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HPTLC A TECHNOLOGY OF INDUSTRIAL UTILITY

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ABSTRACT

Analysis of pharmaceutical and natural compounds and newer drugs is commonly used in all the stages of drug discovery and development process. High-performance thin layer chromatography is one of the sophisticated instrumental techniques based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation, and so on enable it to be a powerful analytical tool for chromatographic information of complex mixtures of pharmaceuticals, natural products, clinical samples, food stuffs, and so on.

INTRODUCTION

Analysis of pharmaceutical compounds and newer drugs is commonly used in all the stages of drug discovery and development process.These analytical technique provide more accurate précised data, not only supporting drugdiscovery and development but also postmarket surveillance.[1]Thin layer chromatography studies are among the key identity tests in most pharmacopoeial monographs. Pharmacopoeial standards are typically used by industry as a basis for meeting QC requirements and current good manufacturing practices (cGMPs). An extension of TLC is high-performance thin layer chromatography (HPTLC) is robust, simplest, rapid, and efficient tool in quantitative analysis of compounds. HPTLC is used for the identification of constituents, identification and determination of impurities, and quantitative determination of active substances. HPTLC is an analytical technique based on TLC, but Some of the enhancements such as the use of higher quality TLC plates with finer particle sizes in the stationary phase which allow better resolution.[2]The separation can be further improved by repeated development of the plate, using a multiple development device. As a consequence, HPTLC offers better resolution and lower Limit of Detection (LODs). The use of modern apparatus such as video scanners, densitometers, and new chromatographic chambers, and more effective elution techniques, high-resolution sorbents with selected particle size or chemically modified surface, the possibility of combining with other instrumental methods, and development of computer programs for method optimization all make HPTLC an important alternative method to HPLC or gas chromatography. Specifically, HPTLC is one of the ideal TLC techniques for the analytical purposes because of its increased accuracy, reproducibility, and ability to document the results, compared with standard TLC. Because of this, HPTLC technologie are also the most appropriate TLC technique for conformity with GMP's.[3]

There are several advantages of using HPTLC for the analysis of compounds as compared to other techniques, like HPLC, spectrophotometry, titrimetry, etc.[4] Some of the advantages of HPTLC are:

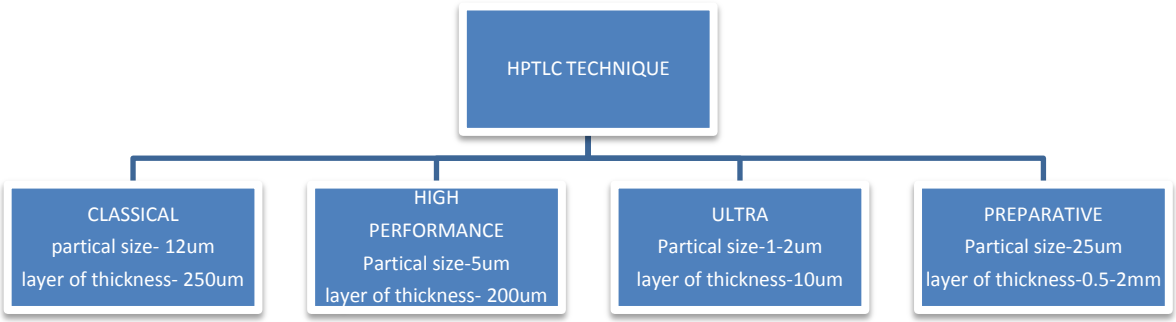
- Ability to analyze crude samples containing multi-components.
- The separation process is easy to follow especially with colour compounds.
- Several samples can be separated parallel to each other on the same plate resulting in a high output, time saving, and a rapid low-cost analysis.
- Choice of solvents for the HPTLC development is wide as the mobile phases are fully evaporated before the detection step.

- Two-dimensional separations are easy to perform. Stability during chromatography should be tested using two-dimensional development.
- Specific and sensitive colour reagents can be used to detect separated spots (Dragendroff reagent/Kedde reagent).
- HPTLC can combine and consequently be used for different modes of evaluation, allowing Identification of compounds having different light-absorption characteristics or different colours.
- Contact detection allows radiolabelled compounds to be monitored and microbial activity in spots to be assessed.

