.0</td>
</tr>
<tr>
<td>Resolution (Rs)</td>
<td>It should be greater than 2 between analyte peak and the peak of nearest eluting interfering substance such as pharmaceutical excipient, impurities, internal standard, degradation product, etc.</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>should not be more than 2</td>
</tr>
<tr>
<td>Theoretical Plates</td>
<td>Generally should be ( &gt;2000 )</td>
</tr>
<tr>
<td>Repeatability</td>
<td>%RSD should not be more than 2</td>
</tr>
</tbody>
</table>

1.2.1 Introduction to Spectroscopy

Ultraviolet & visible spectrometers could be used for several decades and over these decades they were most helpful equipment for ascertainment in pharma laboratories & this technique have various supplications because of their simplexes, versatility, moderate speed, accrues & economic in order to analyzing the samples. Ultraviolet and visible spectrometry were falls in the 200-800 nm range of electromagnetic spectrum which includes 200-400 nm wavelength in ultraviolet regions and 400-800 nm wavelength in visible regions which gives the basis of colorimetric method of analysis. The term ‘spectroscopy’ could be used for broad word which could comprise every analytical strategy which could be related to interaction of light with matter. Spectrophotometry could be one of the part of spectroscopy in which one could easily determines the absorption of photon of light or radiation which could be absorbed by molecules that are in a gas or vapor state or dissolved molecules/ions.
Quantitative analytical methods based on the measurements of UV-Visible radiation are the most popular and widely used in routine laboratory practice. Commercial instruments available today are very cheap and easy for operation. Spectrophotometric techniques are non-time consuming and non-labor consuming. The economic aspects of these techniques are worth significant. This is one of the cheapest technique available for the routine analysis, with comparative accuracy and precision, is the basic need of every laboratory. However, the simple technique of zero order (basic) has the limitation of low selectivity. The absorbance measurement is affected by interferences of other components of the sample because the recorded UV-Visible spectrum will be the sum of absorbance values of analyte and matrix.

Usually, the registered absorbance bands are well defined but disturbed by the background interferences. An isolation of analyte from one matrix to another matrix by solvent extraction may be the solution to this problem but increases the chance of loss of analyte, contamination of analyte and time consuming. One of the best and simple tools to increase the sensitivity and selectivity is derivatization of spectra. This modification allows eliminating spectral background and matrix interferences. Derivative spectroscopy retains the entire principles of conventional spectroscopy, for example, reliance of derivatization form (compared to absorbance value in zero order) on amount of analyte and law of additivity i.e. Beer’s Law (Owen A.J., 1996)

**General methodology for the development of new visible spectrophotometric method**

For developing & analyzing drug product which having unknown strength of any chemical entity one could be go for absorption spectrophotometric method of analysis & prime requirement for absorption spectrophotometric method of analysis are selection of suitable wavelength so that absorption of active content could be easily measured at selected analytical wavelength. The analytical wavelength can be chosen either from literature or experimentally by means of a scanning spectrum in the UV-visible region. For enhancing the responsiveness of absorption spectrophotometric procedure sign to sound proportion could be minimized by choosing the analytical wavelength of which could be capable of producing maximum absorbance. After selection of the suitable analytical wavelength, colour producing agent and absorbing product would be stabilized for certain time duration. Always the preparation of standards and unknowns should be on a definite time schedule.
Absorption spectrum

The absorption spectrum is a graphical representation of the amount of light absorbed by a substance at definite wavelengths. To plot a absorption curve, the values of the wavelengths are laid off along the axis of abscissas and the values of the absorbance along the axis of ordinates. A characteristic of an absorption spectrum is a position of the peaks (maxima) of light absorption, which is determined by the absorptivity at definite wavelengths.

Lambert’s Law

Lambert’s Law define as when beam of one phasic radiation could be allowed to enter to homogeneous absorbing channel & momentum of reducing in the strength of monochromatic light & width of channel are related to strength of incidence radiation.

Beer’s law plot

A standard specimen of the analyte is taken and the solutions of it with known concentrations are prepared. The absorbances of all the solutions are measured at a definite wavelength and calibration curve is plotted by lying off the known concentrations along the axis of abscissas and the absorbances corresponding to them along the axis ordinates. The calibration curve is used for identifying unknown strength of analyte from the sample under investigations. (Kirk G.F., 1966)

Fig. no. 1.2.1: Electronic spectrum
Fig. no. 1.2.2: Electronic transition

Divergence from Beer’s law

From Beer’s law give the idea about plot which could constructed by taking absorbance versus concentration and straight line passing from zero point should be obtained but in some cases there would be the chances of deviation in plot of absorbance versus concentration which could fails to obeys beer’s law. Such types of deviations occurs in plot of absorbance versus concentration may in both the direction either in positive or in negative according to the sample under investigation.

Deviations occur in the plot of absorbance versus concentration i.e. beer’s law due to following circumstances:

1. Deflection would be happened due to use of color sample which containing the solute which could be easily liberated in sample mixture because of process called dissociates & ionization.

2. Beer’s law will hold over full limit of strength which could be gives clue regarding the framework of ions which produced color during dissolution stage and if these ions could be have other impurities then it could affect the radiation which being absorbed by molecules and strength of sample mixture may be change.
3. Deviation may occur if polychromatic light used instead of monochromatic light.

4. Deviation may occur due to improper width of slit & because of improper slit width then unpleasant light may enters and act on detecting device and produced large divergence or changes in absorbance of sample at elevated strength.

5. Deflection could be also happened because of presence of other foreign particle which could be affect and enhancing the absorbing radiation. Due to this there may be chances of wrong determination of absorbance intensity which could be penetrating to sample.

6. The deflection could be also happened because of sample under investigation going to forms the polymers.

Beer’s law cannot be applied to suspensions but the latter can be estimated calorimetrically after preparing a reference curve with known concentrations (Skog D. A., 1980).

