INTRODUCTION:

PHARMACEUTICAL INDUSTRY AND QUALITY MANAGEMENT OF FIXED DOSE COMBINATIONS.

Healthcare encompasses everything that prevents ill health and promotes well-being. Therefore, total living environment such as proper nutrition, potable water, adequate shelter, clothing, clean air, hygiene and sanitary facilities, medical care and medicines are all needed to promote good health. The role of pharmaceutical industry is limited to providing an important component to the total healthcare package namely the medicines of drugs. Over the years, the pharmaceutical industry has played a vital role in human battle against diseases, disability and sufferings at global level, besides serving the nation, the Indian Pharmaceutical industry has also made significant contributions in elevating the economy to the country; this has been feasible, primarily due to innovational inputs of research and development of leading pharmaceutical companies.

There has been spectacular monetary growth involved during the last two decades in the drug industry. The success in the utility of the pharmaceutical industry depends on safe and effective drug therapy which is possible only with strict observation of Good Manufacturing Practices (GMP) of drugs and careful monitoring of their active ingredients in the pharmaceutical formulations.

The pharmaceutical science has therefore become increasingly important and speedily advancing all over the world. More than one third of all new drugs introduced worldwide during last decade were fixed Dose Combination (FDC) preparation.

OBJECTIVES:

- Study to develop High performance thin layer chromatography method for the separation and determination of the combination drugs.
- To separate combination drug and determine it qualitative and quantative method.
- HPTLC method development for combination drug is very helpful because we get quick results and it is a less expensive method.
- HPTLC method will help industries for analysis of combination drugs.

WORK PLAN & METHODOLOGY
**METHODOLOGY**

- Selection of suitable detection wavelength
- Optimization of mobile phase composition
- Optimization of chromatographic condition
- To determine linearity range of combined drug
- By proposed method, perform analysis of combination drug in mixture form.
- To validate the developed methods by using different statistical parameters

**WORK PLAN**

- In first six months referencing will be completed
- In next three month bulk drug and sample collection takes place
- After that lab work will be completed within six month
- After paper publication thesis will be submitted

**DRUG CLASSIFICATION:**

1) According to structural unit

2) According to therapeutic action

On the basis of therapeutic action the drug can be classified as follows.

1) Functional or Pharmacodynamic agents:

These are drugs which act on various functions of the body, these drugs may further classified as follows:

a) Non selective CNS

b) Selective modifiers on CNS -tranquilizers

c) CNS stimulants-antidepressants

d) Cholinergic and anticholinergic agents

e) Adrenergic stimulants, blocking agents

f) Histamine and antihistamines agents

 g) Local anesthetics

 h) Cardiovascular agents
2) Chemotherapeutic agents are used in the treatment of the infectious diseases. The term chemotherapy means treatment of diseases due to parasitic microorganism by chemical substances which have specific toxicity for those microorganisms without causing injury to the host.

a) Organometalic compounds
b) Anthelmintic agents
c) Antimalarilas
d) Antiprotazoas
e) Antiseptics
f) Antifungal
g) Antibacterial
h) Antibacterial agents
i) Anti-tubercular and antileprosy drugs
j) Antineoplastic agents
k) Antibiotics

3) Vitamins

Vitamins are of following types:

1) Water soluble vitamins e.g. vitamin of group B and group C

2) Fat soluble vitamins: vitamin A, D, E and K.

3) In soluble in both fat and water: vitamin H Hormones

These are required in small quantities and synthesized by organism. They are responsible for the metabolism, growth and reproduction.

1) Thyroid hormones and antithyroid hormones

2) Pituitary and hypothalamic hormones

3) Pancreatic hormones
4) Hypoglycemic agents

5) Sex hormones

6) Adrenal cortex hormones

It is evident that pharmaceutical products have been enormous assets in therapy and prevention of human disease. It is perhaps worth knowing a few of this specific drug benefits according to a convenient classification curative, corrective (Pharmacodynamic) palliative, substitutive, preventive (Prophylactic), diagnostic, supportive and restorative types of the drugs.

**CURATIVE DRUGS**

Practically every drug that exerts a curative action is chemotherapeutic agent directed towered the treatment of infectious disease.

With the modern chemotherapeutic agent, infectious diseases are no longer the terrifying threat that they were in past. Bacterial septicemia and meningitis that were once considered to be 100% fatal can now be cured with regularity.

**CORRECTIVE (PHARMACODYNAMIC) DRUGS**

These groups of drugs acts directly on the body to help correct physiological or biochemical abnormalities. None is capable of curing a disease, but they can reverse pathological processes to the extent that patient can enjoy a long and productive life. The list of Pharmacodynamic agents includes general aesthetics, hypnotics, anticonvulsants, local anesthetics, and new muscular blocking agents, drugs for Parkinson’s antihypertensive agents, diuretics, and anti-inflammatory agents.

**PALLIATIVE DRUGS**

Palliative drugs are used to treat symptoms. They contribute to comfort of patients without carrying any biochemical and physiological abnormality. Some palliative drugs are used for relief of pain in minimal disorders while others block the pain of major disease such as cancer. Others may be used to inhibit a runny nose, a cough or to help alleviate congestion. For example Grosgrain acetate for Palliative treatment of advanced breast cancer in women.

**SUBSTITUTIVE DRUGS**

Substitutive drugs comprise natural or synthetic substances for the treatment of diseases
associated with their deviancy in the organism. Many of these are endocrine or hormonal substances. Many deficiency states can be treated endocrine or hormonal substances. Many deficiency states can be treated effectively to correct manifestations of the deficiency. E.g. Cirtucisteriuds.

RESTORATIVE DRUGS

Drugs to help the body to its normal health state are called restorative. These drugs are usually used during convalescence to aid nature in its reconstructive processes. The vitamins and minerals are common substances commonly used for this purpose.

SUPPORTIVE DRUGS

They are used to sustain the patient until other measures can be instituted which will either cure or alleviate the conditions. The tranquilizers, vitamins and antibiotics are examples.

The human body’s natural recuperative powers will overcome most illness, but with help they will do the job effectively and the less time than alone.

DIAGNOSTIC DRUGS

Drugs used to aid the physicians in deciding what is causing the patients symptoms are known as diagnostic drugs. These drugs not only help the doctors to determine the reason for the patient’s illness, but are also benefit in locating the exact cure of the body affected 66.

SOURCES OF DRUGS:- The various sources of drugs are as:

Minerals: e.g. Magnesium sulphate Animal: e.g. Insulin, Heparin Plant: e.g. Morphine, Quinine Synthetic: e.g. Aspirin Micro-organism: e.g. Penicillin and other antibiotics General engineering: e.g. Human insulin and human growth hormone genes, majority of drugs currently used in therapeutic are synthetic drugs.

CHOICE OF THE METHOD DEPEND UP ON THE

I. Availability of the instrument (s)
II. Experience of analyst in using particular instrument
III. Expected concentration range
IV. Required accuracy and precision
V. Potentional interference

VI. Time and expenses required for number of samples to analyzed.

HPTLC Sample Applicator

**Volumetric methods:**

This method is used to determine unknown concentration of an known analyte. Solution which is present in burette is called as titrant and solution which is present in conical flask is called as titrand. Concentration of titrant is known is called as standard solution. For example HCl Vs NaOH (strong acid Vs strong base). In above case, HCl is titrand and NaOH is titrant. Phenolphthalein is used as indicator in this titration.

1) **Gasometric method:**

Gas phase is measured in this method. Gas phase is measured at definite pressure and temperature. For example analysis of carbon dioxide.

2) **Titrimetric method:**

In this method the volume of solution of known concentration required to interact with desired constituent or with another substance chemically equivalent to it is measured. This is achieved by making use of number of types of well known reactions a few of them can be summarized as:

3) **Neutralization reaction or acidimetric and alkalimetry**

This includes titration of free base or those formed from salt of weak acids or those formed by the hydrolysis of salt of weak base with a standard base. The reaction involves combination of hydrogen and hydroxide ions to form water .e.g. USP
Describes acidimetric assay of amminophylline, caffeine and sodium benzoate injection, etc. Alkali metric assay of -Busulfalin, benzoic acid and salicylic acid ointment etc.