HPTLC method may help to minimizes exposure risk of toxic organic effluents and significantly reduces its disposal problems, consequently, reducing environment pollution.[4-14]

CLASSIFICATION OF HPTLC

HPTLC techniques may be classified into four classes i.e. Classical, High performance, Ultra and Preparative thin-layer chromatography. They differ with classical TLC in the particle size distribution and thickness of the sorbent layers. The mean particles sizes are 12, 5, 25 μm for classical, high-performance and preparative thin-layer chromatography, respectively, whereas Ultra-thin layer chromatography does not have particles but a monolithic layer with 1–2 um macropores.[15] Another difference is the thickness of the sorbent layers which is 250 um, 200 um, 10 um and 0.5–2 mm, for classical, high-performance, ultra-thin and preparative sorbent layers, respectively.[5]



KEY FEATURE OF HPTLC

1. Simultaneous processing of sample and standard - better analytical precision and accuracy less need for Internal Standard.
2. Several analysts work simultaneously.
3. Lower analysis time and less cost per analysis.
4. Low maintenance cost.
5. Simple sample preparation - handle samples of divergent nature.
6. No prior treatment for solvents like filtration and degassing.
7. Low mobile phase consumption per sample
8. No interference from previous analysis – fresh stationary and mobile phases -no contamination.
9. Visual detection possible - open system[16]

COMMON METHODOLOGY FOR HPTLC ANALYSIS

Method development in thin-layer (planar) chromatography is one of the most significant steps for a qualitative and quantitative analysis. During establishing a new analytical procedure, always starts with wide literature survey[17] i.e. primary information about the physicochemical characteristics of sample and nature of the sample (structure, polarity, volatility, stability and solubility). It involves considerable trial and error procedures.[18]General steps involved in HPTLC method developments are as follow:[7,16,19]

1. Sample preparation.
2. Selection of chromatography.
3. Plate, prewashing, conditioning.
4. Sample application
5. Pre-conditioning.
6. Mobile phase.
7. Chromatography development.
8. Detection spot.
9. Scanning & documentation

Quantitation: HPTLC method validation for pharmaceutical analysis:

- Specificity
- Linearity
- Range
- Accuracy
- Precision
- Detection Limit, Quantitation Limit
- Robustness

BASIC STEPS:

Selection of the stationary phase –

During method development, stationary phase selection should be based on the type of compounds to be separated.[20] HPTLC uses smaller plates (10*10 or 10*20 cm) with significantly decreased development distance (typically 6 cm) and analysis time (7–20 min). Precoated plates - different support materials - different Sorbents available.80% of analysis - silica gel GF is used. Example-Basic substances, alkaloids and steroids - Aluminum oxide Amino acidsdipeptides, sugars and alkaloids – cellulose Non-polar substances, fatty acids, carotenoids, cholesterol - RP2, RP8 and RP18. HPTLC plates provide improved resolution, higher detection sensitivity, and improved in situ quantification and are used for industrial pharmaceutical densitometric quantitative analysis.[14, 21]

Activation of pre-coated plates

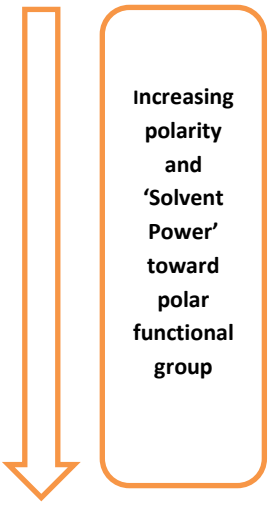
Freshly open box of plates do not require activation Plates exposed to high humidity or kept on hand for long time to be activated By placing in an oven at 110-120ºc for 30’ prior to spotting Aluminum sheets should be kept in between two glass plates and placing in oven at 110-120ºc for 15 minutes.[22]

Sample Preparation

A good solvent system is one that moves all components of the mixture off the baseline, but does not put anything on the solvent front. The peaks of interest should be resolved between Rf 0.15 and 0.85. The elution power of the mobile phase depends on a property called eluent strength which is related to the polarity of the mobile phase components.[7]The more nonpolar the compound, the faster it will elute (or the less time it will remain on the stationary phase) and the more polar the compound the slower it will elute (or more time on the stationary phase). The following chart is helpful in predicting the order of elution[5, 11]

Table 1: Common Mobile Phases listed by Increasing Polarity

S. No	Solvent	Eluent Strength
1	N- Pentane	0.00
2	Hexane	0.01
3	Cyclohexane	0.04
4	Carbon tetrachloride	0.18
5	Toluene	0.29
6	Chloroform	0.40
7	Methylene Chloride	0.42
8	Tetrahydrofuran	0.45
9	Acetone	0.56
10	Ethyl Acetate	0.58
11	Aniline	0.62
12	Acetonitrile	0.65
13	Ethanol	0.88
14	Methanol	0.95



Application of sample and standard

Usual concentration range is 0.1-1µg /µl above this causes poor separation.