**Fig. no. 1.2.3:** Single beam UV-Visible Spectrophotometer
1.2.2 Introduction of chromatography

Chromatography was invented by the Russian botanist at the beginning of 20th century and name of the scientist was Mikhail Tswett. In this technique, quantifying the ingredient could be looks like colored bands on separating phase i.e. column, therefore in Greek terminology it could be explained like “Chroma means color & graphein means write & their supplication could be encourages in last fifty decades due to ont only quantifying the chemical species but also need in the areas of characterization of difficult sample mixture. These strategies could be related to similar medium of quantification depending upon the reactivity of analyte between two phases but one could be differentiate it with column chromatography in which solvent part would be pumped from packed column with high pressure. The main advantages of HPLC compared to classical chromatography are improved resolution of separated substances, faster separation time and increased accuracy, precision and sensitivity with which the separated substances may be qualified.

Types of Chromatography

Chromatography characterized as a separation method based on the differential migration of solute through a system of two phases, one is mobile phase another one is stationary phase.

Chromatography is mainly divided into two categories:

- Adsorption Chromatography: Separation is mainly due to the interaction between solute and surface on the adsorbent. In this immobile part and also called as stationary part could be solid in nature & mobile part could be solvent in nature or made of different composition of solvent depending upon the polarity of sample under investigation e.g.: TLC, HPTLC and GC
- Partition Chromatography: Determination of analyte under investigation could be related to partition amongst two different parts i.e. immobile & solvent part which could be mobile in nature. In this mode, both stationary phase and mobile phase are liquids e.g.: HPLC, GLC and PC.
1.2.3 Introduction to HPLC

Liquid chromatography could be explained as analytical procedure or method or instrumental technique helped to separates & analyzing active pharmaceutical ingredient that could be able to dissolve in appropriate solvent. There would be the chances of improper result due to difference in absorbance when taken sample solution could be in contact with other phase which leads to interaction between different solutes which could be interact with other phase due to partitioning & differs in the size of active content of medicament & these types of differences allow the separation of active content of medicament of the sample mixture from each other by using these differences to determine the transit time of solutes through column.

Liquid chromatography consists of stationary phase which include solid component in column for adsorption & in case of ion exchange chromatography different groups of ions present on resins which exchanges the ions according to their affinity toward the resins and for separating porous inert particles size exclusion chromatographic techniques consist of liquids on an inert solid support. The sample mixtures which are to be analyzed were placed into the stationary phase i.e column and then allow to place more and more solvent in order to performed separation easily as per their affinity toward the stationary phases and then sample mixture were allowed to flow as per different rates due to the differences in their portioning behavior among above said two different stages. The components are separated by collecting aliquots of the column eluent as function of time. (Sethi P. D., 2001)

High performance liquid chromatography could be widely used analytical technique because of its sensitivity, accuracy, suitability for separating non-volatile components or thermally fragile ones and complex mixtures in many areas of science. The implementation of these instrumental processes could be elevated explosively in recent years because of framing novel chromatographic strategies helped to pharma companies for easy interpretation of sample mixture whose characterization could be difficult by other available procedure & techniques & growing requirement of scientist for better methods for characterizing complex mixtures.
Basic HPLC Instrumentation (Smith F. J., 1999)

As shown in Fig: 1.2.4 basic HPLC instrument are

**Mobile phase reservoir**

In order to store appropriate strength solvent for continuous carrying out operation of the HPLC system mobile phase reservoir must be equipped in such a way that it could be easily degassing HPLC system & removes the HPLC solvent under the influence of external environment condition.

**Solvent delivery system**

Role of solvent delivery system is to pumped suitable amount solvent part easily transported with high pressure from various reservoir point & then transfer that mobile phase into the column at fixed time. Depending upon the nature of substance to be eluted normal phase & reversed phase separation method were used for eluting active content of drug substance. In case of normal phase technique elution of power could be enhance by enhancing duality of solvent but in case of reversed stage analysis elution power could be decline by enhancing the duality. In solvent delivery system degasser could be needed in order to detach dispersed air & other gases which could be likely to be present in solvent part from solvent there for high quality of solvent could be available i.e. HPLC solvents & solvent could be purified carefully in order to diminished certain type of impurities & smaller particle for prevention of clogging of the HPLC column from these particulate matter.

Fig. no. 1.2.4: Diagram of HPLC equipment
Pump
The pumps which are most prime requirement HPLC system could be directly affects the performance & retention time, reproducibility which lead to error in measurement. There were three kinds of pumps were likely to be available HPLC for pumping solvent composition into HPLC process.

- **Displacement pump:** due to limited capacity of 250 ml these type of pump were rarely used because it produces flow which unable to maintains proper viscosity & backpressure.

- **Reciprocating pump:** It could be widely used due to having small internal volume and these pumps have ability to produce high output pressure up to 10,000 psi at a constant flow rates.

- **Pneumatic or constant pressure pump:** Due to limited capacity and their flow depends on mobile viscosity and couldn’t able to work at high pressure these type of pump not used. But in some circumstance work with this type of pump could be give optimum result when pressures up to 2000 psi were desirable.

Sample injection system
Sample injection system should be such that easy & proper carrying out function by inserting the sample into pressurized column with narrow plug in order to minimize the effect of peak broadening & peak splitting and moreover sample injection system must have no void volume so that above said effect may be minimize easily. Insertion of sample solution into pressurized column could be done by three ways:

**Loop injection:** By using loop injection one could inserted fixed strength of mobile phase into the pressurized column with the help of fixed volume loop injector.

**Valve injection:** By using valve injection one could inserted variable strength of mobile phase into the pressurized column with the help of an injection valve.

