GENERAL INTRODUCTION

1.1 Introduction to Drugs

The word drug is derived from the French word drogue, which means a dry herb. In general, a drug may be defined as a substance used in the prevention, diagnosis, treatment or cure of diseases in man or other animals. According to world health organization (WHO), a drug may be defined as any substance or product which is used or intended to be used for modifying or exploring physiological systems or pathological states for the benefit (physical, mental as well as economical) of the recipient. Alternatively, a drug is any substance that, when absorbed into the body of a living organism, alters normal bodily function. Drugs are synthesized in bulk and used for their therapeutic effects in pharmaceutical formulations. In the development of drugs, pharmaceutical scientists have explored numerous approaches to find and develop drugs that are now available in pharmaceutical formulations suitable for the treatment of diseases and often for the maintenance of human health. The methods of quality control and conditions of storage of pharmaceutical formulations are the important contents placed in many text books of pharmaceutical chemistry **[1-8].** These biologically active chemical substances are generally formulated into convenient dosage forms such as tablets, capsules, dry syrups, liquid orals, creams or ointments, parenterals (injections in dry or liquid forms) lotions, dusting powders, aerosols, metered dose inhalers and dry powder inhalers etc. These formulations deliver the drug substances in a stable, nontoxic and acceptable form, ensuing its bioavailability and therapeutic activity.

Drugs are classified into various categories according to the chemical structure and therapeutic action. Chemotherapeutic agents such as antibacterial, trypanocides, antiprotozoals, antifungals, anthelmintics, antiseptics, antitubercular, antilepral drugs, antineoplastic agents, disinfectants and antiviral drugs which are used to kill the invading organisms without harmful effects on the tissues of the patient. Pharmacodynamic agents may be further subdivided into different classes like central nervous system modifiers, adrenergic stimulants, blocking agents, cholinergic, anticholinergic agents, cardio-vascular agents, diuretics, anti-inflammatory agents, immuno suppressive agents, antispasmodics, antihypertensive, antidepressants,

antihistamines, anticoagulants and antipsycotic agents. Antihypertensive drugs are used to control blood pressure. An antidepressant is a psychiatric medication used for alleviating major depression and dysthymia (milder depression). These are classified as selective serotonin reuptake inhibitors, neither serotonin nor epinephrine reuptake inhibitors, noradrenergic and specific serotonergic antidepressants, norepinephrine reuptake inhibitors, norepinephrine-dopamine reuptake inhibitors, tricyclic antidepressants, monoamine oxidase inhibitors **[9]**. The vital role of the adrenal cortex is due to its ability to produce a group of steroidal hormones **[10]**. In addition to the naturally occurring corticosteroids many synthetic steroids with similar properties have been introduced. The pharmaceutical properties of corticosteroids also make them useful in the treatment of rheumatoid arthritis, bronchial asthma and bronchial hypersensitivity **[11]**. The anti-inflammatory corticoids represented by hydrocortisone and related synthetic analogs have gained an unchallenged position in modern therapeutic practice. The therapeutic effect of steroids depends on their stability **[12,13]**.

Anti cancer drugs are used to control the growth of cancerous cells. Altretamine, asparaginase, bleomycin, busulfan etc., are used as anti-cancer drugs. HIV is a virus that attacks the body's defence against infection and illness the immune system. Abacavir, didanosine, emtricitabine, lamivudine etc., are used as anti-HIV drugs. Drugs used in diabetes treat diabetes mellitus by lowering glucose levels in the blood. Except insulin, liraglutide and pramlintide, all are administered orally and are thus also called oral hypoglycemic agents. Sulfonyl urea, metformin etc., are used as anti diabetic drugs. Anti depressants are the most prescribed therapy for depression. They increase the concentration of one or more brain chemicals (neurotransmitters) that nerves in the brain use to communicate with one another. The neurotransmitters affected by antidepressants are norepinephrine, serotonin and dopamine.

The following drugs are selected for the development of their stability indicating RP-HPLC and UV spectrophotometric methods. Everolimus, belinostat, ceritinib and ibrutinib are used as anti cancer drugs, saroglitazar is used for the treatment of diabetic dyslipidemia, cobicistat and elvitegravir are used for the treatment of HIV, dapoxetine hydrochloride is used for the treatment of premature ejaculation and azelnidipine is a cardiovascular agent.

1.2 Profile of the selected drugs

Everolimus: Everolimus (ERL) is a class of medications called kinase inhibitors used for treatment of different cancer. It is a 40-O-(2-hydroxyethyl) derivative of sirolimus and works similarly to sirolimus as an inhibitor of mammalian. Everolimus is (1R,9S,12S,15R,16E,18R,19R,21R,23S,24E,26E,28E,30S,32S,35R)-1,18-dihydroxy-12-{(1R)-2-[(1S,3R,4R)-4-(2-hydroxyethoxy)3-methoxy cyclohexyl]-1-methylethyl}-19,30 dimethoxy 15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-aza-tricyclo[30.3.1.0] hexa triaconta-16,24,26,28-tetraene-2,3,10,14,20 pentaone. The chemical structure of ERL is represented in **Fig 1.1.**

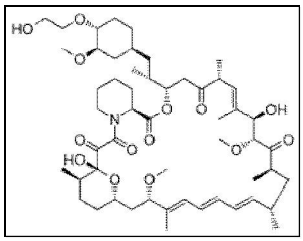


Fig 1.1: Chemical structure of Everolimus

Belinostat: Belinostat (PXD101, trade name beleodaq) is a drug under development by topotarget for the treatment of hematological malignancies and solid tumors. It is a histone deacetylase inhibitor. The chemical name of belinostat (BLT) is (2E)-N-Hydroxy-3-[3-(phenyl sulfamoyl)phenyl] prop-2-enamide. The chemical structure of BLT is represented in **Fig 1.2.**

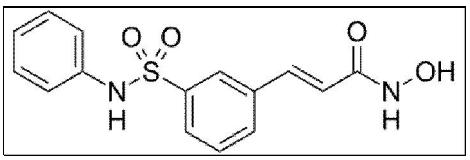


Fig 1.2: Chemical structure of belinostat

Ceritinib: Ceritinib(CRB), 5-chloro-N^2-[2-iso propoxy-5-methyl-4 (4-piperidinyl) phenyl]-N^4-[2-(isopropyl sulfonyl)phenyl]-2,4-pyrimidine diamine, is an anaplastic lymphoma kinase (ALK) inhibitor which induces complete tumour regression in a xenograft model of EML4-ALK-positive lung cancer. The alternative names of ceritinib are LDK 378, NVP-LDK 378, zykadia^{TM}. The chemical structure of CRB is represented in **Fig 1.3.**

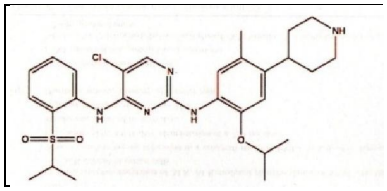


Fig 1.3: Chemical structure of ceritinib

Saroglitazar: Saroglitazar (lipaglyn) is indicated for the treatment of diabetic dyslipidemia and hyper triglyceridemia with type 2 diabetes mellitus not controlled by statin therapy. Saroglitazar is (2S)-2-ethoxy-3-[4-(2-{2-methyl-5-[4-(methyl sulfanyl) phenyl]-1H-pyrrol-1-yl}ethoxy)phenyl] propanoic acid. The chemical structure of SAR is represented in **Fig 1.4.**

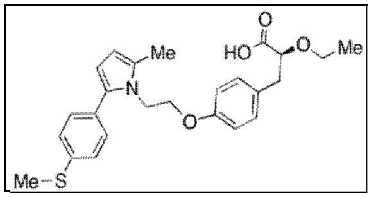


Fig 1.4: Chemical structure of saroglitazar

Cobicistat: Cobicistat, CBT [1,3-thiazol-5-ylmethyl N-[(2R,5R)-5-[[(2S)-2-[[methyl- [(2-propan-2-yl-1,3-thiazol-4-yl) methyl] carbamoyl] amino]-4-morpholin-4-ylbutanoyl] amino]-1,6-diphenylhexan-2-yl] carbamate], is used in the treatment of human immunodeficiency virus (HIV). The chemical structure of CBT is represented in **Fig 1.5.**

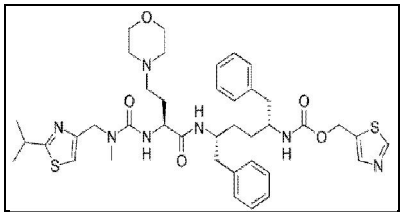


Fig 1.5: Chemical structure of cobicistat

In combination with three other drugs elvitegravir, emtricitabine, and tenofovir, cobicistat is used in the treatment of HIV. The combination of the four drugs is popularly known as quad – pill.

