HPTLC (HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY): AN ENHANCED TECHNIQUE FOR SUBSTANCES SCREENING

Abstract

HPTLC (High performance thin-layer chromatography process represents a cutting-edge analytical method that offers exceptional separation as well as qualitative or quantitative analysis features for a variety of substances, including herbal and botanical dietary supplements, nutraceuticals, traditional western medicines, traditional Chinese medications, and Ayurvedic (Indian) medicines. Numerous articles have emphasised HPTLC's advantages over Efficiency and turnaround time for analyses are superior to High-Performance Liquid Chromatography (HPLC). HPTLC runs as a standalone offline process with distinct stages. The ability to analyse complex samples with multiple components, the application of numerous samples and standards using the spray-on technique, a variety of solvents for development, the processing of Standards and samples are treated equally on the same plate for some of its important advantages are enhanced quantification precision and accuracy, a variety of selective detection methods, including sequential in situ spectrum recording for successful outcomes. Additionally, HPTLC permits limitless storage of the entire sample on the layer, decreasing exposure hazards and significantly minimising difficulties with the disposal of harmful organic effluents, hence reducing the likelihood of environmental pollution. Given these advantages, HPTLC-based approaches are evolving into crucial tools for routine research.

Keywords: High-Performance Thin-Layer Chromatography (HPTLC) Methodology, Chromatogram.

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I. INTRODUCTION

High-performance thin-layer chromatography (HPTLC) is a more advanced and automated version of thin-layer chromatography. Both the quantitative and qualitative analytical objectives can be fulfilled by this contemporary instrumental approach. Because it enables the various stages of the process to be carried out independently, HPTLC offers a flexible, adaptable, and economical method. The advantages of this sophisticated technique include (Sherma 2007)

1. Several analysts can use the system at once.
2. The system is technically simple to use and to learn.
3. The maintenance cost is low.
4. Less time is spent on analysis, which lowers the cost per analysis.
5. The selection of the solvents for the mobile phase is not constrained by criteria for ultra-high purity or low UV transparency. Corrosive & UV-absorbing mobile phases are options.
6. There is a broad selection of stationary phases with distinctive selectivity for mixture constituents. TLC/HPTLC plates, which serve as the chromatographic layer, are disposable and do not require regeneration.
7. The mechanism is open, allowing for visual detection.
8. There is no need for prior treatment of solvents such as filtration and degassing.
9. Each analysis uses fresh stationary and mobile phases, eliminating the possibility of interference from previous analyses. There is no carry-over, ensuring no contamination.
10. The system can chromatograph numerous samples at once, which allows for high sample throughput.
11. Cleanup of samples is rarely required.
12. Due to the fact that all the TLC/HPTLC plate maintains sample fractions, the same sample can be densitometrically evaluated multiple times under various conditions without having to redo the chromatography procedure.
13. Since there is less mobile phase in each sample, there is less money spent on buying and discarding solvent. Additionally, it lessens environmental contamination and the dangers associated with exposure to harmful organic effluents.
14. The method provides analysis sensitivity limitations that are typically in the nanogram (ng) to pictogram (pg) range.
15. There are numerous general and specialised detection methods available.
16. On a single TLC/HPTLC plate, standards and samples are chromatographed & quantified using the same experimental conditions, resulting in high quantification accuracy and precision. (Patel et al., 2010)

II. HPTLC METHODOLOGY

The analytical goal, which may involve the quantitative or qualitative identification of components, Before HPTLC is used the separate separation of two separate components and multicomponent combinations, or the optimisation for analysis a period of time must be established. It is essential to have a fundamental grasp of the sample's properties, including it’s the solubility parameter, the structure, polarity, volatility, and stability, in order to use HPTLC to analyse pharmaceuticals in multicomponent dosage forms. The process of developing a method frequently involves a lot of trial and error. To utilise HPTLC to study

pharmaceuticals in multicomponent dosage forms, it is crucial to have a fundamental understanding of the sample's properties, including its structure, polarity, volatility, stability, and solubility parameter. A lot of trial and error is typically a part of the method development process.