**On column injection:** One of the important method for inserting variable strength of mobile phase with help of high quality needle like syringe into septum.
Column packing

Column packing used in HPLC system must be having narrow particle size distribution so that easy chance of elution of drug content from the column. Narrow particle size distribution could be achieved by making column packing in terms of porous bed & porous bed layer having particle size small & rigid particle & there were three different kinds of column filling & supporting strategies in HPLC.

- Porous, polymeric beds
- Porous layer beds
- Totally Porous silica particles (diameter <10 µm)

Detectors

Detectors detect various compounds as they elute out from column. The detector gives response in terms of a millivolt (mv) signal that is then processed by the computer (integrator) to obtain you a chromatogram. Basically detector consists of a flow-cell through which the mobile phase and resolved sample moves optic shine through the detector cell and variation in optical properties are detected. (Chatwal G. R., 2002)

The Photo Diode Array Detector (PDA) is the most used detector in LC today. The PDA gives a three dimensional view of chromatogram (Intensity Vs Time) and Spectra (Intensity Vs Wavelength) simultaneously. It can be called as spectro chromatogram. The detailed analysis of the data reveals more information on the complexity of co elution and helps in identifying the merged peaks and gives information on peak purity.

Bulk property detectors

Refractive indices could be a kind of bulk properties measurement detecting devices available in few HPLC process to gives clues regarding differential measurement & it could be useful in normal elution methodologies but not suitable for gradient elution and is less sensitive.

Solute property detectors

This measures physical or chemical property that is specific to the solute only. Ex: U.V detector, conductivity detector. These can be used for gradient elution.
Recorders

Recorders are used to record the responses obtained from detectors after amplification. They record the baseline and all the peaks obtained with respect to time (Smith F. J., 1999).

Types of HPLC (Kazakevich Y., 2007)

Reverse phase chromatography

Reverse phase chromatography could be at most famous analytical approaches in pharma companies & would be highly utilized in order to assessing the impurity & active determination of pharma related products & superiority of HPLC methods has become increasingly important. The necessity of procedure could be usually required during quantifying phase of chemical entities but during early stages, main focus could be done on these methodologies to obtained high throughput & rapid turn round time and after completion of development stage then simpler strategies could be utilized in later stage of pharma related quantifying.

Normal phase chromatography

In case of normal phase chromatographic technique immobile parts which are used must be always polar & solvent phases are in non-polar in nature. According to affinity of active drug content of medicament drugs were separated as per their affinity and gives the appropriate retention time. Affinity of active drug content of medicament occurs mainly due functional groups in the analyte molecule & steric factors.

Size exclusion chromatography

It is the process by which mixture of compounds with molecular sizes are separated by using gels. The gel used acts as molecular sieve. It can be separated by steric and diffusion effects of pores in the gels. The compound can separate according to the molecular sizes and immobile parts could be porous matrix. The stationary phase could be wide pore gel that can separate molecules on the basis of their size and shape, the largest molecules traveling most rapidly through the system.

Eg: separation of proteins and polysaccharides.
Ion exchange chromatography

In ion exchange chromatography stationary phase consist of an ion exchangeable resin which responsible for separation by the strength of the interactions between solute ions and the exchange sites on the resin. An ion exchange resin consists of an insoluble, rigid three-dimensional matrix, for example polystyrene cross-linked with a small amount of divinyl benzene to produce mechanical stability. The surface of this matrix contains ionizable sites that can carry a positive or a negative charge. Each of these sites also requires an oppositely charged ion for overall neutrality. If the ionizable sites are positively charged, the counter ion is an anion and the resin will exchange anions from solution.

Chiral chromatography

Chirality plays an important role in pharmaceutical industry. It is mainly because of the enantiomers. They exhibit different pharmacological and toxicological properties in living systems.

1.2.4 Introduction to HPTLC

In present research work, analysis is performed using HPTLC method. Because according to details literature survey there were many spectroscopic and HPLC method were available for analysis of combination of drug composition selected. But no any analytical method available for superior analysis selected composition using HPTLC method. There for in this research work HPTLC method selected in order to buildout & quantifying HPTLC procedure to estimate selected combination of drug composition.

There are only very few methods available in order to identify & analyzed chemical entity from drug component must be selective in nature so that selective chemical entity present in drug and dosage from could be analyzed easily. But separating and analyzing active content of dosage in presence of interfering substance could be a rate limiting step in the research. Above said interfering impurity could be analyzed and identify with help of chromatographic techniques and those chemical entity similar in their structure could be separated from homogeneous sample mixture by using chromatographic techniques. Moreover separation of active drug content
from dosage form could be separated according to their affinity between two phase’s i.e. mobile phase & stationary phase. Based on the separating principals different modes of chromatographic were used. During early stage of drug discovery there would be the large numbers drugs were available and they are similar in their structure and must be important to separate them there for chromatographic methods were important tool in order to study the impurity profile of drug substance. So without chromatographic method one couldn’t easily perform the purity testing of active drug component. During the developed stage of drug component it could be very important to develop & validated analytical methods because by developing and validating analytical method related to drug component could be help to manufacturing department for manufacturing active drug component.