4) **Complex formation reactions:**

These reactions are dependent on the composition of ions other than hydrogen and hydroxide to form a soluble slightly dissociated ion or compound e.g. USP describes a complexmetric assay of calcium gluconate, zinc oxide suspension, aluminum and magnesium oral suspension and calcium laevulin injection etc.

5) **Precipitation reaction:**

Insoluble metal salt is called as a precipitate and the reaction is called as precipitation reaction. For example if silver nitrate is treated with chloride ions it gives AgCl which is precipitate. These depend on the combination of ions to form a precipitate e.g. US describes precipitation titration of Acetochloine chloride, Amminophylline, Benzyl hexachloride.

6) **Oxidation - reduction reaction:**

Under the heading the reaction involves the change of the oxidation number or transfer of electrons amongst the reacting species is included. The standard solutions are either oxidizing or reducing agents e.g. USP describes titration of ferrous fumarate, ascorbic acid, ferrous sulphate, and Meclorethamine hydrochloride. In all these reactions end point is detected generally by change in colors given by the indicator used. Similarly there is another class of titrimetric reactions via non aqueous titrimetric method. In these methods dry organic solvents are used for the titration purpose. These methods are considerably accurate precise and widely applicable e.g. assay of chlorcyliazine. Titrimetric methods need simpler apparatus and are quickly performed. Tedious and difficult separation are avoided these factors are responsible for the wide use of titrimetric analysis in drug analysis even in an era of modern technique.

C) **Chromatography:**

It has taken major strides and plays important role in Quantitative analysis Ion exchange chromatography, liquid-liquid extraction, paper chromatography find extensive use in industry for drug analysis HPTLC, HPLC and GC is one of breakthrough in the field of chromatography, other Instrumental methods like UV, NMR, IR, Mass spectroscopy X ray diffraction colorimetry have proved very useful and effective in drug analysis.
Advances in column technology high pressure pumping system and detectors have transformed liquid chromatography in a high speed, highly efficient method of separation. This method is termed as high performance liquid chromatography.

HPLC basically consist of high pressure pump, injector, chromatographic column, detector, amplifier, and recorder. High pressure pumping system delivers the mobile phase solvent from the solvent reservoir to the column through pressure tubing and fittings.

The columns usually have small internal diameter. They may be heated to give efficient separation but rarely heating above 60°C is required because of potential difficulties from arising stationary phase durations or mobile phase volatility at higher temperature.

On type consist of packing of microparticlals about 30 µm in diameter and having a solid center and thin porous crust. Some of these peculiar supports can be preactivated to give them adsorptive properties and other types can be coated with a chemically bonded stationary phase.

**Gas chromatography**

Gas Chromatography (G.C) or Gas liquid chromatography (GLC) is used for the separation and analysis of compounds those changed into vapor without any decomposition in analytical chemistry. G.C is used for testing the purity of a particular substance. It helps to separate the different components from the mixture. It also helps to quantify those components too. G.C has the capacity to identify the compounds. In preparative chromatography, the pure compound can be prepared with the help of G.C.

Separation of volatile substance is done by this technique. In this case mobile phase is gas and stationary phase is liquid or solid. If stationary phase is liquid then it is called as Gas-Liquid and if it is solid then it is called as Gas- solid Chromatography. Silica gel is used for column packing. The development of conventional TLC into modern instrumental technique started with introductions of so called HPTLC in 1974, the speed of migration of a liquid flowing in TLC layer by capillary action decreases with the square of the distance. Accordingly, on HPTLC layers due to their shorter optimum separation distance, chromatography on HPTLC layers take place in the fast capillary flow range of the respective developing solvent. This accounts for practically all advantages of HPTLC over conventional TLC. HPTLC in short may be
described as a separation technique where separation is achieved by employing the solute molecules on a precoated plate with the automatic device (Linomat IV), separating them (in AMD) and evaluating quantitatively by densitometry (scanner II) from others. Good separation reduces selectivity problems and permits the use of maximum detector sensitivity with minimum fear of interferences. The normal chemical or instrumental methods cannot however readily distinguish mixtures and pure materials. Therefore in analysis of combination preparations, the applicability of these instrumental methods, chromatographic technique deserve special recognition as they are capable of separating the sample into various components prior to their estimation. Because of this interest and the nature of the chromatographic methods they invariably turnout to be the referee methods in the case of drug analysis. They attained incredible prominence in very small time. The commendable and acceptable features of these techniques which triggered this explosive growth includes high resolving power, fast synthesis, continuous monitoring and simplicity of operation, precise identification based on accurate quantitative measurement, repetitive analysis automation of complete analysis and data handling operations. In the field of pharmaceuticals, these techniques have been adopted to monitor the control of synthesis, isolation of natural compound, control of microbiological processes, study of metabolism of drugs, stability testing, routine quality control of the materials, exponents and formulation.

Literature survey however, shows very meager information as regards the determination of FDC sincere attempts have therefore, been undertaken by us to develop analytical methods using chromatographic techniques such as HPLTC for simultaneous evolution of some combination drugs from pharmaceutical preparations. The work in this thesis involves HPTLC determination of some multiaction combination drugs in formulations. Therefore, HPTLC technique has been discussed in detail in the preceding paragraphs.

**Column Chromatography:**

The method used for the purification of a compound from the mixture of compounds in chemistry by column chromatography. This method is used for the preparative application to developed microgram to kilogram separation of the compound. There are various type of glass column with a diameter of 50mm and height of 50cm to 1m columns are used in preparative chromatography. We generally used two methods to
prepare a column and those are wet method and dry method for packing the preparative column. In this chromatography method, compounds are isolated by using different stationary phases and by the use of different eluents and flow of the mobile phase. In this process the mixture of the compounds separated one by one with their polarity and solubility. In this chromatographic method the eluent comes out through the column, we collect that each volumes, automatically or manually for the checking of the purity of that volume in analytical HPLC and subject to get solid for the characterization of that required molecules.

Features of this method are:

- It is a system with low delay volume optimized for high purity, recovery and desired throughput.
- Delay volume calibration is very easy and very fast system set up automatically.
- Only the desired fractions collected based on time, peak and/or masses or manually.
- Recovery collection ensures that the costly sample can never be wasted.
- Software solution helps us to configure our own purification system.
- All the equipments, column, supplied and supported by a single vendor.
- It has mass based fraction collector with universal detection capabilities.
- CAN network for data processing is system intelligence in the time for precise and instantaneous fraction collection.
- It is very safe in handling because it has the detection technology of leak handling and forced fume extraction of the solvents.

**Flash Chromatography:** The purification of organic compounds done by distillation, extraction and re-crystallization technique. In this modern time we use flash chromatography to separate the organic compounds in a shorter time. In the conventional column chromatography the sample purified by putting the sample in the top of the column containing some solid support, generally silica gel, after that solvent put into the column and solvent runs under the help of gravity to separate the organic compounds. When the organic compounds travelled through the column it separated and collected separately in the bottom of the column. But this technique is very slow and time consuming. Therefore the scientist prepare an automated machine to minimize the time and that machine is named as Flash chromatography. This helps
to increase the flow of the solvent by using air pressure and contained a modern
detector for the identification of different peaks. It decreases the time and helps to get
the good purity products.

The medium pressure chromatography is known as Flash chromatography. It was
first popularized by Clark Still of Columbia University. It is an alternative method for
the slow and often inefficient gravity led chromatography. The difference between
Flash chromatography and conventional techniques are:

- Flash chromatography uses slightly smaller silica gel particles (250-400mesh).
- Flow of the solvent regularized by the pressurized gas of pressure
  50Psi through the column of stationary phase.

Solvent system can be selected through the TLC of 0.15 to 0.20 Rf values of the
compounds. One solvent of higher polarity than the other, usually the best in the
Binary solvent system. The polarity of the solvent system is determined by the ratio of
the solvents and that’s why the compounds are properly separated. The binary solvent
system with increasing polarity is hexane/ ethyl acetate, dichloromethane/ methanol,
ether/ hexane, dichloromethane/hexane. In my research work, I have used the
Teledyne Combiflash Chromatography.