Automatic applicator- nitrogen gas sprays sample and standard from syringe on TLC plates as bands

Band wise application better separation high response to densitometer[22]

Mobile phase selection and optimization-

The selection of mobile phase is based on adsorbent material used as stationary phase and physical and chemical properties of analyte. [11,12]

Normal phase

Stationary phase is polar then Mobile phase is non-polar.

Non-polar compounds eluted first because of lower affinity with stationary phase

Polar compounds retained because of higher affinity with the stationary phase

Reversed phase

Stationary phase is non-polar then Mobile phase is polar

Polar compounds eluted first because of lower affinity with stationary phase

Non-Polar compounds retained because of higher affinity with the stationary phase. [22]

The Table 2 gives the details of mobile phase generally used in detection of some chemical compounds.

Table 2: Generally used Mobile phase in detection of some chemical compounds

Sr. No.	Chemical Compounds	Mobile Phase
1.	Polar Compounds Anthraglycosides, Arbutin, Alkaloids, Cardiac Glycosides, Bitter Principles, Flavonoids, Saponin	Ethyl Acetate: Methanol: Water [100:13.5:10]
2.	Lipophilic Compounds Essential oils, Terpenes, Coumarin, Napthoquinons, Velpotriate	Toluene: Ethyl Acetate [93:7]
3.	Alkaloids	Toluene: Ethyl Acetate: Diethyl Amine [70:20:10]
4.	Flavonoids	Ethyl Acetate: Formic Acid: Glacial Acetic Acid: Water [100:11:11:26]
5.	Saponin	Chloroform: Glacial Acetic Acid: Methanol: Water [64:32:12:8]
6.	Coumarin	Diethyl Ether: Toluene [1:1] Saturated with 10% Acetic Acid
7.	Bitter Drug	Ethyl Acetate: Methanol: Water [77:15:8]
8.	Cardiac Glycosides	Ethyl Acetate: Methanol: Water [100:13.5:10] OR [81:11:8]
9.	Essential Oil	Toluene: Ethyl Acetate [93:7]
10.	Lignans	Chloroform: Methanol: Water [70:30:4] Chloroform: Methanol [90:10] Toluene: Ethyl Acetate [70:30]
11.	Pigments	Ethyl Acetate: Formic Acid: Glacial Acetic Acid: Water [100:11:11:26]
12.	Pungent Testing	Toluene: Ethyl Acetate [70:30]
13.	Terpenes	Chloroform: Methanol: Water [65:25:4]

Pre- conditioning (Chamber saturation)

Un- saturated chamber causes high Rf values Saturated chamber by lining with filter paper for 30 minutes prior to development - uniform distribution ofsolvent vapours - less solvent for the sample to travel - lower Rf values[22]

Chromatogram Development (separation)-Although chromatogram development is the most crucial step in the HTLC procedure, important parameters are generally overlooked.[11] HPTLC plates are developed in twin-trough chambers, or horizontal-development chambers. In general, saturated twin-trough chambers fitted with filter paper offer the best reproducibility. Twin-through chamber avoids solvent vapor preloading and humidity.[23-25]

Detection and visualization

Detection of separated compounds on the sorbent layers is enhanced by quenching of fluorescence due to UV light. This process is commonly called Fluorescence quenching.

Detection under UV light is first choice-non-destructive and spots of fluorescent compounds can be seen at 254nm (short wave length) or at 366 nm (long wave length) Spots of non-fluorescent compounds can be seen -fluorescent stationary phase is used - silica gel GF. Non UV absorbing compounds like ethambutol, dicylomine dipping the plates in 0.1% iodine solution. When individual component does not respond to UV- derivatisation required for detection.[22]

Visualization at white light

Zone containing separated compounds can be detected by viewing their natural color in daylight (White light).[21]

Derivatisation

Derivatization can be defined as a procedural technique that primarily modifies an analyte's functionality in order to enable chromatographic separations. Derivatization can be performed either by immersing the plates or by spraying the plates with a suitable reagent(Table 4). [11,12,26,27]

For better reproducibility, immersion is the preferred derivatization technique.