Proper selection of chromatographic procedure for deducing any complicated concoction could be an important task for easy and smooth way for identification and characterization of active drug component. While selecting chromatographic method one would be thought on chemical composition, nature, physical & chemical parameters and physical state of test combination under investigation because chemical properties of analytes may be consist of various and drug under investigation may have different chemical behavior as pertaining to acidic or basic behavior. Based on the chemical nature of active drug component & physical properties of active drug component applicable chromatographic technique must be selected and it could be help in determination of physicochemical behavior of active drug substance like stable constant of complicated blends & free energy. (Frank A. S., 1997)

High performance thin layer chromatography (HPTLC) techniques, an automated type of thin-layer chromatography (TLC) that gives the idea regarding the superior separation power using optimized coating material. By using these techniques automated procedure of solvent part supplementing, film contingency, precised analyte transferring & chromatogram development scanning & photo affirmation because it could help in separation of active drug component with higher efficiencies with analysis in shorter period of time by using only low volume of solvent phase with easy data acquisition and processing. The procedure could be utilized to validate concurrently deducing of more than two analyte mixture from dosage regimen (Srivastava M. M., 2011; Sethi P. D., 2013).
With use of modern apparatus composed in HPTLC system like scanners, densitometers & chromatographic chambers, sufficient rinsing part & high proposition with chosen fragment acreage it could be possible to combined it with instrumental procedure & availability of computer related programs for strategies cumulating there for it could be very significant substitute procedure to HPLC or gas chromatography (Srivastva M. M., 2011). Moreover high performance thin layer chromatography techniques are important tool for developing the any analytical method due to its ability to give the accurate and precise results during analysis and ability to document the results easily by comparing with standard TLC. Due to these reason high-performance thin-layer chromatography techniques was most appropriate TLC technique for conformity as per good manufacturing practices norms.

**Features of HPTLC**

Because of convenience, due to off line adjustment one could be chose this or it could be alternative to other those who requires the plan schedule and adjustment with on line procedure like HPLC (Patel B. R., 2011) & features of this HPTLC have been outlined as following:

- Due to open component system it could be possible for visual observation & detection of analytes in terms of bands.
- Because of availability of readymade TLC plates with optimized absorbent layers with uniform particle size distribution as the stationary phase.
- Automatic sample application of samples on the TLC plates may minimized the errors occurs due to sampling.
- Automatic detection of analytes from chromatogram using UV and florescent detectors detector system.
- Even in minute quantities of sample mixture could be detected using HPTLC.
- Compound which are very complex or those in very scarce quantities can be analyzed.
- Simultaneous process of sample and standard can be done under similar condition gives better analytical precision and accuracy.
- Detection is visual and derivatization is simple (Patel B. R., 2011).
1.2.5 HPTLC methodology

Procedure for assessing analyte from multi combinations by above said technique desires prime idea & requires detail knowledge regarding utilization of of above said instrument and one could have comprehension regarding the nature, framework, polarity & volatility of analyte under investigation. Procedure divination includes appropriate trial and error methodologies and at most complex happening lies in deciding its starting point and determining the kind of mobile phase. During demonstrating novel analytical process, one would have always initiated with vast literature survey in order to acquire more and more knowledge regarding HPTLC for solving particular problem facing during ascertaiment. (Sharma B. K., 1995)

Choosing immobile or motionless part would be quite simple & easy. Silica gel can be advantageously chosen in such a way that it could be easily separated all kind of drug samples. Optimization of mobile phase in HPTLC methodology would be an important task & it can be done by following method.

- In first method based on selection of suitable solvent having the ability to give have typically detachment efficacy for required analytes.
- The second method for optimization of drug component based on strength & polarity of mobile phase by reducing and elevating solvent capabilities by taking hexane or water for appropriate intent.
- The third method for optimization of drug component related to taking appropriate blends of applicable solvents from available solvents of above said two procedures which would then classified by taking or using acids or alkalis.

Active drug component from dosage form could be detected by using two modes i.e. fluorescence mode or absorbance mode and if active drug component from dosage form couldn’t detect easily then one have to change either stationary phase or mobile phase or may need help of pre or post chromatographic derivatization.

Optimization of mobile phase in HPTLC methodology could be done only after appropriate chromatograms were obtained and which could be obtained by changing the composition of solvent system. By doing the above said methodology it could lead to obtained reasonable chromatogram with desired peak shape and well resolution of all the drugs under the investigation. (Srivastva M. M., 2011).
Sample Preparation

Constituent arrangement in HPTLC could be hardly essential cleaning because sheet of immobile part which could be utilized in HPTLC were not reused again for deducing the analytes & it could be possible to utilized analytes without any kind of pretreatment. Pharma related composition having high strength of active drug component could be dissolved in appropriate solvent so that it would be completely solubilize the active drug component and remove undissolved excipients to give sample mixture which could be directly applied on HPTLC plate. In certain cases, sonication of sample may be required to ensure complete solubility of sample, as well as filtration or centrifugation to remove un-dissolved excipients (Camag., 2012).

Selection of Stationary Phase

Precoated plates: Different support material-different sorbents available

80 % of analysis: Basic substances, alkaloids and steroids, Aluminum oxide silica gel GF, Amino acids, dipeptidees, sugars and alkaloids- cellulose

Non-polar substances, fatty acids, caratenoids, cholesterol-RP-2, RP-8 and RP-18,
Sample application

Linomat 5, Automatic TLC Sampler (ATS4)

Sample applicator could be selected based on the type of sample solution analysed & by selecting the automated sampler certain factors could be take into consideration like analytical task nature & time & workload and type of separation layer & it would be also depends on amount of test solution to be applied on TLC plates and number of sample under investigation.

In order to obtained good resolution of drug sample under investigation it could be very important to restrict the size of the sample origin in the direction of chromatography to a minimum. The high strength of sample solution could be applied spot wise (in one stroke) are 5 µl on conventional layers and 1 µl on HPTLC layers. Spot wise application of larger volumes requires a device with controllable delivery speed. (Peter E. W., 2005),

Some region widening that could be produced in chromatography during exercising by contact spotting could be removed & in some important cases like trace deducing, huge analyte strength or analytes with huge matrix content would be sprayed in terms of rectangles bands before chromatographic divination and could be focused onto narrow spot with one of shorter expansion stage by taking huge solvent of high eluting concentration.
Table no. 1.2.4: Sample application parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interval from bottom circumference of sheet in mm for TLC</td>
<td>8</td>
</tr>
<tr>
<td>Minimal interval from left and right circumference of sheet in mm</td>
<td>10</td>
</tr>
<tr>
<td>Minimal space in mm among spots in mm</td>
<td>4</td>
</tr>
<tr>
<td>Band length in mm</td>
<td>8</td>
</tr>
<tr>
<td>Maximal diameter of supplication of band in mm</td>
<td>5</td>
</tr>
</tbody>
</table>

**Processes in the Developing Chamber**

It could be very important to know the various parameters pertaining to developing the TLC plate. First of all in order to developed chromatogram TLC plate should be place in the developing chamber in such a way that it must contains appropriate volume of solvent system and almost around 2 mm marked at the end of TLC plate must be sufficiently dipped in the selected solvent phase so that solvent system could be run smoothly and reached to desired distance with help of capillary action to give optimum result.