It is essential to have a fundamental grasp of the sample's properties, including its structure, polarity, volatility, stability, and solubility parameter, in order to use HPTLC to analyse pharmaceuticals in multicomponent dosage forms. The process of developing a method frequently involves a lot of trial and error.

Choosing the starting point is frequently the most difficult component, especially in terms of picking the right mobility phase. Either the fluorescence mode or the absorbance mode can be used to identify analytes. However, a number of strategies can be used if analyte detection is subpar. Examples include altering the mobile phase, stationary phase, or using derivatization techniques prior to or after chromatography.

It is possible to alter the stationary phase, the chromatographic system's immobile component, to improve detection. This may entail utilising a different kind of column altogether, altering the composition of the stationary phase, or changing its kind. It is feasible to improve the detection of the target analytes by choosing and they would benefit more from a fixed phase.

The mobile phase, which transports the analytes through the chromatographic apparatus, can also be altered to enhance detection. Analyte separation and detection can be affected by changing the buffer concentration, pH, or solvent composition. It is feasible by enhancing the mobile phase conditions, the detection method's sensitivity and selectivity may be increased.

Techniques for pre- or post-chromatographic derivatization may be required in some circumstances to make analyte detection easier. To improve the analytes' detectability, derivatization entails chemically altering them either before or after the chromatographic separation. By adding particular reagents that react with the analytes to form fluorescent or absorbing compounds that make them easier to detect, this can be accomplished.

In general, it is advised to investigate adjustments in the stationary phase, mobile phase, or take into account applying pre or post chromatographic derivatization procedures if the detection of analytes utilising fluorescence or absorbance modes is not optimal. The precision and sensitivity of analyte detection in chromatographic analyses can be considerably increased by using these methods.

Table 1: Comparison between HPTLC and TLC on the Basis of Parameters (Patel and Patel 2008)

Dimensions	HPTLC	TLC
Mean particle size	5 to 6 mm	10 to 12 mm
Technique	Automated&instrumental	Manual
Layer thickness	100 mm	250 mm
Plate height	12 mm	30 mm

Efficiency	Due to the lower particle size that was produced,	Less
Analysis time	There is a 3-5 cm reduced migration distance and a considerable reduction in analysis time.	Slower
Separation	3 to 5 cm	10–15 cm
Solid support	a variety of stationary phases are available, for reversed-phase modes, C8, C18, and silica gel are included.	Silica gel, Kiesulguhr&alumina
Development Chamber	a novel kind that requires less mobile phase	New type that requires less amount of mobile phase
Sample Spotting	Automatic spotting	Manual spotting
Starting Spot's	1to 1.5 mm	3to 6 mm
Sample Volume	0.1to 0.5 ml	1 to5 ml
Separated spot's diameter	2to 5 mm	6to 15 mm
Sample tracks/plate	<36 [72]	<10
Scanning	A more advanced kind of densitometer called a UV/visible/fluorescence scanner may be used to qualitatively and quantitatively scan the whole chromatogram.	Impossible
Detection limits [Absorption]	100 to 500 pg	1 to 5 ng
Time of separation	3 to 20 min	20 to 200 min

1. **Stationary Phase:** The most advanced TLC technique now used is known as HPTLC. It make use of specialised plates that have a tiny particle size distribution. This property makes it possible to build homogeneous layers with smooth surfaces. HPTLC uses smaller plates than conventional TLC, often measuring 10x10 or 10x20 cm, which leads to shorter analysis periods (between 7 and 20 minutes) and development distances of about 6 centimetres. HPTLC plates provide higher in situ quantification capabilities, increased resolution, and improved detection sensitivity. They find extensive application in the industrial pharmaceutical sector for densitometric quantitative analysis.