Table 3: List of common derivatization reagents

Sr.no	Color reagent	Chemical compound	Colour
1	Dragendroff Reagent It forms complex reaction with some nitrogen containing compounds	Alkaloids	Red-brown Zone (vis)
2	Natural products- Polyethylene Glycol reagent i.e. Diphenylboric acid -2-aminoethyl ester forms complexes with 3-hydroxyflavones via condensation reaction	Flavonoids	Intense yellow, Orange and Green Fluorescent zones in UV 366 nm
3	Vanillin Sulphuric Acid OR Anisaldehyde Sulphuric Acid	Bitter Principle	Red-brown, Yellow-brown, Dark green Zone (vis)

4	10 % Ethanolic KOH	Anthrones (Aloin, Cascarosides)	Yellow zones (vis) Yellow Fluorescence (UV 366 nm)
5	Ninhydrin Reagent	Amino acids, peptides, amines and amino-sugars	Yellow, brown to pink and violet (vis)
6	Iodine It produce iodine reaction possibly result in an oxidative products	Indole, quinoline derivative, thiols and all organic compounds	Dark zone (UV 254)

QUANTIFICATION

Generally quantitative evaluation is performed by measuring the zones of samples and standards using a densitometer or scanner with a fixed sample light beam in the form of a rectangular slit. Sample and standard should be chromatographer on same plate-after development chromatogram is scanned TLC scanner III scan the chromatogram in reflectance or in transmittance mode by absorbance or by fluorescent mode. Scanning speed is selectable up to 100 mm/s .[7,11]Spectra recording is fast 36 tracks with up to 100 peak windows can be evaluated. Calibration of single and multiple levels with linear or non-linear regressions are possible. When target values are to be verified such as stability testing and dissolution profile single level calibration is suitable. Statistics such as RSD report automatically concentration of analyte in the sample is calculated by considering the sample initially taken and dilution factors.[22]

DOCUMENTATION

Plates with imprinted identification code supplier name. Item number, batch number and individual plate number - avoid manipulation of data at any stage - coding automatically gets recorded during photo documentation.

Multiple Detection

Separation of Herbal sample and reference substances, white light (left), UV 254 nm (center), UV 366 nm (right) in addition to the evaluation of chromatograms via images there is a broad array of other detection modes available. Detection is a very flexible and independent step. Multiple detection is possible without repeating the chromatography.

Scanning densitometry

Allows measuring the absorption and/or fluorescence of underivatized or derivative substances at wavelengths between 200 and 800 nm. Up to 31 wavelengths can be evaluated and spectra of any peak can be recorded. Following integration densitometric data can be quantitatively evaluated. Biological tests can be performed directly on the HPTLC plate. Bacteria, enzymes, yeast, fungi, etc. can be used as test organisms.[22]

HPTLC-MS

It allows the hyphenation of a high resolution planar separation with modern mass spectrometers for identification and quantitation of substances. Technologies for available interfaces include elution and desorption approaches. Multi-wavelength scan evaluation of UVspectra toxicity screening with Vibrio fischeri (Bioluminex TM, Chromadex) and identification by HPTLC-MS.[22]

Difference between HPTLC AND TLC

Table No.4 Difference of HPTLC and TLC - Particle and Pore size of Sorbents

Sr. no		HPTLC	TLC
1	Layer of Sorbent	100µm	250µm
2	Efficiency	High due to smaller particle size generated Separations	Less
3	Separations	□ 3- 5 cm	□ 10- 15 cm
4	Analysis Time	Shorter migration distance and the analysis time is greatly reduced	Slower
5	Solid support	Wide choice of stationary phases like silica gel for normal phase and C8 , C18 for reversed phase modes	Silica gel , Alumina & Kiesulguhr
6	Development chamber	New type that require less amount of mobile phase	Less amount
7	Sample spotting	Auto sampling	Manual spotting
8	Scanning	Use of UV/ Visible/ Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer	Not possible

Table 5: Differences between HPLC and HPTLC[28,29]