**Thin Layer Chromatography:**

To isolate a combination of the molecules, a chromatographic technique is used and
that is known as Thin Layer Chromatograph (TLC). Thin Layer Chromatography is
done on a glass, plastic or aluminum sheet, and these are coated with thin layer of
adsorbent materials. The adsorbent materials are aluminum oxide, silica gel or
cellulose. This layer given upon the sheet is known as stationary phase.

Sample put up on the plate, a solvent or solvent mixture known as the mobile phase, is
drawn up the plate with the help of capillary action. Different types of analytes run
through the TLC plate at different rates and therefore separation is obtained.

Thin layer chromatography has various types of applications for the determination of
components that are contained in the plants. TLC also helps us to monitor organic
reactions. The many more to monitor for detection and analyze as ceramides, fatty
acids, Pesticides or insecticides in food and water. TLC also helps to analyze dye
composition of fibers in forensic and compounds present in a given substance. It helps
to analyze radiochemical purity of radiopharmaceuticals. An automated method is developed to quantify accurately the different compounds with the help of TLC and this method is called HPTLC or High pressure thin layer chromatography.

**HPTLC**

One type of column chromatography is known as high performance liquid chromatography or high pressure liquid chromatography and it is used for identification, separation and quantification depending upon their polarities and the interaction with the stationary phases of the column, in biochemistry and analytical chemistry. HPLC contains of one pump that helps to move the mobile phases and analytes through the column. HPLC uses different types of stationary phases as hydrophobic, saturated carbon chain and a detector for the finding of retention time of the analytes. The detector provides spectroscopic data of the analytes. The flow rates of the mobile phase, the different ratios of the solvents and the interaction strength with the stationary phase, changes the retention time of the analytes. The low level of impurities and for the proper identification of those impurities, it is not preferable to check in thin layer chromatography. Some of the pharmaceutical products can be analyzed with this technique and those are indoramine, tolnaftate, ifosamide, carmustine, amiodarone and bromazepam. The identification of epoxy alkaloids from Datura stramonium isolation is also done by TLC and HPTLC methods. Sulfur and nitrogen mustards residue can be determined with the help of TLC. The degradation products of rifampicin such as hydrazones, rifampicine quinine can be identified by the high performance thin layer chromatographic technique. Hence TLC and HPTLC are used for the regular quality control and the analysis of stability of the pharmaceutical products and this technique is used in the developing world.

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K_m = \frac{[S]}{[X]}
\]

In case of HPTLC, separation of solutes takes place by a dynamic differential migration process in a system. Cost per analysis is very low as compared to other methods. The distribution equilibrium is described by the distribution coefficient and Where X is the concentration of the component of the stationary phase at equilibrium and \(xm\) is its concentration in the mobile phase.
HPTLC method development takes place in twin through chambers. Horizontal development chambers are also used in method development. Twin through chambers are made in such a way that it requires very low volume of proposed mobile phase or different conditioning liquid. In method development vapors or gas is produced which Affects the result of the mobile phase development. The gas phase depends on the saturation condition of the mobile phase and also depends on the size and type of twin through chamber used in method development. Unsaturated chambers are avoided during mobile phase development because it may affect the reproducibility of the result. Two dimensional methods are used for the complex separation in which sample mixture is injected on the lower side of the HPTLC plate. Plate is developed with first and second mobile phase step by step.

Selection and optimization of mobile phase depends on analyst’s own experience. Literature review about particular drug is important for selection and optimization of mobile phase, also trial and error method is important. Mostly diethyl ether, methylene chloride, chloroform are used with hexane in combined, together in case of normal phase. Selection of mobile phase is also depends on nature of drug means physical properties of drug. If water is used with methanol then method is considered as Reverse phase method. Lab. Of CAMAG suggested four steps for selection and optimization of mobile phase. In first step use of seven or twelve solvents take place and then proper solvent is selected for drug which separates that drug powerfully. After this solvents are decided on the basis of Rf values they produce. The difference between two drugs or Rf values must be less than 0.5. Solvents are classified according to their Rf values as:

1. Group A solvents------- $0.2 < \text{Rf} < 0.8$
2. Group B solvents------- $\text{Rf} > 0.8$
3. Group C solvents------- $\text{Rf} < 0.2$

After this, hexane or water is used to decrease or increase Rf value. Strength of the B group solvent is decreased by solvent which is weak in strength solvents i.e. hexane or cyclohexane. If that solvent runs below the solvent front then the solvent is diluted first, then 1:4 ratio is used for trial. If samples have higher Rf values but they get separated then 1:1 ratio is used for trial. Acid is used to increase the strength of the solvents. These are called as Polar modifier. Generally acetic acid or formic acid are used as a modifier. Sometimes bases are also used as modifiers. Generally diethyl
amine or ammonia is used as a base modifier. There are used to avoid tailing in the method development. First used 1:1 ratio, if result is improved and if result is not better then use another ratio of the solvent. First avoid combination because it disturbs the resolution. Try one combination at a time. If problem get solved then quit the procedure. If not then try another method. Solvent from group B or group C are combined at first level. At third level, 1:1 ratio is good but addition of 10% of the B group C solvent. If Rf value is satisfactory then go with fourth level. In fourth level, minor adjustment takes place. In fourth level, acid or base is used to avoid tailing in Rf values. If analytical results are not satisfactory then try with different stationary phases. For multi component drugs, mobile phase is prepared in a small bottle with lid to small amount of solvent is used.

**Supercritical Fluid Chromatography:**

Supercritical fluid chromatography (SFC) is used for the normal phase chromatography. It is very helpful for the purification and analysis of small to reasonable molecular mass. This method also used in those materias those are not stable thermally. It is also used for the isolation of sterio molecules. SFC method is also similar to HPLC. In this method one of the mobile phases is carbon dioxide and therefore the total system in the chromatographic flow path should be pressurized.

**HPLC PROCEDURES:**

The genotoxic impurities of non-volatile are analyzed by HPLC isolation procedure. Reverse phase HPLC (RPLC) is used mainly for the isolation of the impurities. The isocratic RPLC procedure is being utilized for the identification of four genotoxic alkyl benzene sulfonates (ABSs). Amlodipine besylate contains methyl, n-propyl, ethyl and isopropyl benzene sulfonates can be identified with the help of isocratic RPLC method. The sulfonate impurities containing phenyl group is also checked with the help of RPLC. The Tosylates moity containing ethyl, methyl and isopropyl functional groups and same functional groups within the besylates will be identified by RPLC technique. Epoxides and hydroxides are checked using HPLC and some of the impurities are checked in RPLC too. For the analysis of different genotoxic impurities of the APIs, HPLC with PDA detector and ELSD (Evaporative Light Scattering Detection) are used. Alkylating impurities are analyzed by RPLC and MS detection. HPLC/ UV technique is also very effective for the checking of alkylating
impurities. The polar molecules and the small molecules are identified by Hydrophilic Interaction Liquid Chromatography (HILC) and obtained a good isolation with retention time. The polar molecules are analyzed with the HILC method with good retention that is more efficient than the RPLC column. Hydrazine groups are checked by the HILC technique and HPLC/ UV and HPLC/ MS methods are also used.

**G.C Method:**

Many volatile small compounds of genotoxic impurities are analyzed by the use of G.C technique. Liquid injection technique and the headspace sampling technique are used for the analysis in G.C. In the time of liquid injection a large volume of non-volatile API attached in the injector or on the column head of the G.C column and because of it this method reduces the quality of the analysis. But in the case of head space injection, it reduces the contamination in the column or injections, because the APIs are not attached with it and hence this technique increases the quality of the analysis.

**TLC/ HPTLC Methods:**

The low level of impurities and for the proper identification of those impurities, it is not preferable to check in thin layer chromatography. Some of the pharmaceutical products can be analyzed with this technique and those are indoramine, tolnaftate, ifosamide, carmustine, amiodarone and bromazepam. The identification of epoxy alkaloids from Datura stramonium isolation is also done by TLC and HPTLC methods. Sulfur and nitrogen mustards residue can be determined with the help of TLC. The degradation products of rifampicin such as hydrazones, rifampicine quinine can be identified by the high performance thin layer chromatographic technique. Hence TLC and HPTLC are used for the regular quality control and the analysis of stability of the pharmaceutical products and this technique is used in the developing world.