Elvitegravir: Elvitegravir (ELT), 6-[(3-Chloro-2-fluorophenyl)methyl]-1-[(2S)-1- hydroxy-3-methylbutan-2-yl]-7-methoxy-4-oxoquinoline-3-carboxylic acid, is a newly introduced HIV-1 integrase strand transfer inhibitor. ELT is an integrase inhibitor used to treat HIV infection. The chemical structure of ELT is represented in **Fig 1.6.**

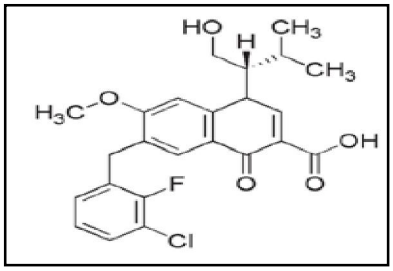


Fig 1.6: Chemical structure of elvitegravir

Ibrutinib: Ibrutinib (IBR) is an anticancer drug targeting β-cell malignancies **(**blood cancer treatment medicines**).** It was approved by the US FDA (2013) and used for the treatment of mantle cell lymphoma and for the treatment of chronic lymphocytic leukaemia.It is an orally administered, selective and covalent inhibitor of the enzyme bruton's tyrosine kinase (BTK). The systematic (IUPAC) name of ibrutinib is given by 1-[(3R)-3-[4-Amino-3-(4-phenoxyphenyl)-1H-pyrazolo [3, 4-d] pyrimidin-1-yl] piperidin-1-yl] prop-2-en-1-one. The chemical structure of IBR is represented in **Fig 1.7.**

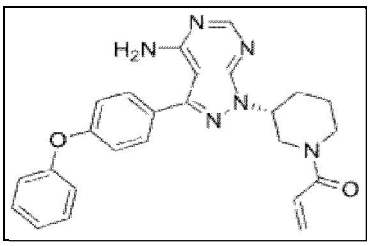


Fig 1.7: Chemical structure of ibrutinib

Dapoxetine hydrochloride: Dapoxetine hydrochloride (DAP), (S)-N,N-dimethyl-α-[2-(1-Naphthalenyloxy) ethyl]benzene methanamine hydrochloride, drug is used for the treatment of premature ejaculation. It acts as selective serotonin re-uptake inhibitor (SSRI) as it is an antidepressant. The chemical structure of DAP is represented in **Fig 1.8.**

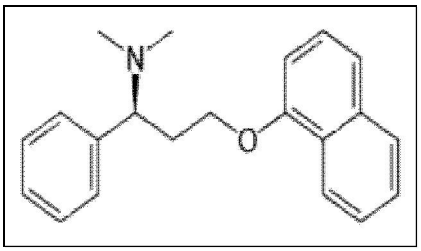


Fig 1.8: Chemical structure of DAP

Azelnidipine: Azelnidipine (AZL) is a white crystalline powder and is used as cardiovascular agent. The therapeutic action of AZL is that it acts as anti-hypertensive agent and also as calcium channel blocker. The presence of impurities in an active pharmaceutical ingredient (API) can have a significant impact on the quality and safety of the drug product. During the analysis of laboratory batches of AZL, four impurities were observed in HPLC method. The recommended dosage of AZL is 16 mg per day. The structure of possible impurities related to raw materials or degradants is identified by the various characterization techniques such as UV, IR, NMR and mass spectrography studies.

In the studies of AZL, four impurities were identified namely, propan-2-yl-2-(4-nitrobenzylidene)-3-oxobutanoate[impurity 1]; propan-2-yl2-(3-nitrobenzylidene)-3-oxobutanoate[impurity 2]; 3-[1-(diphenyl methyl)azetidin-3-yl] 5-propan-2-yl 2-amino-6-methyl-4-(4-nitro phenyl)-1,4-dihydro pyridine-3,5-dicarboxylate [impurity 3]; and 3-[1-diphenyl methyl)azetidin-3 yl] 5-propan-2-yl 2-amino-6-methyl-4-(2-nitro phenyl)-1,4-dihydro pyridine-3,5-dicarboxylate [impurity 4]. The chemical structures of impurities of AZL were represented in **Fig 1.9 (a), (b), (c) and (d)** respectively.

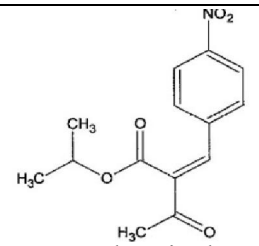


Fig 1.9 (a): Chemical structure of para impurity of AZL

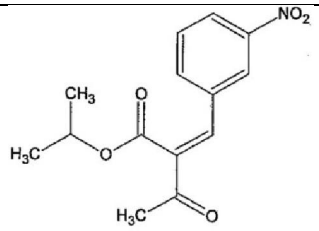


Fig 1.9 (b): Chemical structure of Azelnidipine intermediate

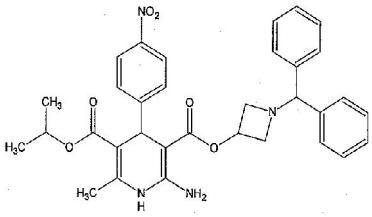


Fig 1.9 (c): Chemical structure of 4-nitro azelnidipine

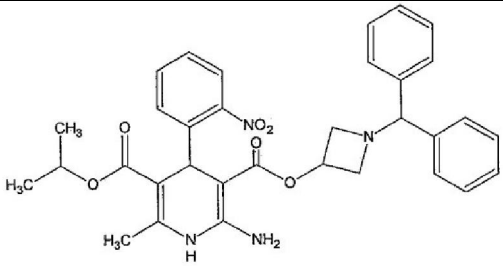


Fig 1.9 (d): Chemical structure of 2-nitro azelnidipine

The physical properties of the chosen drugs were presented in **Table 1.1.**

Table 1.1: Physical properties of chosen drugs

Physical properties	Everolimus	Belinostat	Ceritinib	Saroglitazar	Cobicistat	Elvitegravir	Ibrutinib	Dapoxetine	Azelnidipine
Chemical formula	$C_{53}H_{83}NO_{14}$	$C_{15}H_{14}N_2O_4S$	$C_{28}H_{36}ClN_5O_3S$	$C_{25}H_{29}NO_4S$	$C_{40}H_{53}N_7O_5S_2$	$C_{23}H_{23}ClFNO_5$	$C_{25}H_{24}N_6O_2$	$C_{21}H_{23}NO.HCl$	$C_{33}H_{34}N_4O_6$
Molecular mass	958.2 g/mol	318.35 g/mol	558.14 g/mol	439.56 g/mol	776.023 g/mol	447.883 g/mol	440.497 g/mol	341.87 g/mol	582.65 g/mol
Appearance	white or off-white crystalline powder	white solid powder	white or light yellow powder	white crystalline powder	white or pale yellow solid	white to pale yellow solid	white to off white solid	white crystalline powder	pale yellow to white crystalline powder
Solubility	soluble in acetonitrile	soluble in dimethyl sulfoxide (DMSO) but insoluble in water	soluble in DMSO, ethanol but slightly soluble in water	DMSO, methanol, ethanol, water and acetonitrile	soluble in water, DMSO, PBS buffer	soluble in DMSO, acetonitrile and methanol	soluble in DMSO, methanol, acetonitrile	DMSO, methanol, ethanol, chloroform, water and acetonitrile	soluble in ethanol, slightly soluble in methanol and water
Formulations	zortres- 1 mg, 10mg 100 mg tab certican 1mg, 10mg, 100 mg tab advacan 0.25 mg, 0.5 mg tab	beleodaq - 500 mg/vial PXD 101 -10 mg, 100 mg, 500 mg, 1 kg	LDK 378- 5 mg, 10 mg, 50 mg, 100 mg zykadia™- 150 mg hard-gelatin capsule	lipaglyn-4 mg tablet	tybost-150 mg tablets quad-pill 50 mg, 100 mg, 150 mg tablets	stribild-150 mg tablets and vitekta - 85 mg, 150 mg tablets	ibruvica 140 mg capsule	sustinex-30 mg, 60 mg capsules prejac-60 mg capsule kutub-30 mg, 60 mg capsules	watsonnoke-30 mg, 60 mg tablets and calblock® - 8 mg, 16 mg tablets
Characterization	anti cancer drug	anti cancer drug	anti cancer drug	anti diabetic drug	anti HIV drug	anti HIV drug	anti cancer drug	anti depressant drug	anti hypertensive drug