   Over 90% of documented cases involving pharmaceutical and Silica gel plates with less polar mobile phases, such as chloroform-methanol, are used in conventional phase adsorption TLC for drug analysis. Reversed-phase TLC also makes use of lipophilic C-18, C-8, or C-2, hydrocarbon-impregnated ceramic silica gel plates, and silica gel phases containing phenyl chemical alterations. In reversed-phase TLC, a more polar water mobile phase, such as methanol-water and dioxane-water, is used. Some types of precoated layer that are available for TLC include those containing bonded amino, cyano, diol, & thiol groups, as well as magnesium silicates, magnesium oxide,

polyamide, a cellulose, kieselguhr, & polar modified silicate gel layers. To separate optical isomers, specialised chiral layer are employed. These include C-18 modified silica gel plates loaded with a Cu (II) salt and an optically active, enantiomerically pure hydroxyproline derivative, or cellulose with mobile phases augmented with chiral selectors such cyclodextrins. Silica layer coated with a chiral selection like the brucine are another illustration. The primary use of these approaches is the separation of the amino acids & their derivatives (Sherma 2007).

2. **A Prewashing Layer:** In order to prevent contamination, plates are typically handled only at their upper edge. Most of the time, plates are utilised without any preparation unless chromatographic contamination causes impurity fronts. However, the layers are frequently prewashed for quantitative analysis and repeatability testing. Methanol is a typical prewashing solvent, and a twin-trough chamber (TTC) measuring 20 cm x 10 cm usually uses 20 cc of methanol per trough. As an alternative, the method's mobile phase or a methanol & ethyl acetate combination can be employed as a prewashing solvent. In every trough of the TTC, a maximum of two 20 cm x 10 cm as well four 10 cm x 10-centimetre plates can be grown back-to-back. The plate has to be removed after development & baked for a period of twenty minutes at 120 degrees Celsius in a sanitised drying oven. The plate should then be adjusted to the laboratory environment, including the temperature and relative humidity, to ensure accuracy and consistency. To accomplish this, put the plate in a suitable container that shields users from dust and pollutants.

Table 1.2: Sample Application Parameters on HPTLC plate

Dimension	HPTLC
The distance between TLC and the plate's lowest edge	8 mm
Measurement from the plate's lower border to the centre of the HDC, or horizontal development chamber	5 mm
initial track's x-position	15mm
maximum application spot diameter	5mm
Minimum separation between bands or dots	2mm
Band length	8mm
Maximum number of grooves on a 20 10 centimetre plate	16
Maximum number of tracks on a 10 10 centimetre plate	7

3. **Cellular Phase:** The adsorbent material used as the stationary stage and the analyte's physical and chemical characteristics are only a few of the factors that influence the selection of the mobile stage for chromatographic analysis. Numerous mobile-phase systems are employed based on their varied selectivity properties. Diethyl ether, methylene chloride, & chloroform are popular mobile-phase solutions utilised in normal-phase thin-layered chromatography (TLC), either alone or in conjunction of hexane as strength-adjusting solvents. Water, methanol, acetonitrile, & tetrahydrofuran are frequently mixed in the mobile phase of reversed-phase TLC, along with a solvent for strength adjustment.

When utilising ion pair on C-18 layers, the mobile phase used may include 25 mM Sodium pentanesulfonate (15.5:4.5) added to a combination of methanol & 0.1 M acetate buffer (pH 3.5). The mobile-phase components must be independently volumetrically determined with the proper volumetric glasses. The elements are tossed together to ensure appropriate mixing. A suitable micropipette can be used to measure volumes under 1 ml, and a graduated volumetric pipette of the right size can be used to measure amounts up to 20 ml. An appropriate-sized graduated cylinder is used for amounts more than 20 ml. Developmental solvents should be prepared in quantities adequate for one working day to reduce volume measurement mistakes.

4. **Sample Preparation and Application:** Samples must be correctly prepared in order to carry out the technique of separation. When the amount of analyte is high enough, pharmaceutical dosage forms may be dispersed in an appropriate solvent that fully dissolves the analyser while leaving excipients that or other undesired components undissolved. This approach yields a test solution that is immediately suitable for HPTLC analysis. Comparatively speaking, HPTLC sample preparation is simpler than that for other chromatographic techniques. However, if the amount of analyte in the sample is low, more processes may be needed, such as grinding, sonication, filtering, extraction, centrifugation, & concentration methods.