**Different modes of HPTLC Separation**

On the basis of chromatographic separation mechanism there types can be classified as:

1) Adsorption

2) Partition

3) Ion exchange.
1) **Adsorption Chromatography**

Adsorption is a surface phenomenon. In adsorption chromatography mobile phase is liquid or gas and stationary phase is solid. In this case, solute gets adsorbed on the surface of the stationary phase.

![Adsorption chromatography](image1.png)

2) **Partition Chromatography**

In Partition on silica gel stationary phase is absorbed. On the surface of the solid support thin film is formed. Liquid is stationary phase which is adsorbed on the surface of the silica gel.

![Partition chromatography](image2.png)

3) **Ion-exchange**

In this case, ions are replaced by ions of like charges in the sample or mobile phase. Electrolyte solution acts as a mobile phase. There are two types of ions:

i) **Anions**

ii) **Cations**

Also there are two types of resins used in ion exchange chromatography as:

i) **Anion exchange resin**

ii) **Cation exchange resin**

**Capacity factor.**

The capacity factor $K$ is defined as $K = \frac{T_r - T_m}{T_m}$
Where $Tr =$ the retention time of the component.

$Tm =$ Retention time of the non retained component (mobile phase)

Resolution

The resolution of a separating stationary phase provides a Quantative measure of the ability to its separate two analyte. Resolution is defined as

$$R = \frac{2(Tr_2-Tr_1)}{W_1+W_2}$$

Where $Tr_1$ and $Tr_2$ are the retention time of the two components and $W_1$ and $W_2$ are the corresponding width of the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the base line.

Separation Factor or selectivity

Selectivity $C$ describes how well a chromatographic system can separate two components and it is defined as $= \frac{Tr_2 - tm}{Tr_1 - tm}$ Thus the resolution for two peaks can be increased by increasing $K$ or by increasing the number of the theoretical plate’s $N$ depending on the purpose of the separation of (quantification and isolation) and the ease with which the change affected.  

Detection of Mobile Phase:

Detection of mobile phase required one’s own experience and Trail & error method. In case of normal phase, stationary phase is polar and mobile phase is non polar that’s why non polar compounds eluted first and polar compounds retained. The reason is lower affinity of non polar phase with stationary polar phase. In case of Reverse phase,
Stationary phase is non polar and mobile phase is polar that’s why polar compounds eluted first and non polar compounds retained. The reason is lower affinity of polar compounds with stationary non polar phase.

**HPLC system:**

The HPLC consists of pumps, an injector, column, mobile phase reservoir, oven, and detector. The injector introduces the sample into the HPLC system. This is either done by hand with a syringe, or automated with an auto-sampler. Figure 2.1 shows a simplified schematic of an HPLC system. There are several different types of pumps available for use with HPLC. They include reciprocating pumps, which are the most common, syringe type pumps, and constant pressure pumps. The reciprocating pumps use a motor-driven piston to pump mobile phase into the column. On the backstroke, mobile phase is pulled in, and on the forward stroke, it is driven out to the column. These have the advantage of being able to achieve a wide range of flow rates. Dual and triple head pumps consist of identical units, which are 120 or 180 degrees out of phase. This type of pump system is significantly smoother. Syringe type pumps, or displacement pumps, have a very small capacity, and are therefore most suited to small bore columns. They consist of a large syringe type reservoir, with a plunger that is activated by a motorized lead screw. The flow rate can be controlled by changing the voltage on the motor.

**Instrument used in HPTLC**

Instrumentation used in HPTLC can be divided into following main parts.

1) Sorbent and silica layers.
2) Sample applicators.
3) Developing chambers.
4) Visualization method.
5) Detectors

1) **The sorbents used in TLC layers**

There are different 25 materials are used as a sorbent in thin layer chromatography. Different solvent have different specific range of application. (E.g. silica gel impregnated with caffeine for polyaromatic hydrocarbons) silica gel and
alumina are split into a number of sorbent depending on the pore size particle size and pH. Selection of sorbent depends on the nature of compounds. Characteristics such as polarity, solubility, insolubility, molecular weight, shape and size of the analyte are important in deciding on the separation mechanism and hence largely define both the type of the sorbent and the solvent used for the preparation of the sample and in development, following are the examples, Silica gel - derivatives food dyes (acidic and basic) carbohydrates. Kieselguhr - Carbohydrates, herbicides, tetracycline. Polyamides - Phenols flavonoids, nitro-compounds. Amino bonded silica gel - Particularly for carbohydrates, sulphonic acids, phenols, carboxylic acids, nucleotides, and nucleosides. Cyano- bonded silica gel - For pesticides steroids and preservatives. Diol based silica gel - For steroids and hormones. Reverse phase (RP-2, RP-8, and RP 18) silica gel for steroids tetracycline’s phthalates, antioxidants lipids barbiturates, aminophenol and fatty acids. Chiral modified silica gel - Enantiomers for amino acids halogenated N alkyl and methyl amino acids and hydroxyl carboxylic acids. Silica gel impregnated with silver nitrate- Lipids and variation in unsaturation and geometric isomers. Silica gel impregnated with caffeine- for polyaromatic hydrocarbons. Silica gel impregnated with boric acids / phosphate- for carbohydrates.

2) Sample applicator:

Adequate sample preparation and careful application of the sample to the HPTLC plate is imperative for good chromatographic separation. Application to the sorbent layer can be performed manually with very simple equipment or automated using sophisticated instrumental methods. As a general rule the more precise the positioning of the sample delivery and delivery rate the more reliable will be the final chromatographic result e.g. manual and instrumental methods.

a) Manual method:

In manual methods before sample is to be applied to the plate the origin point should be marked in some way so that migration distance can be determined after development. The method of application of the sample solution on the plate by using a standard glass capillary of set volume. A capillary of 1-2 uL will give a spot of size of approximately 3-4 mm, calibrated glass capillaries. The micro syringe allow a variable volume to be applied, with all the applicators the capillaries or syringe
needle that a flat ended is used.

Hamiltonian Syringe

b) Instrumental method:

In manual instrumental technique the sample can be introduced to the layer surface at precisely the location desired in one smooth dosage of the required volume and with minimal or no damage to the layer e.g. Nanomat 4 with capillary holder.

With this instrument capillary sizes from 0.5-5 uL can be used and spots can be applied to all sizes of HPTLC plates. The capillary holder in the position by a permanent magnet to dispense the content of the capillary the applicator press down, the pipette touches the layer surface at constant pressure and the pipette is discharged. Variable volume dosage units have also been used with this device. The volume dispense are 50-230 nL, also for spot application to HPTLC a fixed volume nano-pipettes are used are made from a reagent resistant platinum-iridium alloy fixed in sealed glass holder. 

Automated techniques

Automated technique can also be used to apply the sample solution spot or bands. The equipment can vary from semi-automated to a complete robotic system, controlled by a pre-set on a computer in automated band application equipment provides a very even narrow application of sample as the sample syringe passes over a pre-determined length on TLC plate at constant speed delivering a pre-set quantity of the sample by a spray in technique. A constant flow of gas ensures that the very thin application of zone is possible and the band is dried quickly when the volatile solvent is used. In this way the surface of TLC never comes in contact with the syringe. Such an automated application 1-20 uL result in extremely thin line with little diffusion on the other side. The equipment can best be described as semi-automated as the syringe is cleaned and refilled with each sample solution manually. For fully automated system program of
sample application can be stored on a personal computer both spot and band
application can programmed with detail number, size and position of application .e.g.
Camag Linomat 5 .The spots can be applied either by this technique or by direct
contact transfer. The samples are prepared in vials with septum seals . According
to the pre set program the robotic arm will move from vial to vial on removing
sample solution to syringe and delivering it on the chromatographic plate at the
predetermined point. Between sample application the robotic arm move the syringe to
a bottle containing an appropriate wash solvent after several flushes the syringe is
retuned to the next sample vial.