1.3 Analysis of pharmaceuticals

Pharmaceutical analysis plays a very vital role in the quality assurance and quality control of bulk drugs and their formulations. Pharmaceutical analysis is a specialized branch of analytical chemistry which involves separating, identifying and determining the relative amounts of components in a sample of matter. It is concerned with the chemical characterization of matter both quantitative and qualitative. Pharmaceutical analysis derives its principles from various branches of sciences like physics, microbiology, nuclear science and electronics etc. Pharmaceutical analysis establishes the relative amount of one or more of these species or analytes in numerical terms **[14,15].** The good manufacturing practices provide minimum quality standards for production of pharmaceuticals as well as their ingredients **[16,17].** Every country has legislation **[18]** on bulk drugs and their pharmaceutical formulations that sets standard and obligatory quality indices for them. These regulations are presented in separate article general and specific relating to individual drugs, and are published in the form of book called "pharmacopoeia" (e.g. indian pharmacopoeia, IP **[19],** united states pharmacopoeia USP **[20],** european pharmacopoeia EP **[21],** united kingdom, BP **[22],** martindale extra pharmacopoeia **[23],** merck index **[24],** etc.

1.3.1 Quantitative analysis of pharmaceuticals

Complete quantitative analysis of pharmaceuticals actually involves the following steps.

(i) Preparation of sample solution: Sample solutions for the analytical investigation can be prepared by dissolving finely powder of the tablets or granules of the capsules in suitable solvent for the samples in solid state.

(ii) Conversion of the analyte into a measurable form: This step is a vital one, in developing any analytical method. Particularly, while dealing with the interferences in pharmaceutical products, one should have sound knowledge about the chemical and structural factors of the analyte and interfering molecules, and also in the selection of appropriate chromogenic reagent, which should form the coloured product with the analyte molecule, and not with the foreign molecule. Any possible

interference from foreign matter is if expected during the course of the colour development, an appropriate clean up procedure should be adopted prior to the analysis.

(iii) Measurements: The measurement step in an analysis can be carried out by chemical, physical or biological means. An important feature of modern pharmaceutical chemistry is the introduction of more refined and sensitive methods of physico-chemical analysis such as spectroscopy and chromatography that enable one to assay the drugs more accurately and with the smallest consumption of the analyte, reagents and time.

(iv) Calculation and interpretation of measurements: In spectrophotometric or chromatographic methods absorbance or peak area is directly proportional to the concentration of the analyte in the solution. Since errors can be made in any measurement, the analytical chemist must consider this possibility in interpreting his results. The methods of statistics are commonly used and are especially useful in expressing the significance of analytical data.

1.4 Study of impurities

The quality of a drug plays an important role in ensuring the safety and efficacy of the drugs. Quality assurance and control of pharmaceutical and chemical formulations is essential for ensuring the availability of safe and effective drug formulations to consumers. Hence analysis of pure drug substances and their pharmaceutical dosage forms occupies a pivotal role in assessing the suitability to use in patients. The impurity profile of pharmaceuticals is of increasing importance as drug safety receives more and more attention from the public and from the media.

In the changed perspectives, not only the content of active ingredient is essential but also a complete detailing of the impurities present or likely to appear during the course of usage has become mandatory. During the manufacturing process, whether by chemical synthesis, extraction, cell culture/fermentation, recovery from natural sources, or any combination of these processes, impurities may arise. Purity of active pharmaceutical ingredient depends on several factors such as raw materials, their method of manufacture and the type of crystallization and purification process.

Concept about purity changes with time and it is inseparable from the developments in analytical chemistry. The pharmacopoeias specify not only purity but also puts limits which can be very stringent on levels of various impurities.

1.4.1 Importance of impurity profile evaluation in pharmaceutical industry

Impurities are extraneous compounds that are not the drug substance (also known as active pharmaceutical ingredient), but arise during the synthesis, extraction, purification, or storage of the drug. Understanding the origin, control, and measurement of impurities is critical to the production of high quality drug substances. In addition to guidance from the local authorities of many countries, a series of guidelines developed in recent years by expert working group of the international conference on harmonization of technical requirements for registration of pharmaceuticals for human use have been increasingly accepted by the pharmaceutical community. The expert working group ICH has defined an impurity as "any compound of the medicinal product which is not the chemical entity defined as the active substance or as an excipient in the product". Similarly impurity profile has been defined as "a description of the identified and unidentified impurities present in the medicinal product" **[25].** Chiral drugs constitute about 56% of the drugs currently in use and about 88% of these chiral synthetic drugs are used therapeutically as racemates. The racemates can exhibit variable metabolic pathways and pharmacologic activity and under some special circumstances, enantiomers as well as polymorphs are also considered as impurities **[26].**

The impurities and /or degradants may evoke any form of adverse response, either pharmacologic or toxicological in patients undergoing medication. Modern separation methods clearly play a dominant role in scientific research today because these methods simultaneously separate and quantify the components. To ensure patient safety, impurity profiling which can be defined as a group of analytical activities aimed at the detection, identification or structure elucidation and quantitative determination of organic and inorganic impurities as well as residual solvents in bulk drugs and pharmaceutical formulations is very essential. Impurities in pharmaceuticals are unwanted chemicals that remain with the APIs or develop during formulation or develop upon ageing of both APIs and formulated APIs to medicines **[27-30].** The presence of these unwanted chemicals even in small amounts

may influence the efficacy and safety of the pharmaceutical products. Different pharmacopoeias such as british pharmacopoeia (BP) and the united states pharmacopoeia (USP) are incorporating limits to allowable levels of impurities present in the APIs or formulations. The ICH has published guidelines on impurities in new drug substances, products and residual solvents **[31-33].** Impurity profile is description of the identified and unidentified impurities present in a typical batch of API produced by a specific controlled production process **[34].**

1.4.2 Formation of impurities

Process impurities arise during the manufacturing of the drug substance, degradation impurities arise during the storage of the drug substance and contaminant impurities are not drug related but are inadvertently introduced during processing or storage, and are not part of the synthesis, extraction, or fermentation process. Impurities that cause the greatest concern are those that are toxic, defined by the USP as impurities that have significant undesirable biological activity **[35]** and host cell contaminants in biopharmaceuticals that have potential risks of allergic reaction or other immune pathological effects **[36].**

Impurities in the starting materials and intermediates: Possibility of impurities in the starting materials may follow the same reaction pathways as the starting material itself, and the reaction products could carry over to the final product as process impurities. Understanding of the impurities in starting materials helps to identify related impurities in the final product, and to understand the formation mechanisms of related process impurities. Starting materials or intermediates are the most common impurities found in every API unless a proper care is taken in every step involved in throughout the multi-step synthesis. Although the end products are always washed with solvents, there are chances of remaining residual un reacted starting materials unless the manufacturers are very careful about the impurities.

Formation of impurities from degradation of the API's: Normally, degradants are chemical breakdown compounds of the drug substance formed during storage. The definition of degradation of the end product in the ICH guideline is a molecule resulting from a chemical change in the substance brought about by overcome or the effect of light, temperature, acid, base and peroxide. The goal of the

stability indicating method is to obtain baseline resolution of all the resulting products (the API and all the degradation products) with no co-elutions **[37,38].** The degradation products generated in the stressed samples are termed as "potential" degradation products that may or may not be formed under relevant storage conditions. The major forced degradation studies are acid, base, oxidative, photolytic and thermal degradation.