The following points were considered with developing the chromatogram in the developing chamber for smooth running of process:

- Equilibrium confirmed among vapor & part of solvent system and these type of established equilibrium called as chamber saturation and it could be depends on vapor pressure of single part of combination composition of gaseous stage which could be differentiate significantly from that of solvent system.
- When TLC plate available in dry condition means that adsorptive saturation equilibrium established as TLC plates could have ability to adsorb modicum from gaseous part & these equilibrium approached in such a way that polar constitute could be retreat from gaseous part & were shacked onto exterior of TLC plates.
- At same time small parts which were already moistened with solvent phase which could be combine with gaseous part & then less polar constituent of the liquid were discharged into gaseous part & which couldn’t as much dominated by vapor pressure as by adsorption forces.
The problems occurring due to this can be avoided by keeping increasing the assembly permeation period & could be attained as follows: preserving assembly high or low totally by taking piece of filter paper moisten with spreading solvent & then wait for certain time amongst introducing of developed solvent into assembly & initiation of chromatographic methodologies.

The privilege of assembly permeation could be such that constituent of spreading solvent would be discharged onto shriveled film by gaseous part & gas phase were forced ahead of mobile phase with invisible solvent front & inconsistency were observed in very polar constituent like H$_2$O, CH$_3$OH, acids & bases & these outcomes in terms of retention factor and which could be slight less in permeation assembly & typically with pre conditioned film than in imperfection chamber.

The consequences of not allowing chamber saturation initiated in non-equilibrium between immobile, solvent & gaseous part there for these reasons it could be very complex to narrate the situation of spreading assembly. Uniform chromatographic outcomes could be only results can only be presumed when every specification were retained as persistent as achievable. Assembly outline & permeation were playing significant aspect in these matters & grievously this expedient that chromatographic outcomes could be dissimilar in every assembly.

**Derivatization**

After the development, it’s an intrinsic benefit of thin layer chromatographic procedure that fragment persist on sheet & could be uninventive after chromatography & by derivatization strategies ingredient could not be replied to visible or UV radiation and could be contributed detectable but in few circumstances chemical moieties or categories of chemical moieties could be recognized by particular testing indicators.

Derivatives of chemical moieties could be prepared & carried out by gas and with the help of liquid by spraying or immersion but reagent which are used to prepare derivative must be homogeneous in nature to easily transfer to the chromatogram. With the help of dipping techniques TLC plate could be transfer into the derivatizing reagent in such a way that homogenous in nature could be achieved. Moreover it could be take into consideration that no fumes were generated at the time.
derivatization technique & exposure to hazardous chemicals could be limited. When derivatizing reagent would be suitable then immersion technique could be prefer over spraying techniques. In many case spraying techniques would be most widely used technique for transfer of derivatizing reagent onto the TLC plate due to simplicity and quickness and very economical as it couldn’t require expensive equipment.

Furthermore sprinkling could be appropriately malleable & crucial when testing indicators have to be executed in series & also throughout strategies expansion, when perceiving for at most suitable testing indicators & sprinkling could be more usually initated. The two principal heating devices were ovens and plate heaters. (Sethi P. D., 1996)

**Table no. 1.2.5: Scan mode with specification**

<table>
<thead>
<tr>
<th>Types of light featured</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-wave UV light – reflection</td>
<td>254</td>
</tr>
<tr>
<td>Long-waver UV light – reflection</td>
<td>366</td>
</tr>
<tr>
<td>White light – reflectance</td>
<td>W</td>
</tr>
<tr>
<td>White light – transmittance</td>
<td>WT</td>
</tr>
<tr>
<td>White light – reflectance &amp; transmittance combined</td>
<td>WRT</td>
</tr>
</tbody>
</table>

Computerized resemblance could be regularly catalogues & could be repossessing at any time for review or to perform a quantitative interpretation & vitality of computerized resemblance acquirement of totally likeness of chromatogram & it could be possible to obtain perceptible apprehension of chromatogram would be the one of prime benefit of Thin-Layer Chromatography over all other chromatographic techniques.
1.3 DRUG PROFILE OF SELECTED DRUG IN RESEARCH WORK

1.3.1 Montelukast sodium

- Montelukast sodium is orally active leukotriene receptor antagonist that inhibits leukotriene receptor.
- **formula**: $C_{35}H_{35}ClNNaO_3S$
- **Molecular weight**: 608.17
- **Chemical name**: $[R-(E)]-1-[[[1-[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]3-[2-(1-hydroxy-methylethyl phenyl)propyl]thio]methyl] cyclopropaneacetic acid, monosodium salt.