Parameter	HPLC	HPTLC
Type	Reverse Phase Chromagraphy	Straight Phase Chromagraphy
Stationary phase	liquid	Solid
Conditioning phase	none	Gas
Separation by	partition	Adsoption
Result	By machine	By machine + eyes
Analysis	On-line	Off-line
Resolution	Very high	Moderate to high
Chromatography System	closed	Open
Separating medium	Tubular Column	Planar layer (plate)
Strongly Retarded Fractions Seen As	Broad peaks	Sharp peaks
Analysis in parallel	No. Only 1 at a time	Yes. Upto 100 samples.
High temp. / pressure	High pressure	None
Time per sample	2-60 min	1-30min
Data obtained from chromatography	Limited to very high	High to very high
Post chromatography derivatisation	Limited possibilities. Cumbersome.	Simple. Possible for every sample. Gives additional information
Fraction collection / micro preparative chromatography	Requires prep. scale chromatograph & fraction collector	Simple. No special requirements
Sensitivity	High to ultra	high Moderate to ultrahigh
Fluorescence data	Possible, optional	Possible, built-in
Detectors	UV, Fluor, electrochem Light scatter , MS	UV - Vis, bioluminescence , MS
Chromatogram image documentation	No	Yes. At 254 & 366 nm & visible
Sample clean-up	Through Column reusable	Not so imp. Layer disposable
Chromatographic fingerprint	Yes, but limited	Yes. Comprehensive
Cost per analysis	Very high	Low
Equipment. maintenance	Very high	Low
Analyst's skills required	High to very high	Low (TLC) to high (HPTLC)

APPLICATION OF HPTLC

HPTLC is one of the most widely applied methods for the analysis in pharmaceutical industries, clinical chemistry, forensic chemistry, biochemistry ,cosmetology, food and drug analysis, environmental analysis and other areas. It's due to its numerous advantages. For example, it is only chromatographic technique offering the option of presenting the results as an image. Other advantages include simplicity, low costs, parallel analysis of samples, high sample capacity, rapidly obtained results, and possibility ofmultiple detection. Le Roux et al [30] evaluated a HPTLC technique for the determination of salbutamol serum levels in clinical trials and established as a suitable method for analyzing samples from the serum. Many lipids have also been analyzed and studied using HPTLC; 20 different lipid subclasses were separated using HPTLC with the reproducible and promising results. Many reports on studies related to clinical medicine have already been published in many journals. HPTLC is now strongly recommended in the analysis of drugs in serum and other tissues.[31]

1. HPTLC IN PHARMACEUTICAL PRODUCTS

HPTLC is also used in analyzing the purity and efficacy of many pharmaceutical preparations and dosage forms. Puranik et al developed and validated a simple, rapid, and accurate chromatographic methods (HPLC and HPTLC) for simultaneous determination of ofloxacin and ornidazole in solid dosage form. The amount of ofloxacin and ornidazole estimated as percentage of label claimed was found to be 100.23 and 99.61% with mean percent recoveries 100.47 and 99.32%, respectively. Both these methods were found to be simple, precise, accurate, selective, and rapid and could be successfully applied for the determination of pure laboratory prepared mixtures and tablets.[32]

2. HPTLC IN NATURAL PRODUCTS

The HPTLC technique is rapid, comparatively simple, robust, and extremely versatile. HPTLC not only confirm but also establish its identity. It is also an ideal screening tool for adulterations and is highly suitable for evaluation and monitoring of cultivation, harvesting, and extraction processes and testing of stability. A simple and reproducible method using HPTLC was successfully performed for the quantitative analysis of above diterpenoids in the root bark of Photiniaintegrifolia. In which Diterpenoids 1β,3α,8β-trihydroxy-pimara-15-ene (A), 6α,11,12,16-tetrahydroxy-7-oxo-abieta-8,11,13-triene (B) and 2α,19-dihydroxy-pimara-7,15-diene (C) were used as chemical markers for the standardization of Photiniaintegrifolia plant extracts.[33]

3. HPTLC IN OTHER FIELDS

In recent years, HPTLC is a globally accepted practical solution to characterize small molecules in quality assessment throughout the developing world. HPTLC is used for purity control of chemicals, pesticides, steroids, and water analysis.[34] HPTLC is also widely used for analysis of vitamins, water-soluble food dyes, pesticides in fruits, vegetables, and other food stuffs.[34]Beate et al[35] reported the analysis of stem cell lipids by offline HPTLC-MALDI-TOF MS. HPTLC is useful in detecting chemicals of forensic concern,including abuse drugs, poisons, adulterations, chemical weapons, and illicit drugs.[36,37]

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