Stereochemistry-related impurities: It is of paramount importance to look for stereo chemistry related compounds, that is, those compounds that have similar chemical structure but different spatial orientation, these compounds can be considered as impurities in the APIs. Chiral molecules are frequently called enantiomers. Since the discovery of difference between thalidomide enantiomers in pharmacological and toxicological actions, discrimination of optical isomers has been one of the major subjects in the field of pharmacy, because optical purities of substrates with asymmetries are critical for the evaluation of their biological activities **[39].** Enantiomers of racemic drugs often show different behaviors in pharmacological action and metabolic process. Often one enantiomer is active and other can be non-active, poorly active or toxic. The pharmaceutical industry has raised its emphasis on the generation of enantiomerically pure compounds before under taking pharmacokinetic, metabolic, physiological and toxicological evaluation in the search for drugs with greater therapeutic benefits and low toxicity **[40,41].** In recent years, stringent regulations for marketing for marketing enantiomeric drugs have been implemented by the regulatory agencies of all the major countries (pharmaceutical consumers) of the world **[42-44].**

1.5 Analytical techniques

Analytical techniques or methods are intended to establish the identity, purity, physical characteristics and potency of the drugs that we use. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long term stability studies. Methods may also support safety and characterization studies or evaluations of drug performance. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients **[45].** Quantification of bulk drugs or

pharmaceutical formulations can be achieved by the introduction of more refined and sensitive methods of physiochemical analysis **[46,47]** such as colorimetry, spectrophotometry covering UV, visible and IR regions, fluorimetry or turbidimetry, NMR and mass, and chromatography **[48-56]** that enables one to assay of drugs more accurately and with the smallest consumption of the analyte, reagent and time. In recent years, several analytical techniques have been evolved that combination of two or more methods into one called "hyphenated" technique Eg., GC-MS, LC-MS etc. Such methods are to be validated demonstrating the accuracy, precision, and specificity, limit of detection, quantification, linearity range and interferences. The validation of analytical procedures is an important part of the registration application for a new drug **[57,58]**. The international chemical harmonization (ICH) has harmonized the requirements in two guidelines **[59,60]**. These guidelines serve as a basis worldwide both for regulatory authorities and industry in proper validation.

Organic impurity levels can be measured by a variety of techniques, currently organic impurities are almost exclusively measuring by using of chromatographic procedures. Chromatographic procedures should involve a separation mode that allows for the resolution of impurities from the drug substance and a detection mode that allows for the accurate measurement of impurities. In general, non-volatile impurities are analyzed by HPLC techniques, among which reversed phase HPLC (RPLC), normal phase (NPLC), LC-MS, GC and GC-MS are the most widely used separation modes **[61,62].** Hydrophilic interaction liquid chromatography (HILIC) seems complementary to RPLC for the retention and separation of small molecule polar analytes, and has thus gained increasing attention recently. Good retention can be achieved for more polar analytes, which is not possible on RPLC columns. In the hydrazine group, the HILIC method was used in addition to the HPLC-UV and LC-MS methods **[63].** However, if the analytes are ionized, ion chromatography (IC) may provide better retention and separation. IC is based on ion-ion interactions between charged analytes and oppositely charged groups embedded in the stationary phase. GC methods are commonly used for the analysis of several volatile small molecules. Some examples include the liquid injection technique and the headspace sampling technique. Liquid injection is prone to contamination in which injection of a large amount of non-volatile API can accumulate in the injector liner or on the head of the GC column, which can cause a sudden deterioration in method performance.

Headspace injection, on the other hand, is desirable because it minimizes potential contamination of the injector or column by avoiding the introduction of a large quantity of API. Jouyban and Kenndler (2008) **[64]** reviewed the applicability of capillary electrophoresis (CE) methods for the analysis of pharmaceutical impurities. FT-IR (fourier transform infrared spectroscopy), NIR (near-infrared spectroscopy), raman spectroscopy, DSC (differential scanning calorimetry), TGA (thermogravimetry) and P-XRD (powder X-ray diffraction) techniques are used for identification and quantification of crystalline phases and polymorphic impurities.

1.5.1 High-performance liquid chromatography

High-performance liquid chromatographic technique **[65,66]** is a widely accepted separation technique for sample analysis, purification and impurity evaluation in a variety of areas including the pharmaceutical, biotechnological, environmental, polymer and food industries. The successful use of liquid chromatography for a given problem requires the right combination of a variety of operating conditions such as the type of column packing and mobile phase, column length and diameter, mobile phase flow rate, column temperature, and sample size. In HPLC, separations are achieved by partition, adsorption or ion-exchange, depending on the nature of interactions between the solute and the stationary phase, which may arise from hydrogen bonding, vanderwall forces, electrostatic forces, hydrophobic forces (or) based on the size of particles (e.g. size exclusion chromatography). When a mixture of components is introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower. The component which has less affinity towards the stationary phase travels faster. Since, no two components have the same affinity towards the stationary phase, the components are separated.

HPLC instrumentation **(Fig 1.10)** is made up of eight basic components: mobile phase reservoir, HPLC pump, sample introduction device, HPLC column, HPLC detector, waste reservoir, connective tubing and a computer, integrator, or recorder.

Mobile phase reservoir: The mobile phase reservoir can be any clean, inert container such as an empty solvent bottle, laboratory flask. This should be with small

covered opening. It should have a cap that allows the tubing inlet line to pass through.

HPLC pump: The purpose of the pump or solvent delivery system is to ensure the delivery of a precise, reproducible, constant and pulse-free flow of mobile phase. There are two classes of HPLC pumps: constant pressure and constant flow pumps. The most common type of HPLC constant flow pump is the reciprocating piston pump, in which a piston is driven in and out of a solvent chamber by an eccentric cam or gear. On the forward stroke, the inlet check valve closes, the outlet check valve opens, and the mobile phase is pumped to the column. On the reverse stroke, the check valves reverse and solvent is drawn into the chamber.

Sample introduction devices: A variety of sample introduction devices exist, manual and automatic that used primarily a valve mechanism. When the valve is in the load position, the sample loop is filled. For best results, a two to five fold excess of sample should be passed through the loop to ensure that the previous sample has been thoroughly purged.

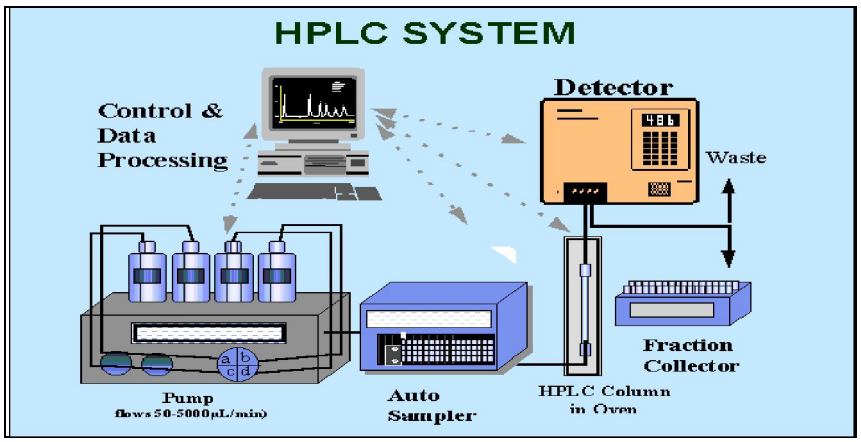


Fig 1.10: Schematic diagram of an HPLC instrument

HPLC column: The column is heart of the HPLC instrument because the separation occurs here. It is generally made of 316-grade stainless steel which is relatively inert to chemical corrosion and is packaged with the desired stationary phase. Common dimensions for analytical scale columns are in the range of 10 to 25 cm long and 3 to 9 mm inner diameter.

HPLC detectors: The important role of the HPLC detector is to monitor the solutes as they are eluted from the column. The detector generates an electrical signal

that is proportional to the level of some property of the mobile phase or solutes. Some characteristics of a good HPLC detector are sensitivity, linearity, predictability in response, reliability, non-destructiveness, ease of use and low dead volume. Over 70% of all of the HPLC detectors are UV absorbance detectors. In order to generate real-time spectra for each solute as it is eluted, a photodiode array is used.