Contact spotting technique

In order to avoid damaging layers with capillaries and at the same time to apply
large volumes as 50 uL as sample spots to the TLC contact spotting can be used.
In this technique the sample is placed in depression or dimple in fluorocarbon polymer
film that has been pretreated with a coating of perflurokerosene (or similar
perflurinated fluid). A symmetrical droplet for in the depression (the depression
is large enough to accommodate at least 50 uL of sample solution.) With the
apparatus covered a gentle stream of nitrogen is passed over the surface
evaporate the solve
After complete evaporation a TLC is placed side down the
sample position and clamped . Nitrogen pressure is applied approximately 1.5 atm. to
transfer the sample to the sorbent layer.

3) Developing chambers.

There is variety of different types of chambers each designed with particular feature
to control to greater lesser extent parameters of the chromatographic development
reproducibility. As a solvent vapors saturation sorbent vapors adsorbed, solvent fumes
remixing and solvent front and edge effect on the chromatographic layer can behave
a bearing on the separation achieved. It is important to eliminate unwanted effects
and to utilize those features that will improve resolution the following types of
HPTLC chambers are,

Nu-chamber (normal flat bottomed, vapor unsaturated glass tank)

Nu-chamber (normal flat bottomed, vapor saturated glass tank)

Twin trough chamber (two compartment tank, saturated or unsaturated) Su-chamber
(sandwich tank unsaturated)
Ss-chamber (sandwich tank unsaturated)

Horizontal chamber (Twin development saturated or unsaturated) U- Chamber (circular HPTLC saturated or unsaturated)

Automated development chamber (ADC) fully environmentally controlled unit)

Forced flow development chamber (OPLC) (TLC development under pressure)

Vario chambers (saturated or unsaturated development using six different mobile phases on one sorbent layer.)

4) Development techniques.

In HPTLC development techniques are very powerful techniques focusing separated zones often improving resolution and spot/band capacity many fold. Multifold development can split into several types. Single mobile phase multiple and continuous development

Two dimensional developments

In this method the first development is allowed to proceed until the mobile phase has migrated to a predetermined point on the sorbent layer. The plate is then removed, dried and re-introduced into the same solvent mixture. Development is again allowed to proceed until the mobile phase has migrated the same distance. The process can be repeated until the sample component have migrated sufficiently to be resolved.

Manual gradient development.

This method is used where the sample contains both polar and non polar analyte. In this method a strong solvent is chosen and allow to migrate halfway up the plate. This solvent is polar enough to cause some migration of the most polar components and for the non polar components to travel with the solvent front. Development is repeated after drying after drying with but this time the solvent front is allowed to migrate entire separation length. The substance separated on the first run remains almost immobile whilst those on the development those on the solvent front now separate on the upper part of the plate. In it usual form gradient development involves a stepwise development but the solvent front is only allowed to travel a short distance. After drying the process is repeated with slightly less polar solvent, allowing solvent front to travel slightly further. The process is repeated 10 to 20 times
reducing the polarity of solvent each time until satisfactory separation is achieved.

**Automated multiple development (AMD)**

As the name suggest AMD is based on the same principle as manual multiple development. In AMD chromatogram is the result of the several automated chromatographic runs. In most cases the number varies between 10 to 40. The migration distance of the solvent front in each run is progressively longer by a constant increment. Typical increment is from 1 to 3 mm. The distance is programmed by computer control instrument. On each solvent run is completed the developing tank is drained and the HPTLC plate is dried by vacuum system. Before the next run begins as solvent is pumped in to the chamber to pre condition the plate.

4) **Detection and visualization**

In this case, UV chamber is used for detection of spots, if UV chamber is not available then destructive technique is used i.e. iodine or ammonia vapor is used.

**Visible detection**

Few compounds are clearly seen in visible light and do not require any further treatment for visualization e.g. Natural and Synthetic dyes.

**Ultra -violet detection**

Separated chromatographic zones on HPTLC layer may appear colorless in normal light but can absorb electromagnetic radiation at shorter wavelength.

These compounds are detected in the range of 200-400 nm. Most commercially UV lamps and cabinets functions at these wavelength.

**Iodine vapor**

This technique is also used for detection of spot but it is very old and destructive technique.

**Fluorescent Dyes:**

These are commonly used for the non destructive detection of lipophilic substances. These are the universal regents used for the detection for the organic compounds e.g. fluorescein, dichlorofluorescein, eosin, rhodamine B, and 6G, berberine and pinacryptol yellow. These used in lipids, purines primidines,
unsaturated compounds, saturated and unsaturated lipids, fatty acids phenols, polyphenols.

HPTLC METHOD DEVELOPMENT

pH indicators

Acidic or basic nature of substances are detected by pH indicators. The primary indicators are used are sulfonthalein. These indicators are used for the mostly for organic acids.

Destructive techniques

Chemical reaction occurring on the chromatographic layer between a reagent and separated analyte that result in a dramatization or in a total change in organic species could be described as destructive.

Thermal activation

In thermal activation developed zones on the TLC or HPTLC plate are heated at high temperatures fluoresce to UV light. In this technique the separation of the moderately polar aminopropyl-bonded silica gel layers have been give the consistent and sensitive result. It is used in detection of catecholamine, fruit acids, and some carbohydrates.
Quantification and video imaging

The separated analyte on the thin layer chromatogram can be quantified in a following ways. It measure the concentration of the chromatographic zones on the developed TLC /HPTLC plate .In this tech the beam of electromagnetic radiation of pre set wavelength (usually UV visible) that moves at pre determined rate across the chromatographic zones or the beam remain stationary TLC/ HPTLC plate moves, It can be scanned a variety of wavelength .In this the scanning the developed tracks result in chromatograms that are very similar to HPLC in Video imaging in this the storage of all the chromatograms on the TLC plate as one computer file the data can be quantified77.