   It is frequently possible to use less refined samples because HPTLC layers are not frequently reused. Solvents that are nonpolar and flammable, such as methanol, ethanol, and chloroform should be used to dissolve the material. For sample application, automated equipment is easily available, especially for quantitative HPTLC. With the Automated TLC Sampling (ATS) 4 and Linomat 5, it is suggested to employ the spray-on technique. The Nanomat or ATS 4 can be utilised for spot application through touch. Using this method, the sample ought to be dissolved in the solvent at the least suitable solvent concentration. To get the highest resolution & sensitivity in a certain chromatographic separation, it is recommended to apply the sample as thin bands. However, it is essential to limit the sample band's spreading during application. Typically, a syringe is used to hold the sample, and a motor is used to empty, while being electronically controlled in terms of delivery volume and speed (refer to Table 1.2 for more details).

5. **Development of Chromatogram:** Although the creation of a chromatogram is an essential phase in the TLC process, some vital factors are frequently ignored. When using HPTLC, the separations are affected by the phase of vapour, which relies upon the kind, size, & degree of saturation of the chamber used during development. Reliable and reproducible TLC separations depend on the control of interactions among these three phases as well as other elements including temperature and relative humidity. HPTLC plates are typically produced in horizontal growth chambers, twin-trough chambers, and flat-bottom chambers.

   To develop plates in a saturated Thin Layer Chromatography (TLC) chamber using TTC (2,3,5-triphenyltetrazolium chloride), follow these steps:

   - First, prepare the desired quantity of mobile phase. Put an inch of filter paper of the right size in the TLC chamber's back trough.

- Gently pour the liquid phase in the TLC chamber, ensuring that the filtration paper is wet and adheres to the rear wall.
- Tilt the chamber used for TLC to one side at a 45-degree angle to allow the amount of solvent in both troughs to equalise.
- After replacing the lid, place the chamber on the bench and give it 20 minutes to equilibrate.
- Line the correct edge of the plate with TLC with a pencil to indicate the required development distance (for instance, 70 mm from the plate's lower edge).
- With the layer & the filter paper opposite one another & the rear of the plate's surface resting on the front wall of the TLC chamber, slide the cover to the side and place the plate containing the TLC in the front trough.
- 7. After replacing the cover, continue working on the plate until solvent front has travelled the designated distance.
- Remove the plate from the chamber, align the chromatography direction, and dry it vertically in a stream of cold air for 5 minutes.
- Discard the leftover mobile phase and filter paper after each development.
- After each development, throw away the filter paper and remaining mobile phase.

6. **Detection:** Once the mobile stage has been thoroughly heated off the created plate, several zones may be visible on the layer. These zones can be identified by their natural colour, fluorescence, fluorescence quenching, or by the formation of coloured, UV-absorbing, and fluorescent zones as a consequence of reagent reaction (a procedure referred to as post chromatographic derivatization).

   UV lights with wavelengths of 254 nm and 366 nm are utilised in cabinets to view zones with fluorescence or zones with quenched fluorescence. The sample is not harmed by detection under UV light; hence it is chosen. The capability to use a diversity of methods for zone detection &identification is one of the main benefits of offline thin-layer chromatography (TLC).

   After the produced layer has been assessed under both long- & short-wave UV light, any of the chromogenic, fluorogenic, and biological detection methods may next be applied. There are many reagents and detection techniques that can be used, and descriptions of these techniques can be found in a variety of literature sources, giving TLC users a wide range of options for zone detection and identification.

7. **Derivatization:** Derivatization is typically required to aid in the visualisation of the target analyses. Derivatization can be done in two ways: by either spraying or submerging the plates in the appropriate reagent. For increased repeatability, immersion-based the derivatization is the suggested technique. The derivatization reaction may occasionally need to be induced or optimised by heating the plates. For this stage to produce consistent results, it is critical to identify the prerequisites and time frame.