Computer, integrator and recorder: A data collection device such as a computer, integrator, and recorder is connected to the detector. It takes the electronic signal produced by the detector and plots it as a chromatogram, which can be evaluated by the user. Recorders are rarely used because they are unable to integrate the data. Both integrators and computers can integrate the peaks in the chromatograms and computers have the further advantage that they electronically save chromatograms for later evaluation.

1.5.1.1 Chromatographic Parameters

Resolution: Chromatographers measure the quality of separation by resolution, R of adjacent bands.

$$R = 2(t_2 - t_1) / (W_1 + W_2)$$

t_1 and t_2 are retention times of the first and second adjacent bands; W_1 and W_2 are base line band widths.

Capacity factor (k): It is the measure of how well the sample molecules are retained by the column during an isocratic separation. It is affected by the solvent composition, separation, aging and temperature of separation. $k = (t_R - t_o) / t_o$

Where, t_R = band retention time and t_o = column dead volume 'o'

Column efficiency (N): It is called as the number of theoretical plates. It measures the band spreading of a peak. When band spread in smaller, the number of theoretical plates is higher. It indicates a good column and system performance. Column performance can be defined in terms of values of N.

column efficiency (N) = $16 (t_R/W)^2$ plate height(H) = N/L (length)

Peak asymmetry/Peak tailing (As): Peak with poor symmetry can result in (i) inaccurate plate number and resolution measurement (ii) imprecise quantization (iii) degraded resolution and undetected minor bands in the peak tail (iv) poor retention reproducibility. Increased peak asymmetry value, k > 1.5 the sign that the column should be changed.

Peak asymmetry (T) = $W_{0.05}/2f$, where $W_{0.05}$ is the width of the peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

Selectivity: It measures relative retention of two components. Selectivity is the function of chromatographic surface (column), melting point and temperature.

$$A = k_2/k_1 = (V_2 - V_o)/(V_1 - V_0)$$

height equivalent to theoretical plate (HEPT) = L/n

Quantization: A critical requirement for quantitative methods is ability to measure wide range of sample concentration with a linear response for each analyte. To achieve the best result with an HPLC method, it is necessary to understand and have a control of the factors that affect quantization. Calculating the following values are used to access overall system performance.

1.5.2 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS method has been applied to the identification of impurities and degradants in API and drug product. These methods gave enhanced sensitivity compared with the standard LC-UV or stand alone MS methods. In pharmaceutical industry LC-MS has become method of choice in many stages of drug development. Recent advances in electro spray, thermo spray, and ion spray ionization techniques offer unique advantages of high detection sensitivity and specificity. Principle of LC-MS is based on the fragmentation of charged ions and detection of the resulting fragments. There are two common atmospheric pressure ionization (API) LC-MS processes: electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Both of these processes are compatible with most chromatographic separations.

In LC-MS analysis, a sample is injected into an LC column after that the sample is ionized by the ion source. Types of sources include electron impact ionization (EI), chemical ionization (CI) and atmospheric pressure ionization (API). In this technique API is mostly used. The vacuum interface ensures the transition of ions from the API source to the mass analyser, which is kept under a vacuum. From the ion source, ions are transferred to mass analyzer where there are separated according to their mass-to-charge (m/z) values. The mass analyser operates under a vacuum to ensure that ions travel with maximum efficiency. The detector measure the abundance of electrons generated from the ions for each m/z ratio. Most MS systems use some type of electron multiplier. Normally pulse counting detector is used.

LC-MS instrument **(Fig 1.11)** consists of three main components namely LC (to resolve a complex mixture of components), an interface (to transport the analyte into the ion source of a mass spectrometer) and mass spectrometer (to ionize and mass analyze the individually resolved components) and a detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present.

Interface: There are many types of interfaces available such as electro spray ionization (ESI)-positive & negative electro spray ionization, atmospheric pressure chemical ionization and atmospheric pressure photo ionization (APPI). APCI sources ionize the samples at atmospheric pressure and then transfer the ions into mass spectrometer. The sample is dissolved in an appropriate solvent and this solution is introduced into the mass spectrometer, APCI source introduce the sample through a series of differentially pumped stages. This maintains the large pressure difference between the ion source and mass spectrometer.

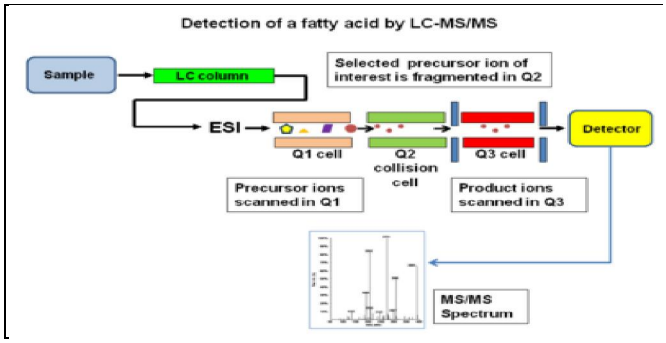


Fig 1.11: Schematic representation of LC-MS instrument

Mass analyzer: In a mass spectrometer, the ions are separated according to mass charge ratio (m/z) using a mass analyzer. There are many different mass analyzers that can be used in LC- MS. Commonly used mass analyzers are quadrupole type (single quadrupole, triple quadrupole, quadrupole-time of flight), time of flight type (TOF type) and ion trap type. The quadrupole analyzer is the most widely used analyzer due to its ease of use, mass range covered, good linearity for quantitative work, resolution and quality of mass spectra all these for a relatively accessible price.

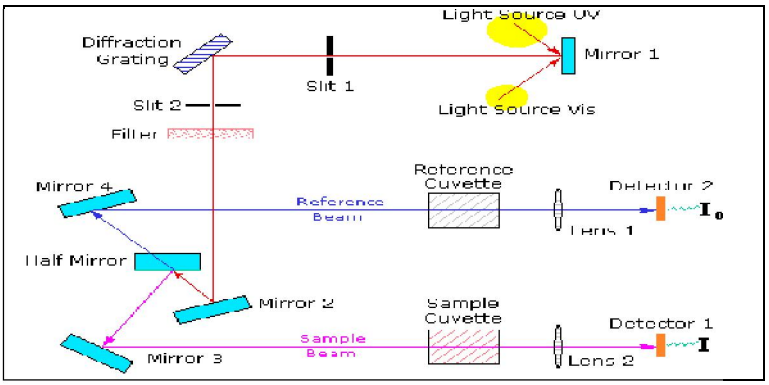
Detector: A series of dynodes maintained at ever-increasing potentials. Ions strike the dynode surface, resulting in the emission of electrons. These secondary electrons are then attracted to the next dynode where more secondary electrons are generated. Ultimately resulting in a cascade of electrons.

1.5.3 UV-Visible spectrophotometry

UV-Visible spectrophotometry is routinely used in analytical chemistry for the quantitative determination of different analytes such as highly conjugated organic compounds and biological macromolecules. The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length **[67].** Thus, for a fixed path length, UV-Vis spectroscopy can be used to determine the concentration of the absorber in a solution. The measuring instrument used in ultraviolet-visible spectroscopy is called a UV-Vis spectrophotometer. It measures the intensity of light passing through a sample and compares it to the intensity of light before it passes through the sample. The ratio is called the transmittance, and is usually expressed as a percentage (%T). The absorbance is based on the transmittance. The absorption in UV or visible range directly affects the perceived colour of the chemicals involved in this region of the electromagnetic spectrum, molecules undergo electronic transitions.