![Structure of Montelukast sodium](image)

**Fig. no. 1.2.9:** Structure of Montelukast sodium

- **Description**: Hygroscopic in nature & optically active, white to off-white powder.
- **Category**: Anti-asthmatic (Leukotriene receptor antagonist)
- **Melting point**: 250 – 256 $^\circ$C
- **Solubility**: Freely soluble in methanol & ethanol, water & insoluble in acetonitrile practically.
- **Partition co-efficient**: 0.37
- **pKa value**: 14.60
- **Brand names**: Montair, Singulair, Singular.
- **Recommended dose**: 4 – 10 mg taken once daily.
Mechanism of action

Montelukast inhibits bronchoconstriction due to antigen challenge and selective leukotriene receptor antagonist of human airway cysteinyl leukotriene receptor by binding to cysteinyl leukotrienes they are correlated with the pathophysiology of asthma, smooth muscle contraction & altered cellular activity associated with the inflammatory process, factors that contribute to the signs and symptoms of asthma.

Pharmacokinetics

- **Absorption:** Absorption of montelukast found to be very rapid after oral administration of tablet of montelukast with oral bioavailability equal to almost around 64%
- **Distribution:** Montelukast having very high binding affinity towards the plasma proteins & gives stable volume of distribution of almost around 9.5 liter.
- **Metabolism:** metabolized in liver by enzyme CYP2C8.
- **Elimination:** Elimination: large amount montelukast excreted through bile and remaining portion of drug excreted by kidney.

Overdosage

There were no any adverse effect were reported for montelukast due to over dosage but with treatment of montelukast some side effect usually found are mydriasis, abdominal pain & , hyperkinesias.
1.3.2 Olopatadine hydrochloride

- Olopatadine hydrochloride is a dibenzoxipine derivative used for systemic treatment of allergic rhinitis, urticaria, and bronchial asthma. It is a histamine H$_1$ receptor-antagonist and is used as an antiallergic and anti-inflammatory agent.

- **Formula**: C$_{21}$H$_{23}$NO$_3$.HCl
- **Molecular weight**: 373.9
- **Chemical name**: 11-[(Z)-3-(Dimethylamino) propylidene]-6-11-dihydrodibenz [b,e] oxepin-2-acetic acid hydrochloride

![Structure of Olopatadine hydrochloride](image)

**Fig. no. 1.2.10**: Structure of Olopatadine hydrochloride

- **Description**: It is a crystalline solid and white powder.
- **Category**: Histamine H$_1$ antagonist
- **Melting point**: 248 – 250°C
- **Solubility**: Freely soluble in ethanol, methanol, diethyl formamide & DMSO.
- **Partition co-efficient**: 0.75
- **pKa value**: 3.78 & 9.76
- **Brand names**: Patanol, Pataday, Patanase.
- **Recommended dose**: 5 – 10 mg taken once daily.
**Mechanism of action**

Olopatadine having the similar structure as like doxepin which is topically effective anti-allergic molecule having the non-steroidal & non-sedating activity which exerts their effects through multiple distinct mechanisms of action by blocking the action of endogenous histamine & stabilized the mast cell & also inhibits the in vivo type one immediate hypersensitivity reaction. Moreover olopatadine also inhibits the release of mast cell inflammatory mediators like histamine, tryptase, prostaglandin & TNFα. Olopatadine is also an inhibitor of pro-inflammatory cytokine secretion from human conjunctival epithelial cells.

**Pharmacokinetics**

**Absorption:** Enough strength of plasma concentration could not be obtained when these drugs used in terms of ophthalmic route. But $T_{\text{max}}$ is 30 to 60 min, $C_{\text{max}}$ is 16 ng/mL, average absolute bioavailability is 57% (intranasal). The oral bioavailability and $C_{\text{max}}$ are not influenced by a standard meal in the morning.

**Distribution:** Protein binding is more than 55% and half-life in plasma was approximately 8 - 12 hours.

**Metabolism:** Olopatadine is not extensively metabolized but sometimes mono-desmethyl and the N-oxide metabolites have been detected at low concentrations in the urine. Excretion of olopatadine through urinary route could be less than 58% & drug metabolized very low in terms of clearance of olopatadine in healthy adults & olopatadine eliminated through kidney by the process of renal excretion.

**Overdosage**

The following Olopatadine intentional overdose side effect reports are blurred vision, keratitis, headaches & taste perversion which were submitted by healthcare professionals and consumers.
1.3.3 Rupatadine fumarate

- Rupatadine is a second generation antihistamine and PAF antagonist used to treat allergies and it has been approved for the treatment of allergic rhinitis and chronic urticaria in adults and children.
- Formula \( C_{30}H_{30}ClN_3O_4 \)
- Molecular weight : 532.04
- Chemical name : 8-Chloro-6,11-dihydro-11-[1-{(5-methyl-3-pyridinyl)methyl]-4-piperidinyldene]-5H-benzo[5,6]cyclohepta[1,2-b]pyridine fumarate

![Structure of Rupatadine fumarate](image)

**Fig. no. 1.2.11:** Structure of Rupatadine fumarate

- Description : It is white to off white crystalline powder.
- Category : Second generation Histamine H1 antagonist
- Melting point : 58 – 61 \(^\circ\)C
- Solubility : Freely soluble in methanol, chloroform, ethanol & DMSO & practically insoluble in water.
- pKa value : 5.6
- Brand names : Rupafin, Rupax, Rinialer.
- Recommended dose : 10 mg taken once daily.

**Mechanism of action**

Rupatadine is a second generation, long acting anti-allergic compound which displays strong antagonist activity towards both histamine H1-receptors and platelet-activating factor (PAF) receptors (dual activity). The PAF induces vasodilation and increased permeability which may be responsible for the appearance of rhinorrhea
and nasal congestion. PAF antagonism represents the likely mechanism behind the inhibition of eosinophil migration which has been suggested to be beneficial in the treatment of chronic urticaria. Rupatadine inhibits the degranulation of mast cells induced by immunological and non-immunological stimuli, and inhibits the release of cytokines in human mast cells and monocytes.

**Pharmacokinetics**

**Absorption:** Rupatadine rapidly absorbed after oral administration following PO administration in all nonclinical species (mouse, rat and dog) and in humans, with Cmax reached within 1 hour of dosing.