Following Tables are used to detect mobile phase as:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>formula</th>
<th>density (g/mL)</th>
<th>Solubility in H₂O¹ (g/100g)</th>
<th>relative polarity²</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetic acid</td>
<td>C₂H₄O₂</td>
<td>1.049</td>
<td>M</td>
<td>0.648</td>
</tr>
<tr>
<td>acetone</td>
<td>C₃H₆O</td>
<td>0.786</td>
<td>M</td>
<td>0.355</td>
</tr>
<tr>
<td>acetonitrile</td>
<td>C₂H₃N</td>
<td>0.786</td>
<td>M</td>
<td>0.460</td>
</tr>
<tr>
<td>acetyl acetone</td>
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<td>0.975</td>
<td>16</td>
<td>0.571</td>
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<tr>
<td>2-aminoethanol</td>
<td>C₂H₅NO</td>
<td>1.018</td>
<td>M</td>
<td>0.651</td>
</tr>
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<td>aniline</td>
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<td>1.022</td>
<td>3.4</td>
<td>0.420</td>
</tr>
<tr>
<td>anisole</td>
<td>C₇H₈O</td>
<td>0.996</td>
<td>0.10</td>
<td>0.198</td>
</tr>
<tr>
<td>benzene</td>
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<td>0.879</td>
<td>0.18</td>
<td>0.111</td>
</tr>
<tr>
<td>benzonitrile</td>
<td>C₇H₈N</td>
<td>0.996</td>
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<td>0.333</td>
</tr>
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<tr>
<td>i-butanol</td>
<td>C₄H₁₀O</td>
<td>0.803</td>
<td>8.5</td>
<td>0.552</td>
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<tr>
<td>2-butanone</td>
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<td>0.805</td>
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<td>Compound</td>
<td>Molecular Formula</td>
<td>Density (g/mL)</td>
<td>Viscosity (cP)</td>
<td>Refractive Index</td>
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<tr>
<td>------------------------------</td>
<td>-------------------</td>
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<td>----------------</td>
<td>-----------------</td>
</tr>
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<td>0.786</td>
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<td>0.389</td>
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<td>0.188</td>
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<tr>
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<td>0.006</td>
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<td>cyclohexanol</td>
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<td>0.509</td>
</tr>
<tr>
<td>cyclohexanone</td>
<td>C₆H₁₀O</td>
<td>0.948</td>
<td>2.3</td>
<td>0.281</td>
</tr>
<tr>
<td>di-n-butylphthalate</td>
<td>C₁₆H₂₂O₄</td>
<td>1.049</td>
<td>0.0011</td>
<td>0.272</td>
</tr>
<tr>
<td>1,1-dichloroethane</td>
<td>C₂H₄Cl₂</td>
<td>1.176</td>
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<td>0.269</td>
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<tr>
<td>diethylene glycol</td>
<td>C₄H₁₀O₃</td>
<td>1.118</td>
<td>M</td>
<td>0.713</td>
</tr>
<tr>
<td>diglyme</td>
<td>C₆H₁₄O₃</td>
<td>0.945</td>
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<td>dimethoxyethane (glyme)</td>
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<td>0.868</td>
<td>M</td>
<td>0.231</td>
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<tr>
<td>N,N-dimethylaniline</td>
<td>C₈H₁₁N</td>
<td>0.956</td>
<td>0.14</td>
<td>0.179</td>
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<tr>
<td>dimethylformamide (DMF)</td>
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<td>0.386</td>
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<td>dimethylphthalate</td>
<td>C₁₀H₁₀O₄</td>
<td>1.190</td>
<td>0.43</td>
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</tr>
<tr>
<td>dimethylsulfoxide (DMSO)</td>
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<td>C₄H₈O₂</td>
<td>1.033</td>
<td>M</td>
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<td>ethanol</td>
<td>C₂H₅O</td>
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<td>M</td>
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</tr>
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<td>ether</td>
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<td>0.713</td>
<td>7.5</td>
<td>0.117</td>
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<tr>
<td>ethyl acetate</td>
<td>C₄H₈O₂</td>
<td>0.894</td>
<td>8.7</td>
<td>0.228</td>
</tr>
<tr>
<td>ethyl acetoacetate</td>
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<td>0.228</td>
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<tr>
<td>Compound</td>
<td>Molecular Formula</td>
<td>Density (g/L)</td>
<td>Solubility (%)</td>
<td>Refractive Index (nD)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------</td>
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<td>----------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>ethylene glycol</td>
<td>C₂H₆O₂</td>
<td>1.115</td>
<td>M</td>
<td>0.790</td>
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<tr>
<td>glycerin</td>
<td>C₃H₈O₃</td>
<td>1.261</td>
<td>M</td>
<td>0.812</td>
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<tr>
<td>heptane</td>
<td>C₇H₁₆</td>
<td>0.684</td>
<td>0.0003</td>
<td>0.012</td>
</tr>
<tr>
<td>1-heptanol</td>
<td>C₇H₁₆O</td>
<td>0.819</td>
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<tr>
<td>hexane</td>
<td>C₆H₁₄</td>
<td>0.655</td>
<td>0.0014</td>
<td>0.009</td>
</tr>
<tr>
<td>1-hexanol</td>
<td>C₆H₁₄O</td>
<td>0.814</td>
<td>0.59</td>
<td>0.559</td>
</tr>
<tr>
<td>methanol</td>
<td>CH₄O</td>
<td>0.791</td>
<td>M</td>
<td>0.762</td>
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<tr>
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<td>C₃H₆O₂</td>
<td>0.933</td>
<td>24.4</td>
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<tr>
<td>methyl t-butyl ether (MTBE)</td>
<td>C₃H₁₂O</td>
<td>0.741</td>
<td>4.8</td>
<td>0.124</td>
</tr>
<tr>
<td>methylene chloride</td>
<td>CH₂Cl₂</td>
<td>1.326</td>
<td>1.32</td>
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<tr>
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<td>C₅H₁₂</td>
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<td>0.004</td>
<td>0.009</td>
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<tr>
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<td>0.814</td>
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<tr>
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<td>0.321</td>
</tr>
<tr>
<td>3-pentanone</td>
<td>C₅H₁₂O</td>
<td>0.814</td>
<td>3.4</td>
<td>0.265</td>
</tr>
<tr>
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<td>0.207</td>
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<td>0.099</td>
</tr>
<tr>
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<td>H₂O</td>
<td>0.998</td>
<td>M</td>
<td>1.000</td>
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<td>D₂O</td>
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<td>M</td>
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<tr>
<td>Solvent</td>
<td>formula</td>
<td>density (g/mL)</td>
<td>solubility in H₂O¹ (g/100g)</td>
<td>relative polarity²</td>
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<tr>
<td>cyclohexane</td>
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<td>0.006</td>
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<td>0.0039</td>
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<td>0.0014</td>
<td>0.009</td>
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<td>0.684</td>
<td>0.0003</td>
<td>0.012</td>
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<td>CCl₄</td>
<td>1.594</td>
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<td>0.052</td>
</tr>
<tr>
<td>carbon disulfide</td>
<td>CS₂</td>
<td>1.263</td>
<td>0.2</td>
<td>0.065</td>
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<tr>
<td>p-xylene</td>
<td>C₈H₁₀</td>
<td>0.861</td>
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<tr>
<td>toluene</td>
<td>C₇H₈</td>
<td>0.867</td>
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<td>0.099</td>
</tr>
<tr>
<td>benzene</td>
<td>C₆H₆</td>
<td>0.879</td>
<td>0.18</td>
<td>0.111</td>
</tr>
<tr>
<td>ether</td>
<td>C₄H₁₀O</td>
<td>0.713</td>
<td>7.5</td>
<td>0.117</td>
</tr>
<tr>
<td>methyl t-butyl ether</td>
<td>C₅H₁₂O</td>
<td>0.741</td>
<td>4.8</td>
<td>0.124</td>
</tr>
<tr>
<td>(MTBE)</td>
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<td></td>
</tr>
<tr>
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<td>0.956</td>
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<td>0.179</td>
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<td>chlorobenzene</td>
<td>C₆H₅Cl</td>
<td>1.106</td>
<td>0.05</td>
<td>0.188</td>
</tr>
<tr>
<td>anisole</td>
<td>C₇H₈O</td>
<td>0.996</td>
<td>0.10</td>
<td>0.198</td>
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<tr>
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<td>C₄H₈O</td>
<td>0.886</td>
<td>30</td>
<td>0.207</td>
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<tr>
<td>(THF)</td>
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</tr>
<tr>
<td>ethyl acetate</td>
<td>C₄H₈O₂</td>
<td>0.894</td>
<td>8.7</td>
<td>0.228</td>
</tr>
<tr>
<td>ethyl benzoate</td>
<td>C₉H₁₀O₂</td>
<td>1.047</td>
<td>0.07</td>
<td>0.228</td>
</tr>
<tr>
<td>dimethoxyethane</td>
<td>C₄H₁₀O₂</td>
<td>0.868</td>
<td>M</td>
<td>0.231</td>
</tr>
<tr>
<td>(glyme)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Chemical Formula</td>
<td>Dens (g cm$^{-3}$)</td>
<td>Visc (cP)</td>
<td>Ref (°C)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------</td>
<td>--------------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>diglyme</td>
<td>C$<em>6$H$</em>{14}$O$_3$</td>
<td>0.945</td>
<td>M</td>
<td>0.244</td>
</tr>
<tr>
<td>methyl acetate</td>
<td>C$_3$H$_6$O$_2$</td>
<td>0.933</td>
<td>24.4</td>
<td>0.253</td>
</tr>
<tr>
<td>chloroform</td>
<td>CHCl$_3$</td>
<td>1.498</td>
<td>0.8</td>
<td>0.259</td>
</tr>
<tr>
<td>3-pentanone</td>
<td>C$<em>3$H$</em>{12}$O</td>
<td>0.814</td>
<td>3.4</td>
<td>0.265</td>
</tr>
<tr>
<td>1,1-dichloroethane</td>
<td>C$_2$H$_4$Cl$_2$</td>
<td>1.176</td>
<td>0.5</td>
<td>0.269</td>
</tr>
<tr>
<td>di-n-butyl phthalate</td>
<td>C$<em>{16}$H$</em>{22}$O$_4$</td>
<td>1.049</td>
<td>0.0011</td>
<td>0.272</td>
</tr>
<tr>
<td>cyclohexanone</td>
<td>C$<em>6$H$</em>{10}$O</td>
<td>0.948</td>
<td>2.3</td>
<td>0.281</td>
</tr>
<tr>
<td>pyridine</td>
<td>C$_3$H$_3$N</td>
<td>0.982</td>
<td>M</td>
<td>0.302</td>
</tr>
<tr>
<td>dimethylphthalate</td>
<td>C$<em>{10}$H$</em>{16}$O$_4$</td>
<td>1.190</td>
<td>0.43</td>
<td>0.309</td>
</tr>
<tr>
<td>methylene chloride</td>
<td>CH$_2$Cl$_2$</td>
<td>1.326</td>
<td>1.32</td>
<td>0.309</td>
</tr>
<tr>
<td>2-pentanone</td>
<td>C$<em>3$H$</em>{10}$O</td>
<td>0.809</td>
<td>4.3</td>
<td>0.321</td>
</tr>
<tr>
<td>2-butanone</td>
<td>C$_4$H$_8$O</td>
<td>0.805</td>
<td>25.6</td>
<td>0.327</td>
</tr>
<tr>
<td>1,2-dichloroethane</td>
<td>C$_2$H$_4$Cl$_2$</td>
<td>1.235</td>
<td>0.87</td>
<td>0.327</td>
</tr>
<tr>
<td>benzonitrile</td>
<td>C$_7$H$_5$N</td>
<td>0.996</td>
<td>0.2</td>
<td>0.333</td>
</tr>
<tr>
<td>acetone</td>
<td>C$_3$H$_6$O</td>
<td>0.786</td>
<td>M</td>
<td>0.355</td>
</tr>
<tr>
<td>dimethylformamide (DMF)</td>
<td>C$_3$H$_7$NO</td>
<td>0.944</td>
<td>M</td>
<td>0.386</td>
</tr>
<tr>
<td>t-butyl alcohol</td>
<td>C$<em>4$H$</em>{10}$O</td>
<td>0.786</td>
<td>M</td>
<td>0.389</td>
</tr>
<tr>
<td>aniline</td>
<td>C$_4$H$_7$N</td>
<td>1.022</td>
<td>3.4</td>
<td>0.420</td>
</tr>
<tr>
<td>dimethylsulfoxide (DMSO)</td>
<td>C$_2$H$_6$OS</td>
<td>1.092</td>
<td>M</td>
<td>0.444</td>
</tr>
<tr>
<td>acetonitrile</td>
<td>C$_2$H$_3$N</td>
<td>0.786</td>
<td>M</td>
<td>0.460</td>
</tr>
<tr>
<td>3-pentanol</td>
<td>C$<em>3$H$</em>{12}$O</td>
<td>0.821</td>
<td>5.1</td>
<td>0.463</td>
</tr>
<tr>
<td>2-pentanol</td>
<td>C$<em>3$H$</em>{12}$O</td>
<td>0.810</td>
<td>4.5</td>
<td>0.488</td>
</tr>
<tr>
<td>2-butanol</td>
<td>C$<em>4$H$</em>{10}$O</td>
<td>0.808</td>
<td>18.1</td>
<td>0.506</td>
</tr>
<tr>
<td>cyclohexanol</td>
<td>C$<em>6$H$</em>{12}$O</td>
<td>0.962</td>
<td>4.2</td>
<td>0.509</td>
</tr>
<tr>
<td>Substance</td>
<td>Molecular Formula</td>
<td>Rf Value</td>
<td>m/z Value</td>
<td>Molarity</td>
</tr>
<tr>
<td>--------------------</td>
<td>-------------------</td>
<td>----------</td>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>1-octanol</td>
<td>C₈H₁₈O</td>
<td>0.827</td>
<td>0.096</td>
<td>0.537</td>
</tr>
<tr>
<td>2-propanol</td>
<td>C₃H₈O</td>
<td>0.785</td>
<td>M</td>
<td>0.546</td>
</tr>
<tr>
<td>1-heptanol</td>
<td>C₇H₁₆O</td>
<td>0.819</td>
<td>0.17</td>
<td>0.549</td>
</tr>
<tr>
<td>i-butanol</td>
<td>C₄H₁₀O</td>
<td>0.803</td>
<td>8.5</td>
<td>0.552</td>
</tr>
<tr>
<td>1-hexanol</td>
<td>C₆H₁₄O</td>
<td>0.814</td>
<td>0.59</td>
<td>0.559</td>
</tr>
<tr>
<td>1-pentanol</td>
<td>C₅H₁₂O</td>
<td>0.814</td>
<td>2.2</td>
<td>0.568</td>
</tr>
<tr>
<td>Acetyl acetone</td>
<td>C₅H₈O₂</td>
<td>0.975</td>
<td>16</td>
<td>0.571</td>
</tr>
<tr>
<td>Ethyl acetoacetate</td>
<td>C₆H₁₀O₃</td>
<td>1.028</td>
<td>2.9</td>
<td>0.577</td>
</tr>
<tr>
<td>1-butanol</td>
<td>C₄H₁₀O</td>
<td>0.81</td>
<td>7.7</td>
<td>0.586</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>C₇H₈O</td>
<td>1.042</td>
<td>3.5</td>
<td>0.608</td>
</tr>
<tr>
<td>1-propanol</td>
<td>C₃H₈O</td>
<td>0.803</td>
<td>M</td>
<td>0.617</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>C₂H₄O₂</td>
<td>1.049</td>
<td>M</td>
<td>0.648</td>
</tr>
<tr>
<td>2-aminoethanol</td>
<td>C₂H₇NO</td>
<td>1.018</td>
<td>M</td>
<td>0.651</td>
</tr>
<tr>
<td>Ethanol</td>
<td>C₂H₆O</td>
<td>0.789</td>
<td>M</td>
<td>0.654</td>
</tr>
<tr>
<td>Diethylene glycol</td>
<td>C₄H₁₀O₃</td>
<td>1.118</td>
<td>M</td>
<td>0.713</td>
</tr>
<tr>
<td>Methanol</td>
<td>CH₄O</td>
<td>0.791</td>
<td>M</td>
<td>0.762</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>C₂H₆O₂</td>
<td>1.115</td>
<td>M</td>
<td>0.790</td>
</tr>
<tr>
<td>Glycerin</td>
<td>C₃H₈O₃</td>
<td>1.261</td>
<td>M</td>
<td>0.812</td>
</tr>
<tr>
<td>Water, heavy</td>
<td>D₂O</td>
<td>1.107</td>
<td>M</td>
<td>0.991</td>
</tr>
<tr>
<td>Water</td>
<td>H₂O</td>
<td>0.998</td>
<td>M</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**Conditions maintained during Rf value determination:**