The basic parts of a spectrophotometer are a light source, a holder for the sample, a diffraction grating in a monochromator or a prism to separate the different wave lengths of light and a detector. The radiation source is often a tungsten filament (300-2500 nm), a deuterium arc lamp, which is continuous over the ultraviolet region (190-400 nm), xenon arc lamp, which is continuous from 160-2000 nm; or more recently, light emitting diodes (LED) for the visible wavelengths. The detector is

typically a photomultiplier tube, a photodiode, a photodiode array or a charge-coupled device (CCD). The scanning monochromator moves the diffraction grating to "step-through" each wavelength so that its intensity may be measured as a function of wavelength. Fixed monochromators are used with CCDs and photodiode arrays. As both of these devices consist of many detectors grouped into one or two dimensional arrays, they are able to collect light of different wavelengths on different pixels or groups of pixels simultaneously. The schematic diagram of UV-Visible spectrophotometer was shown in **Fig 1.12.**



ometer

ds, although the

absorba les are typically

placed i ly rectangular in

shape, ecomes the path

length, L, cuvettes in some

instruments. The type of sample container used must allow radiation to pass over the spectral region of interest. The most widely applicable cuvettes are made of high quality fused silica or quartz glass because these are transparent throughout the UV, visible and near infrared regions. Glass and plastic cuvettes are also common, although glass and most plastics absorb in the UV, which limits their usefulness to visible wavelengths **[68].** Specialized instruments have also been made. These include attaching spectrophotometers to telescopes to measure the spectra of astronomical features. UV-visible micro spectrophotometers consist of a UV-visible microscope integrated with a UV-visible spectrophotometer. A complete spectrum of the absorption at all wavelengths of interest can often be produced directly by a more

sophisticated spectrophotometer. In simpler instruments the absorption is determined one wavelength at a time and then compiled into a spectrum by the operator. By removing the concentration dependence, the extinction coefficient (ε) can be determined as a function of wavelength.

1.5.4 Infra Red spectroscopy (IR)

IR spectroscopy is an absorption phenomenon and to absorb infrared radiations, the molecule should exhibit a change in electric dipole moment, μ, during molecular motion. The polar interactions, such as hydrogen bonding, hydration, proton conduction, conformational changes, order/disorder and metal coordination etc., can be studied by IR spectroscopy. The rotational and vibrational frequencies of molecules are in the infrared region of the electromagnetic spectrum and therefore infrared spectroscopy (IR) can be employed to study molecular rotations and vibrations in these frequency ranges. The frequencies are expressed in IR and raman spectroscopies in wave numbers, (cm^{-1}).

Sample preparation: Gaseous samples require a sample cell with a long path length to compensate for the diluteness. The path length of the sample cell depends on the concentration of the compound of interest. A simple glass tube with length of 5 to 10 cm equipped with infrared-transparent windows at the both ends of the tube can be used for concentrations down to several hundred ppm. White cells are available with optical path length from 0.5 to 100 meters. The schematic diagram of IR spectrophotometer was shown in **Fig 1.13.**

Liquid samples can be sandwiched between two plates of a salt (commonly sodium chloride, or common salt, although a number of other salts such as potassium bromide or calcium fluoride are also used). The plates are transparent to the infrared light and do not introduce any lines onto the spectra.

Fig 1.13: Schematic diagram of IR spectrophotometer

Solid samples can be prepared in a variety of ways. One common method is to crush the sample with an oily mulling agent (usually nujol) in a marble or agate mortar, with a pestle. A thin film of the mull is smeared onto salt plates and measured. The second method is to grind a quantity of the sample with a specially purified salt (usually potassium bromide) finely (to remove scattering effects from large crystals). This powder mixture is then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer can pass. A third technique is the "cast film" technique, which is used mainly for polymeric materials. The sample is first dissolved in a suitable, non hygroscopic solvent. A drop of this solution is deposited on surface of KBr or NaCl cell. The solution is then evaporated to dryness and the film formed on the cell is analysed directly. This technique is suitable for qualitative analysis. The final method is to use microtomy to cut a thin (20–100 μm) film from a solid sample.

It is important to note that spectra obtained from different sample preparation methods will look slightly different from each other due to differences in the samples physical states.

Uses and applications of IR spectroscopy: Infrared spectroscopy is a simple and reliable technique widely used in both organic and inorganic chemistry, in research and industry. It is used in quality control, dynamic measurement, and monitoring applications such as the long-term unattended measurement of CO_2 concentrations in greenhouses and growth chambers by infrared gas analyzers. It is also used in forensic analysis in both criminal and civil cases, for example in

identifying polymer degradation. It can be used in determining the blood alcohol content of a suspected drunk driver.

With increasing technology in computer filtering and manipulation of the results, samples in solution can now be measured accurately (water produces a broad absorbance across the range of interest and thus renders the spectra unreadable without this computer treatment).

Infrared spectroscopy is also useful in measuring the degree of polymerization in polymer manufacture. Changes in the character or quantity of a particular bond are assessed by measuring at a specific frequency over time. Modern research instruments can take infrared measurements across the range of interest as frequently as 32 times a second. This can be done whilst simultaneous measurements are made using other techniques. This makes the observations of chemical reactions and processes quicker and more accurate.

Infrared spectroscopy has also been successfully utilized in the field of semiconductor microelectronics: for example, IR spectroscopy can be applied to semiconductors like silicon, gallium arsenide, gallium nitride, zinc selenide, amorphous silicon, silicon nitride etc.

1.5.5 Nuclear magnetic resonance spectroscopy

NMR spectroscopy is useful in detecting and distinguishing between nuclear particles present in a sample. The fundamental concept of it is that an alternating magnetic field can induce transitions between the spin levels of a nucleus placed in a fixed magnetic field. In this respect NMR spectroscopy differs from ultraviolet and infrared spectroscopy which analyse light and the radiations emitted or absorbed by the electrons surrounding the nucleus.

High resolution nuclear magnetic resonance spectroscopy is a relative newcomer to process chemistry though it is highly exploited in petroleum product research. Proton NMR, in particular, contains a great deal of information about the chemical composition of complex sample mixtures. Peak position is fundamentally related to the connectivity of the nuclei and splitting patterns (sometimes represented

as broadness of features) contain information about the connectivity of neighbouring nuclei. FT-NMR spectra obtained at small tip angles, are linear and additive in nature. Narrow aromatic features are low in polynuclear aromatics while the broad aromatic signature is representative of fuels that contain significant percentages of polynuclear aromatics. Aromatics content and aliphatic/aromatic side chain length are directly related to the fuel cetane number and other properties. The chemical information is so well resolved in NMR spectra that early research into the cetane determination of fuels by NMR was performed *without* chemometrics (ratios of integrated frequency intervals were used) with analytical *reproducibility's* of 1.3 cetane number. NMR is free of the baseline drift issues that plague near-IR. A permanently installed flow through probe means that mechanical positioning and alignment issues do not limit calibration accuracy through time. Finally, the NMR measurement is not susceptible to light scattering and other optical disturbances common in optical spectroscopy. The schematic diagram of NMR spectrophotometer was shown in **Fig 1.14.**

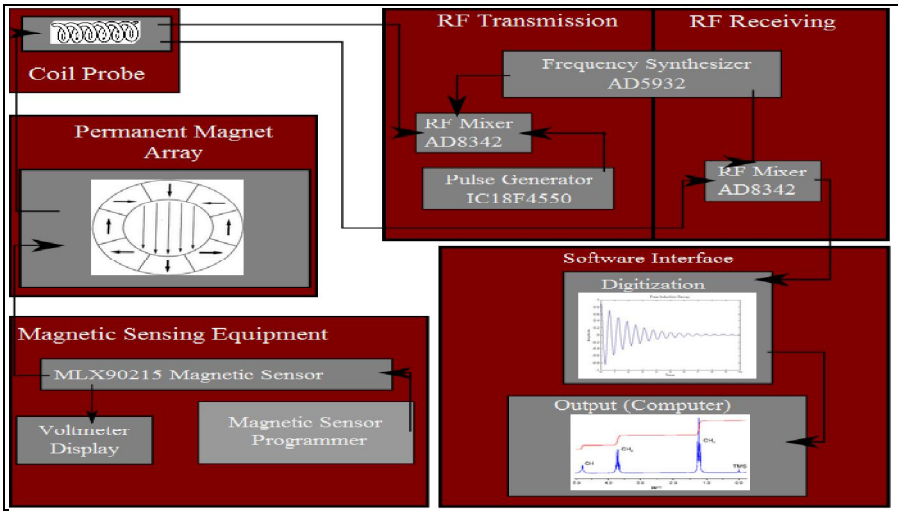


Fig 1.14: Schematic diagram of NMR spectrophotometer

The signal-to-noise ratio of NMR can be enhanced by averaging multiple measurements obtained from repeated pulsing and measurement, though the signal to noise is not likely to meet or exceed that of NIR. Finally, another opportunity for extracting even more information from the petroleum distillate samples may exist in the use of multiple pulse techniques.