8. **Immersing:** There is 200 ml of reagent in the immersion device tank. Place the plate in the immersion device's holder, set the parameters in accordance with the recommended procedure, and push the start button to begin the process. Let any surplus reagent drop off the plate before using a paper towel to gently wipe the back of the plate. The plate should

be taken out of the holder, dried with a cold air burst, and then positioned vertically in the chromatography direction.

9. **Spraying:** Only 50 ml of the reagent should be added to the sprayer container to charge it. The plate should be positioned within the spray cabinet such that it is up against some filter paper. Continue to spray the plate in both horizontal and vertical directions, making sure the reagent is dispersed equally over the plate's surface. In order to finish drying the plate, usage cold air.

10. **Heating**
    - Switch on the surface heater & modify the settings.
    - After the internal temperature has stabilised, carefully lay the serving platter on the heating surface.
    - Permit the plate to stay on the heater for the allotted amount of time.
    - After the stipulated period of time has gone, carefully remove the plate that was heated from the heater.

11. **Quantification:** In modern usage, High-Performance thin-layered chromatography (HPTLC) quantitative analyses are performed in situ, and the sample and reference zones are measured using a chromatogram spectrophotometer, which is additionally referred to as a densitometer or scanner. This device makes use of a constant sample light beam with a rectangular slit. The quantitative evaluation is typically finished using an TLC Scanner 3 device and win CATS application for data processing. Since spectra recording may be done quickly, it facilitates effective data collection. Both single and multiple levels can be accommodated during the calibration process, which uses linear or nonlinear regressions as needed. When confirming goal values single layer calibration is ideal for dissolution profiles and stability testing. The initial sample volume and dilution parameters are considered in the calculation to calculate the analyte concentration in a sample.

12. **Documentation:** Utilising a digital documentation system, each individual plate is subjected to documentation using three different forms of light: UV radiation at 254 and 366 nm, as well as white light. This result is appropriately recorded if a particular light fails to produce information that is useful. Additionally, pictures are taken both before and after derivatization if a plate is subjected to it.

13. **Validation of Method:** The process of verifying a method of analysis cannot be separated from its creation since until verification has been completed, the analyst will not be able to assess whether the created technique or its performance characteristics are acceptable. Any analytical procedure's suitability for the purpose for which it is designed must be determined by objective validation data. Validation is a critical stage in assessing the dependability and repeatability of the technique since it helps confirm that the proposed method is suitable to be utilised on a certain system. Verification in analytical labs is required by several regulations and standards, which is crucial. These include the International Conference of Harmonisation (ICH), good clinical practises (GCP), good laboratory practises (cGMP), and current good manufactured practises (cGMP). Additional regulatory requirements are imposed by the United States Pharmacopoeia (USP), the Food and Drug Administration (FDA), the Environmental Protection Agency

(EPA), along with various quality as well as accreditation norms like the International Standard Organisation (ISO) 9000 sequence, an ISO 17025, the European Norm (EN 45001), or others. Three things are necessary for the confidence of analytical data: the reliability of the tools used, the solidity of the methodologies used, and the appropriate training for the analysts engaged. Various validation criteria are frequently tracked to assure dependability. These parameters include empathy (the capacity to measure relatively small concentration variations), robustness (the results when the method is complemented by different analgesics (K)), selectivity, equilibrium prior to, during, and following growth, linearity of the calibration chart, the range of the amount over which the substance being measured can be quantified, restricts of detection for precise and accurate quantification, accuracy (which indicates systematic errors), precision (which suggests random mistakes), and accuracy (indicating systematic errors).

14. **Specificity:** In order to discover any potential interference from formulation constituents, the sample solutions are analysed to determine the specificity of the devised approach. By contrasting the sample spot's the authenticity of the specimen's spot is confirmed by comparing the values of the retardation factor (Rf) with the values of the standard.