1. Polarity of solvent affects the Rf value of this experiment. Hence non polar solvent is used in this experiment because both the drugs are polar.

2. Poor spotting is avoided in this experiment because there is a possibility of weak or poor separation. Sometime absorptivity of some drugs are too weak, at that time over spotting is necessary to avoid poor separation.
3. Room temperature is maintained during experiment because temperature also affects on the experiment. Temperature increases Rf value is also increases. Rf value is independent on the temperature if miscible solvents are used.

4. Multiple solvents are used for this experiment to determine mobile phase.

**VALIDATION OF ANALYTICAL PROCEDURE USED IN THE EXAMINATION OF PHARAMACEUTICAL MATERIAL.**

**Characterizes of an Analytical procedure**

The properties that may be need to be specified for the analytical procedure are listed below and defined, with an indication of how may be determined.

Not all the parameters are applicable to every test or to every material. Much depends on the purpose for which the procedure is required. The characteristics applicable to various analytical procedures are given as.

**Accuracy**

In case of accuracy closeness of experimental values are compared with the true values. The concept of accuracy is as similar to the bull’s eye experiment. In case of analytical chemistry no one knows the perfect true value but some values or standard values are given by the scientist are considered as true and experimental values are compared with these values.

**Precision**

It is the degree of agreements among test results. In this case, experimental values are close to each other. The student of analytical chemistry knows that good precision doesn’t mean good accuracy.

**Repeatability (within laboratory variation)**

Precision is repeated by the analyst under same condition and with short time interval. In this case experiment is carried out under same laboratory and reagents, samples etc are same.