1.6 Analytical method development

Analytical method development and validation are key elements of any pharmaceutical development to identify, quantity or purifying compounds of interest. Effective method development ensures that laboratory resources are optimized, while methods meet the objectives required at each stage of drug development. Understand its physical and chemical characteristics allow selecting the most appropriate HPLC method development from vast literature. Information concerning sample, for example, molecular mass, structure and functionality, pKa values and UV spectra, solubility of compounds should be compiled. The requirement of removal of insoluble impurities by filtration, centrifugation, dilution or concentration to control the concentration, extraction (liquid or solid phase), derivatization for detection etc. should be checked. For pure compound, determine sample solubility whether its organic soluble or water soluble, as this helps to select the best mobile phase and column to be used in your HPLC method development. The three critical components for a HPLC method are: sample preparation (% organic, pH, shaking/sonication, sample size, sample age), HPLC analysis conditions (%organic, pH, flow rate, temperature, wavelength, and column age) and standardization (integration, wavelength, standard concentration, and response factor correction). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization **[69-75].**

1.6.1 Selecting the separation mode

The nature of the sample determines the best approach to select the mode of separation in method development. Samples are classified as polar or non polar, large portion of analytes are water soluble. Reversed phase chromatography (RPC) is the first choice for most the polar compounds. Wide ranges of stable stationary phases and simple mobile phases are available for many applications in RPC.

1.6.2 Mobile phase

Mobile phase used for liquid chromatography typically are mixtures of organic solvents like acetonitrile (ACN), methanol (MeOH), tetrahydrofuran (THF) and water or aqueous buffers. Mixture of acetonitrile and water is the best initial choice for the mobile phase during method development. Retention can be preferably adjusted by changing mobile phase composition or solvent strength in RPC. A change in organic solvent type is often used to change peak spacing and improve resolution. Characteristics of mobile phases are; it is essential to establish that the drug is stable in the mobile phase; the mobile phase should have a pH 2.5 to 7.0 to maximize the lifetime of the column, mobile phase must be water soluble, low viscosity and uncreative in nature, excessive salt concentration should be avoided as high salt concentration can result in precipitation, and mobile phase must have low UV cut off and minimize the absorbance of buffer. The selection of solvents for this purpose is guided by solvent properties that are believed to affect selectivity, acidity, basicity and dipolarity. A retention range of 0.5 to 20 is allowable for sample to be separated using isocratic condition.

1.6.3 pH of buffer solution

pH is the important consideration in method development. Acidic compounds are retained at low pH while basic compounds are more retained at higher pH. The neutral compounds remain unaffected. The pH range 4-8 is not generally employed because slight change in pH in this range would result in a dramatic shift in retention. However, by operating at pH extremes (2-4 or 8-10), not only is there a 10-30 fold difference in retention that can be exploited in method development **[76,77].** Some acidic or basic samples undergo a change in absorbance as pH is varied and band spacing will occur. Method development can proceeds by investigating parameters of chromatographic separations first at low pH and then at higher pH until optimum results is achieved.

1.6.4 Column and column packing material

The column is the heart of liquid chromatography separation process. The availability of a stable, high-performance column is essential in developing a rugged,

reproducible method. Most columns for liquid chromatography method development use straight lengths of stainless steel tubing with highly polished interior walls. Stainless steel is useful with all organic solvents and most aqueous buffers. The HPLC column packed with stationary phase of C_{18}-bonded silica (C_{18} column) and C_8-bonded silica (C_8 column) is used in RP-HPLC separation of a wide range of organic compounds. The choice of common packing material and mobile phases depends on the physical properties of the drug. Reverse phase columns differ by the carbon chain length, degree of end capping and percent carbon loading. The use of silica-based packing is favoured in most of the present HPLC columns due to several physical characteristics. Silica substrates are available in spherical or irregular shapes and can be prepared with different surface areas, pore sizes and particle sizes, which make them suitable for most HPLC applications. Totally porous silica particles with 5 µm diameter provide the desired characteristics for most HPLC separations. Column having 3 µm particles means fast separation is going on. Column having 1.5 µm means very fast separation will take place.

Separation of many samples can be enhanced by selecting the right column temperature. Higher column temperature reduces system backpressure by decreasing mobile phase viscosity, which in turn allows use of longer columns with higher separation efficiency. However, an overall loss of resolution between mixture components in many samples occurs by increasing column temperature. The optimum temperature is dependent upon nature of the mixture components. The overall separation can be improved by simultaneous changes in column temperature and mobile phase composition **[78-80].** Plate number, peak asymmetry, selectivity, column back pressure, retention, bonded phase concentration and column stability are the column specifications. The separation selectivity for certain components vary between the columns of different manufacturer as well as between column production batches from the same manufacturer.

1.6.5 Selecting a detector

Various detectors such as UV/visible, photodiode array detector, fluorescence detector, conductivity detector, refractive index detector, electrochemical detector, mass spectrometer detector, evaporative light scattering detector. In most of the HPLC method development UV & PDA detector (200 to 400 nm) is generally used,

selection of detector is based on chemical nature of analytes and potential interference. Good analytical results will be obtained only by careful selection of the wavelength used for detection. The wavelength chosen for UV detection must provide acceptable absorbance. The mobile phase must transit sufficiently at the wavelength used for detection.

1.7 Analytical method validation

Analytical method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Validation is a procedure having of documental evidence to demonstrate the procedure (method/process) is able or not to produce the expected results under the stated experimental conditions. For this a number of definitions can imply but the final concept of the validation is to assure the fitness of the procedure for its intended purpose. Assurance of the procedure will be demonstrated by establishing the suitable parameters (nothing but experimental conditions) of validation. These parameters shall be selected with the consideration of intended application, pharmaceutical or industrial guidelines, conditions to be challenged, time, chemistry of the substances. In pharmaceutical industry the validation exercise is mainly applicable to process validation, analytical method validation), cleaning validation and instrument validation-DQ (design qualification)/IQ (installation qualification)/OQ (operation qualification)/PQ (performance qualification)/MQ (maintenance qualification), personal validation. Typical validation characteristics which should be considered are listed below.

1.7.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). Specificity can be evaluated with PDA detector by comparing collected spectrum across a peak. It indicates that homogeneity of peak and also evaluated using modern LC-MS/GC-MS techniques. Specificity is calculated and documented in a separation by the resolution, measuring of theoretical plates and tailing factor.

1.7.2 Precision and accuracy

The purpose of carrying out a determination is to obtain a valid estimate of a 'true' value. When one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first time to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important critical for judging analytical procedures by their results.

Precision: Precision refers to the reproducibility of measurement within a set, i.e., to the scatter of dispersion of a set about its central value. One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set. Standard deviation has the same units as the property being measured.

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

The square of standard deviation is called variance (S^2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e. S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision. Precision criteria for an assay method are that the instrument precision and the intra-assay precision, RSD will be ≤ 2 %.

% relative standard deviation = (standard deviation/mean) X 100

Accuracy: Accuracy normally refers to the difference between the mean of the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method. For assay methods, spiked samples are prepared in triplicate at three levels across a range of 50-150 % of the target concentration. The percent recovery should then be calculated. The accuracy criterion for an assay method is that the mean recovery will be 100 ± 2 % at each concentration across the range of 80 – 120 % of the target concentration.

Absolute method: The test for accuracy of the method is carried out by taking varying amounts of the constituents and proceeding according to specified instructions. The difference between the means of an adequate number of results and amount of constituent actually present, usually expressed as parts hundred (%) is termed as % error. The constituent in question will be determined in the presence of other substances, and it will therefore be necessary to know the effect of these upon the determination. This will require testing the influence of a large number of probable compounds in the chosen samples, each varying amounts. In a few instances, the accuracy of the method controlled by separations (usually solvent extraction or chromatography technique) involved.

Comparative method: In the analysis of pharmaceutical formulations (or solid laboratory prepared samples of desired composition), the content of the constituent sought (expressed as percent recovery) has been determined by two or more (proposed and official or reference) supposedly "accurate" methods of essentially different character can usually be accepted as indicating the absence of an appreciable determinate error.

1.7.3 Limit of detection (LOD)

The limit of detection of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Several approaches for determining the limit of detection are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable. Signal-to-noise (S/N) approach can only be applied to analytical procedures which exhibit baseline noise. The ICH has recommended a S/N ratio 10:1.