15. **Sensitivity:** Its method's sensitivity is evaluated based on its limits of detection, or LOD, and quantification (LOQ). Scanning a blank spot (solvent) six times yields the noise level. A series of drug solutions at various concentrations are placed to a plate and analysed to get the LOD and LOQ. The noise level is multiplied by three to get the LOD, then by ten to determine the LOQ. The known sample concentrations are diluted to empirically confirm the LOD and LOQ, the average answers must be about 3-10 time the average or standard deviation (SD) for the responses obtained from six repeat measurements.

16. **Accuracy:** A three-level recovery study is used to gauge the method's accuracy. The preanalyzed formulations are mixed with three different concentrations of a standard medicine (80%, 100%, and 120% of the drug) to conduct the recovery studies. Six more analyses are performed on the resultant mixes.

17. **Precision:** Measuring both intraday & interday precisions enables the analysis of precision. Using calibration curve solutions, sample of the biomarker at low, medium, as well as elevated concentrations are evaluated to determine the intraday precision. The examination of accuracy is made possible by measuring simultaneous intraday and interday precisions. To measure the intraday precision, specimens of the analyte at medium, low, and elevated levels are assessed using calibration curve solutions.

18. **Repeatability:** The repeatability of the peak area measures is evaluated by examining data from tests on various concentrations of analyte in the low, middle, and elevated regions of the calibration curve. Seven times of this procedure are performed while keeping the plate in the same place. Similarly, by identifying samples that have a comparable range to the calibration curve, the repeatability of sample application is evaluated. To assess the repeatability, these samples are spotted seven times, and each spot is examined once.

19. **Retardation Factor:** According to the supplied formula, the factor of retardation (Rf) measures the degree of separation brought on by the solvent's passage through the sorbent layer. The course of development time + the solvent front's velocity, also known as the velocity coefficient, determines its value.

$$Rf = \frac{\text{Migration distance of the substance}}{\text{Length of solvent front migration from source}}$$

20. **Peak Purity:** The comparison of the spectra at the top's start (s), peakiest point (m), & peakiest point (e) reveals the purity of the peak. Cross-referencing the slope of the peak's maximum spectra with the peak's initial slope is done as part of the purity test. A reference point for statistical calculations is provided by the correlation [r(m, e)] between the spectra collected at the peak's maximum and those from the peak's downward slope or peak end. The smallest test value that is considered acceptable is 2.576, according to the reference spectra for statistical computation (Patel et al. 2008). This value is crucial for evaluating various validation parameters, and the summarized acceptance criteria can be found in Table 1.3.

Table 1.3: Basic Standards for Judging the Effectiveness of Validation Studies have been Established (Ferenczi-Fodor Et Al. 2001; Pa Tel Et Al. 2010).

Parameter-Characteristics	Testing for impurities Acceptance standards	Assay
Remaining plot	No pattern	No pattern
RSD residuals level		<1.5%
	Impurity level <0.5%	< 10%
	Impurity level <0.5%	< 5%
Intercept Y-axis	<25%	<2%
Precision Repeatability	Impurity level 0.1–0.2%	RSD < 20%
Correlation coefficient	r > 0.99	r > 0.998
	Impurity level 0.1–0.2%	RSD <5%
	Impurity level 0.1–0.2%	RSD <10%
Range	quantification limit to 120% of the stated impurity limit	80–120%

III. CONCLUSION

High-Performance Thin-Layer Chromatography (HPTLC) stands as enhanced and versatile technique for substance screening, offering numerous advantages that make it an indispensable tool in various fields. Its ability to separate, identify, and quantify a wide range of compounds with high precision and sensitivity makes it invaluable in pharmaceuticals, food and beverage analysis, forensic sciences, and environmental monitoring. The rapid analysis time, cost-effectiveness, minimal sample preparation, and ability to analyze complex mixtures further underline its significance. HPTLC's adaptability to different detection methods and compatibility with various sample types make it an attractive choice for researchers and analysts alike. As technology continues to evolve, HPTLC remains a reliable and robust option for substance screening, contributing significantly to the advancement of scientific research and analytical capabilities.

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