**Distribution:** Plasma protein binding by rupatadine was high, ranging from 98–99% in rats, dogs and widely distributed in the human body. Highly bound to plasma proteins (99%).

**Metabolism:** It was found to principally involve hydroxylation of the methyl group in the pyridine ring with subsequent oxidation to acid and then further hydroxylation at various positions and dealkylation of the tertiary amine (generating desloratadine, 3-, 5-, and/or 6-hydroxy-desloratadine and other metabolites). Cytochrome P450 (CYP)3A4 was identified as the P450 isoform chiefly responsible for the metabolism of rupatadine in in vitro experiments, with CYP2C19 and 3D6 also potentially involved.

**Elimination:** Excreted via urine and faeces. Significant biliary excretion was demonstrated in rats (16% of an oral dose was recovered in bile and 74% of an IV dose).

**Overdosage:**

The following Rupatadine intentional overdose side effect reports were submitted by healthcare professionals and consumers were Sleepiness, Weakness, Dry mouth, Pharyngitis, Dyspepsia and Increase in appetite.
1.3.4 Doxofylline

- Doxofylline is a new generation long acting oral methyl xanthine derivative. It’s mainly used for maintenance therapy in patients suffering with asthma and Chronic Obstructive Pulmonary Disease (COPD).
- Formula: \( \text{C}_{11}\text{H}_{14}\text{N}_{4}\text{O}_{4} \)
- Molecular weight: 266.25
- Chemical name: 7-(1,3-dioxolan-2-ylmethyl)-1,3-dimethy-3, 7-dihydro-1H-purine-2,6-dione

![Structure of Doxofylline](image)

**Fig. no. 1.2.12:** Structure of Doxofylline

- Description: It is off white to white crystalline powder.
- Category: New generation Xanthine bronchodilator
- Melting point: 144 – 146 °C
- Solubility: Soluble in water, methanol, 0.1N hydrochloric acid, 0.1N sodium hydroxide, acetone, benzene And chloroform.
- pKa value: 9.8
- Brand names: Doxof, Doxima, Doxoyent.
- Recommended dose: 400 mg taken once daily.

**Mechanism of action**

Doxofylline falls under categories of methylxanthines which could be known for inhibitors of enzyme called phosphodiesterase and it is a new generation of methylxanthines derivative which having long acting effect on human body. It is very
important class of drug used in treatment & management of asthma and chronic obstructive pulmonary disease.

**Pharmacokinetics**

Due to greater half-life of new generation methylxanthines it could be very in producing the constant plasma concentration by thrice dose of daily regimen & therapeutic effectiveness of these new generation methylxanthines would be very wide as compare to theophylline & serum levels were also stable there for dose not require to monitored the serum concentration during pharmacokinetic study. After intravenous (I.V.) administration of 100 mg to 5 healthy volunteers, distribution of doxofylline in plasma followed a bi-compartmental model. During the distribution phase, the plasma area under the curve (AUC) was only a modest portion of the total AUC; plasma clearance was somewhat high, ranging from 444 mL/min to 806 mL/min; apparent volume of distribution was about 1 L/kg. The mean half-life after I.V. administration was about 65 minutes (from 40–96 minutes).

**Overdosage:**

Due to overdosage of new generation methylxanthines bronchodilating effect could be prolonged and very safe drug for smokers but other new generation methylxanthines like theophylline have least adenosine interactions i.e., no CNS and CVS side effects & there couldn’t sleep disturbances & gastric secretions effect were produced by using new generation methylxanthines i.e. doxofylline.
1.3.5 Acebrophylline

- Acebrophylline is obtained by targeted salification of the ambroxol base, a mucolytic and expectorant and theophylline 7- acetic acid, a xanthine derivative with specific bronchodilator activity.

- **Formula**: \( \text{C}_{22}\text{H}_{28}\text{Br}_2\text{N}_6\text{O}_5 \)
- **Molecular weight**: 616.30
- **Chemical name**: 4-[(2-amino-3,5-dibromo-phenyl)methyl Amino] cyclohexan-1-ol; 2-(1, 3-dimethyl-2, 6-dioxo purin-7-yl) acetic acid.

![Structure of Acebrophylline](image)

**Fig. no. 1.2.13**: Structure of Acebrophylline

- **Description**: Acebrophylline is a white fluffy powder.
- **Category**: Bronchodilator, Anti-inflammatory & Mucoregulator.
- **Melting point**: 210 – 212 \(^\circ\)C
- **Solubility**: Sparingly soluble in water and acetonitrile and Soluble in methanol.
- **pKa value**: 8.03
- **Brand names**: Doxof, Doxiba, Doxovent.
- **Recommended dose**: 100 mg taken twice a day.

**Mechanism of action**

It is made of theophylline acetate & ambroxol which composed of two different type of pharmacological activity such as bronchodilator effect and mucolysis effect. Bronchodialating activity is due to inhibition of enzyme phosphodiesterases which is present inside the cell due to decreasing the activity of phosphodiesterases.
there is elevation of cyclic AMP level while in case of mucolytic activity affects the motility. Acebrophylline increases synthesis & release of alveolar surfactant which results into triple action i.e. mucoregulation, stimulation of bronchoalveolar clearance & anti-inflammatory antireactive effect

**Pharmacokinetics**

- Absorption: acebrophylline absorbed in intestine after oral administration
- Distribution: it widely distributed in the body
- Metabolism: metabolized in the liver
- Excretion: excreted through urine

**Overdosage:**

With the treatment of acebrophylline adverse/side effect are reported are epigastric pain, vomiting, diarrhoea abdominal discomfort, constipation, heart burn, difficulty in breathing, leukocytosis & nasal inflammation. If chills and fever occur, the drug should be immediately discontinued. Serious side effects are possible in rare cases & you will need immediate medical care should be given to the patient.
1.3.6 Ebastine

- Ebastine is a second generation non-sedating H₁ receptor antagonist and used in the treatment of allergic rhinitis and chronic idiopathic urticaria.
- Formula: \( C_{32}H_{39}NO_2 \)
- Molecular weight: 469.66
- Chemical name: 1-[4-(1,1-Dimethylethyl)phenyl]-4-[4-(diphenylmethoxy)piperidin-1-yl]butan-1-one.