1.7.4 Limit of quantification (LOQ)

The limit of quantification of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable. Signal-to-noise

approach can only be applied to analytical procedures that exhibit baseline noise. Based on the standard deviation of the response and the slope, the limit of quantification (LOQ) may be expressed as LOQ = 10 σ/S, where σ=the standard deviation of the response, S = the slope of the calibration curve. For instrumental techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (S_a), which may be related to LOD and the slope of calibration curve, *b,* by

$$\mathbf{LOD = 3\ S_a / b} \qquad \mathbf{LOQ = 10\ S_a / b}$$

1.7.5 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The correlation coefficient (r), y-intercept (a), slope of the regression line (b) and residual sum of squares should be submitted. A plot of the data should be included. In addition, analysis is the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

1.7.6 Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

1.7.7 Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days etc.

1.7.8 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (eg., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used. Examples of typical variations are influence of variations of pH in a mobile phase, influence of variations in mobile phase composition, different columns (different lots), temperature and flow rate.

1.7.9 Selectivity of the method

The selectivity of the method is ascertained by studying the effect of a wide range of excipients and other additives usually present in the pharmaceutical formulations to be determined under optimum conditions. Initially, interference studies are carried out by the determination of fixed concentration of the drug several times by the optimum procedure in the presence of a suitable (1-100 fold) molar excess of the foreign compound under investigation and its effect on the absorbance of the solution is noticed. The foreign compound is considered to be interfering at these concentrations if it constantly produces an error of less than 3.0% in the absorbance produced in pure solution.

1.8 Statistical evaluation

1.8.1 Calibration

Calibration is one of the most important steps in bioactive compound analysis. A good precision and accuracy can only be obtained when a good calibration procedure is used. In instrumental methods, the concentration of a sample cannot be

measured directly, but is determined using another physical measuring quantity 'y' of a solution. An unambiguous empirical or theoretical relationship can be shown between this quantity and the concentration of analyte. The calibration between y = g(x) is directly useful and yields by inversion of the analytical calculation function. The calibration function can be obtained by fitting an adequate mathematical model through the experimental data. The most convenient calibration function is linear, goes through the origin and is applicable over a wide dynamic range. In practice, however, many deviations from this ideal calibration line may occur. For the majority of analytical techniques the analyst uses the calibration equation, $Y = a + bx$.

1.8.2 Linear regression analysis method of least squares

Least-squares regression analysis can be used to describe the relationship between response (y) and concentration (x). We adopt the convention that the x values relate to the controlled on independent variable (eg. the concentration of a standard) and the y values to the dependent variable (the response measurements). This means that the x values have no error. On the condition that the errors made in preparing the standards are significantly smaller than the measuring error. The values of the unknown parameters must be estimated in such a way that the model fits the experimental data points (x_i, y_i) as well as possible. The true relationship between x and y is considered to be given by a straight line. The signal y_i is composed of deterministic component predicted by linear model and a random component e_i. One must now find the estimates of a and b of the two values α and β. This can be done by calculating the values a and b for which e_i^2 is minimal. The component e_i represent the differences between the observed y_i values and the predicted y_i values by the model. The e_i are called the residuals, a and b are the intercept and slope respectively. The standard error on estimation is a measure of the difference between experimental and computed values of the dependent variable. Standard deviations on slopes (S_b) and intercepts (S_a) are quoted less frequently, even though they are used to evaluate proportional differences between or among methods as well as to compute the independent variables such as concentration etc. It is important to understand how uncertainties in the slope are influenced by the controllable properties of the data set such as the number and range of data points and also how properties of data sets can be designed to optimize the confidence in such data.

$$b = \frac{n\sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n\sum_{i=1}^{n} x_i^2 - \left[\sum_{i=1}^{n} x_i\right]^2} \qquad a = \frac{\sum_{i=1}^{n} y_i \sum_{i=1}^{n} x_i^2 - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} x_i y_i}{n\sum_{i=1}^{n} x_i^2 - \left[\sum_{i=1}^{n} x_i\right]^2}$$

1.8.3 Correlation coefficient (r)

To establish whether there is a linear relationship between two variables x_i and y_i, use Pearson correlation coefficient r. The value of r must lie between +1 and -1; the nearer it to ±1, the greater the probability that a definite linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1 indicate negative correlation. Value r of that towards zero indicate that x and y are not linearly related.

$$r = \frac{\left[\sum^{n}(x_i - \bar{x})(y_i - \bar{y})\right]/(n-1)}{\left[\sum^{n}(x_i - \bar{x})^2 (y_i - \bar{y})^2\right]/(n-1)^2}$$

1.9 Objective of the present investigation

The main objective of the present investigation is to develop stability indicating reverse phase high performance liquid chromatographic (RP-HPLC) method for the determination of everolimus (ERL), belinostat (BLT), ceritinib (CRB), saroglitazar (SAR), cobicistat (CBT), elvitegravir (EVT), ibrutinib (IBR) and dapoxetine hydrochloride (DAP) in pure and pharmaceutical formulations and identification and characterization of four impurities of azelnidipine (AZL). The study of forced degradation under stress conditions is also included in the investigation. In the present study, the author has developed and validated around eight reverse phase HPLC methods in all for the estimation of some chosen drugs in bulk samples and pharmaceutical formulations in dosage forms.

There are no analytical methods available in the literature for the determination of belinostat, saroglitazar and ibrutinib drugs. Hence, the author has attempted to develop stability indicating RP-HPLC method for their determination.

Chapter I includes the general introduction on HPLC and spectroscopic methods. The results of a stability indicating RP-HPLC method for the determination of everolimus (ERL) in pure and pharmaceutical formulations and forced degradation of ERL under various stress conditions are included in chapter II. Since, no stability indicating method has been reported individually for its determination so far, the developed method is validated with respect to specificity, precision, linearity and accuracy. Chapter-III incorporates the results of the developed HPLC method for the assay study of belinostat (BLT) API and tablet dosage form under forced degradation of BLT under stress conditions. The results of an isocratic HPLC method developed for the estimation of ceritinib (CRB) under forced degradation conditions at the 100% level are described in chapter-IV. The chromatographic assay is found to fulfil all the requirements to be identified as a reliable and feasible method including accuracy, specificity, linearity and precision data.

Chapter-V describes a rapid, accurate and precise stability indicating RP-HPLC method for the determination of saroglitazar (SAR) in bulk sample and pharmaceutical dosage form. The author developed the conditions for its sensitivity and studied the robustness, ruggedness and forced degradation of SAR. A new, simple, fast, accurate, efficient and reproducible reverse phase HPLC method was developed and validated for the estimation of cobicistat (CBT) in bulk samples and pharmaceutical formulations. The developed method was validated with respect to specificity, precision, linearity and accuracy and the results are included in chapter-VI. A simple, economic, rapid, precise, isocratic and accurate RP-HPLC method with good sensitivity were developed and validated for the estimation of elvitegravir (ELT), ibrutinib (IBR) and dapoxetine hydrochloride (DAP) in bulk and pharmaceutical dosage forms, with short retention time, according to ICH guidelines, and a detailed description of the methods are discussed in chapters-VII, VIII and IX respectively.

The author has also successfully identified and characterized the four impurities of azelnidipine (AZL) namely para impurity, azelnidipine intermediate, 4-nitro azelnidipine and 2-nitro azelnidipine and well separated by using UV, MS, IR and NMR spectroscopic methods and the results are included in chapter-X.

Even though, UV spectrophotometric methods are available for certain chosen drugs, no attempts have been made to develop conditions for the determination of belinostat, ceritinib and ibrutinib by UV spectrophotometrically. Since these methods do not use any reagents for the development of colour, it would be convenient to determine them without using any reagent in UV region. Hence, the author has attempted to develop conditions for UV spectrophotometric methods for the determination of BLT, CRB and IBR drugs. The detailed description of the methods are discussed in chapter-XI (A), (B) and (C).

Finally, the author has described the summary and conclusions of all the nine drugs used in the present investigation. The references which are used in general introduction, RP-HPLC methods for the assay of ERL, BLT, CRB, SAR,CBT, ELT, IBR and DAP, impurity profiling of AZL drug and UV spectrophotometric methods for the development of BLT, CRB and IBR drugs are given at the end of the thesis.