**Reproducibility**

Precision is done by analyst under different set of condition. In this case, experiment is carried out in different laboratories.
Robustness:

There are some important parameters in Robustness as:

1. The stability of analyte
2. Effect of temperature
3. Effect of humidity
4. Shape of spot, shape of size
5. Eluent composition
6. Sample volume, geometry of chamber

Robustness must be considered before method development but it should not consider in mobile phase optimization. Optimization of mobile phase is one of the part of the method and robustness is the test of effect of some factors on the method.

Most important parameter in HPTLC is Robustness. In this case, mobile phase is changed by small quantity.

Most important parameter in HPTLC is Robustness. In this case, mobile phase is changed by small quantity. In this case; it is the ability of the procedure to provide analytical result of acceptable accuracy and precision.

Ruggness:

In this case, developed analytical method is checked by two or three analysts.

Linearity and range

In linearity minimum of 5 concentrations are needed. In this case, appropriate volume from standard drug stock solution is to give series of spots covering the linear range. The correlation coefficient and other factors should be submitted. Standardization may provide by means of the calibration curve, if the relationship between responses and concentration is not linear\(^\text{36}\).

Selectivity

Reference standards and samples are analyzed and specificity is determined. Rf values of reference standards and samples are compared and combination drugs bands are confirmed. Uv spectra of these bands are determined and compared. Peak start, Apex peak and end peak of spectra are compared and peak purity are accessed.

Limit of detection: In case of LOD i.e. (limit of detection), lowest concentration of
an analyte is detected but it is not necessary to quantify it. It gives u a test whether an analyte is below or above the value. In case of LOQ i.e (Limit of quantitation), lowest concentration of analyte is analyzed and it is quantified hence the name LOQ, acceptable accuracy and precision is determined in this case. In case of LOD and LOQ sample solution n≥3 is applied in decreasing order of concentration and also the same volume of a sample solution is applied as a blank. After this peak area is plotted against concentration applied then we get the graph. LOD and LOQ is depends on signal to noise ratio. Results of this should be noted as applied mass on the plate not the concentration.

Limit of detection can be determined as a signal to noise ratio usually 2:1 to 3:1. It is determined by following formula as:

\[
LOD = 3.3(\text{SD}/S).
\]

\( \text{SD} \) = Standard deviation

\( S \) = Slope of the calibration curve.

**Limit of quantification**

LOQ can be determined as a signal to noise ratio, usually 10:1. Limit of detection is calculated as follows:

\[
LOQ = 10(\text{SD}/S).
\]

In this case, final result is depends on an instrumental reading. Sometimes the detection is approximately twice the limit of detection\(^7\).

**Why HPTLC?**

1.We can easily separate isomers by using this method but in other cases like in HPLC, isomer separation is not easy because chiral columns are very expensive.

2.This method is less expensive and works with high speed.

3.Method development is very easy in this case as compared to other method like HPLC.

4.Time required for HPTLC method development in half day but in case of HPLC 2-7 days are required.

5.In HPTLC method development, solvent required is very less as compared to other
methods.

6. In HPTLC, it is easy to achieve ruggedness but it is very difficult to achieve ruggedness in case of HPLC. HPTLC method gives more information about the drug.

7. In HPTLC, it is easy to prepare sample solution.

**HYPOTHESIS:**

To develop HPTLC method for combination drugs. The properties that may be need to be specified for the analytical procedure are listed below and defined, with an indication of how may be determined. Not all the parameters are applicable to every test or to every material. Much depends on the purpose for which the procedure is required. The characteristics applicable to various analytical procedures are given as.

**Accuracy**

In case of accuracy closeness of experimental values are compared with the true values. The concept of accuracy is as similar to the bull’s eye experiment. In case of analytical chemistry no one knows the perfect true value but some values or standard values are given by the scientist are considered as true and experimental values are compared with these values.

**Precision**

It is the degree of agreements among test results. In this case, experimental values are close to each other. The student of analytical chemistry knows that good precision doesn’t mean good accuracy.

**Repeatability (within laboratory variation)**

Precision is repeated by the analyst under same condition and with short time interval. In this case experiment is carried out under same laboratory and reagents, samples etc are same.

**Reproducibility**

Precision is done by analyst under different set of condition. In this case, experiment is carried out in different laboratories.

**Robustness** Most important parameter in HPTLC is Robustness. In this case, mobile phase is changed by small quantity. In this case; it is the ability of the procedure to provide analytical result of acceptable accuracy and precision.
Recovery study (Accuracy)

In case of accuracy closeness of experimental values are compared with the true values. The concept of accuracy is as similar to the bull’s eye experiment. In case of analytical chemistry no one knows the perfect true value but some values or standard values are given by the scientist are considered as true and experimental values are compared with these values.

**Standard addition method**

Recovery is determined by adding known amount of standard drug to known amount of tablet. In this case negative and positive changes are observed.

The recovery was calculated by using the following formula.

Where,

\[ A = \text{Total drug estimated in mg.} \]

\[ B = \text{Amount of drug contributed by tablet powder (As per proposed method).} \]

\[ C = \text{Amount of pure drug added} \]

\[ \text{Recovery} = \frac{A - B}{C} \times 100 \]

**Ruggedness:**

In this case, developed analytical method is checked by two or three analysts.

**Linearity and range**

In linearity minimum of 5 concentrations are needed. In this case, appropriate volume from standard drug stock solution is to give series of spots covering the linear range. The correlation coefficient and other factors should be submitted. Standardization may provide by means of the calibration curve, if the relationship between responses and concentration is not linear\(^7\).

**Selectivity**

Reference standards and samples are analyzed and specificity are determined. Rf values of reference standards and samples are compared and combination drugs bands are confirmed. Uv spectra of these bands are determined and compared. Peak start, Apex
peak and end peak of spectra are compared and peak purity are accessed.

**Limit of detection:**

Limit of detection can be determined as a signal to noise ratio usually 2:1 to 3:1. It is determined by following formula as:

\[
\text{LOD} = 3.3(\frac{\text{SD}}{S})
\]

SD = Standard deviation

S = Slope of the calibration curve.

**Limit of quantification**

LOQ can be determined as a signal to noise ratio, usually 10:1. Limit of detection is calculated as follows:

\[
\text{LOQ} = 10 \left( \frac{\text{SD}}{S} \right)
\]

In this case, final result is depends on an instrumental reading. Sometimes the detection is approximately twice the limit of detection.

**SOPE AND IMPORTANCE OF HPTLC METHOD:**

1. This HPTLC method development is less expensive and works with high speed.
2. As compared to the HPLC and GC method we can use this method for quantitative analysis of this combination.
3. Time required for HPTLC method development is half day but in case of HPLC 2-7 days are required.
4. For this method very less solvent is used that’s why this method is in the favor of Green chemistry.
5. This method gives more information about drug. It is easy to achieve ruggedness in case of this method but not very easy in case of HPLC method.
6. Very easy to prepare sample solution for this method but not for others.
7. Chromatographic system in this method is open, you can see what is happening in this method with naked eyes.
8. Results can be seen by machine and eyes.
9. In this method analysis takes place offline.
10. Time per analysis required for this method is 1-3 min but in case of other method it is 2-60 min.
11. There is no need to maintain eqpt. Each and every time.
12. This method is ideal for screening.
13. Stationary phase cost in HPTLC method development is Rs.3/sample and Rs 120/plate. But in case of HPLC method cost required for stationary phase Rs 15/Injection i.e 15000 column/500 Injection. (Best case)
14. Stationary phase cost in HPTLC method development is Rs.6/sample and Rs 120/plate. But in case of HPLC method cost required for stationary phase Rs 50/Injection i.e 25000 column/500 Injection. (Worst case)
15. System set up cost is nil in case of this method development.
16. System washing cost is nil in case of this method development.
17. Sample cleanup cost is nil in case of this method development.
18. Mobile phase cost required for this method development is negligible (1 ml/sample).
19. Gas Phase cost required for this method is Rs.2/-. N₂ gas is used as a sample applicator.
20. Cost required per sample analysis is Rs 8-10 but in case of HPLC Cost required for HPLC is Rs 30-75.
21. By this method we can analyze different samples at a same time but this not possible in case of HPLC.
22. In parallel analysis we can analyze 100 samples but in case of HPLC only 1 sample analysis is possible.