![Fig. no. 1.2.14: Structure of Ebastine](image-url)

- Description: It is white or almost white, crystalline powder.
- Category: Antihistamine
- Melting point: 84 – 86 °C
- Solubility: Practically insoluble in water, very soluble in methylene chloride & soluble in methanol.
- And chloroform.
- \( pKa \) value: 8.43
- Brand names: Evastine, Kestine, Ebastel.
- Recommended dose: 10-20 mg taken once daily.

**Mechanism of action**

Ebastine is a type of non-sedating second generation antihistamine which is used for allergic disorders. Medical trials showed that it could effectively decrease symptoms of intermittent or seasonal allergic rhinitis and chronic idiopathic urticaria.
by blocking histamine receptors. Upon ingestion, this medicine undergoes a pharmacological process in which it is converted to its active form carebastine. It then starts to work by means of selectively inhibiting the histamine H1 receptors, and regular dosing will help to consistently retain these effects. Resulting from this, the effects that histamine has on the nasal lining (such as inflammation) are prevented, as the histamine released from the mast cells is unable to work on its receptors.

**Pharmacokinetics**

After drug under oral route of administration it could be under goes first pass metabolism with the help of enzyme cytochrome P450 3A4 which were present in liver and converted into carebastine which could be an active carboxylic acid metabolite. This conversion is practically complete. Plasma levels of carebastine showed a first-order decrease with apparent half-lives of 13.8 to 15.3 h. The Cmax and AUC of carebastine increased in proportion to the dose. Urinary excretion of carebastine during 72 h after single administration accounted for 1.3-1.8% of the dose. Food intake did not affect the pharmacokinetics and gastrointestinal absorption of ebastine.

**Overdosage:**

With the treatment of ebastine adverse/side effect are reported are Headaches, Indigestion, Drowsy feeling, Pain in the stomach and Dryness occurring in the mouth while other side effects could occur due to over dose such as weakness, nosebleeds, difficulty sleeping as well as some reactions which are not listed here. Serious side effects are possible in rare cases, and you will need immediate medical care should this occur.
1.4 AIM AND OBJECTIVE OF RESEARCH WORK

In the current scenario most of all the manufacturing units have sophisticated instruments like UV, HPLC and HPTLC; it is advantage that we can develop sensitive methods to determine anti-allergic drugs in combined dosage forms using these instruments. One of the most critical factors in developing pharmaceutical drug substance and drug products today is ensuring that HPTLC analytical test methods that are used to analyse the product generate meaningful data. Rapid strides have been made in the area of analysis of pharmacologically active molecules. Recent advancements in the area of modern analytical techniques include HPLC, LC – MS, GLC, GC – MS and Ion pair chromatography. These methods are time consuming and require sophisticated instruments and chemicals, which are too costly to afford especially by small – scale industries.

As per vast literature survey it could be known that many methods were reported like Spectrophometric Method(UV), High performance Liquid Chromatography(HPLC), High performance Thin layer chromatography(HPTLC), Flourimetry, Stability indicating method & Extraction technique available for some of Antiallergic drug in either alone or in combination with other drugs but high performance thin layer chromatography method not available for selected Anti-allergic combination. Therefore proposed work is done to develop a simple, sensitive, accurate and reproducible HPTLC method for estimation of some anti-allergic drugs in bulk and their combined dosage form.

- Separate of the combination drugs and determination of the separated drugs by the application of Qualitative and Quantitative analytical method.
- Analytical method development by HPTLC for the combination drug is very helpful for the quick results and the cost of analysis is very less.
- HPTLC method will be helpful to the pharmaceutical manufacturers for the analysis of different combination drugs.

HPTLC method development for combination drug is very helpful because we get quick results and it is a less expensive method.
1.5 PLAN OF RESEARCH WORK

Whole study is divided into different phase and planned to generate data from laboratory i.e.,

- Literature review - It will performed as per National, international journals and E- journals.
- Collection of various API and Dosage form from industry and market.
- Identification of drugs by determination of solubility, λmax, I.R spectra, Melting point etc as per requirement of study.
- Selection of suitable common solvent for solubility:- Solvent will be selected on the basis of solubility and stability of drugs. E.g., water, methanol etc.
- Mobile phase optimization to achieve resolution between both combination drugs.
- Validation of method and assay of combined dosage form by developed method.
- Development of methods using various approaches as per requirement and all HPTLC methods will be validated as per the ICH guidelines.
- To perform analysis of marketed formulations containing above combination by developed HPTLC method.
- It is also planned to use the available literature for interpreting the data.

The whole study is divided into five phases after doing literature survey to generate data. Following are the five phases:

<table>
<thead>
<tr>
<th>Phase – I</th>
<th>It is planned to Collection of various API and Dosage form.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase – II</td>
<td>In this phase, Identification of various API by determination of solubility, λmax, I.R spectra, Melting point etc.</td>
</tr>
<tr>
<td>Phase – III</td>
<td>After phase II, Selection of suitable solvent and mobile phase optimization will be carried out.</td>
</tr>
<tr>
<td>Phase – IV</td>
<td>In this phase Validation of developed method as per ICH guidelines.</td>
</tr>
<tr>
<td>Phase – V</td>
<td>Analysis of marketed formulation by developed method will be carried out after phase IV</td>
</tr>
</tbody>
</table>

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