**HPTLC (High-Performance Thin-Layer Chromatography): An Enhanced Technique for Substances Screening**

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**Abstract**

High-Performance Thin-Layer Chromatography (HPTLC) is an advanced analytical technique that provides exceptional separation and qualitative and quantitative analysis capabilities for a variety of compounds, including herbal and botanical dietary supplements, nutraceuticals, conventional western medicines, conventional Chinese medicines, and Ayurvedic (Indian) medicines. Numerous articles have emphasised HPTLC's advantages over High-Performance Liquid Chromatography (HPLC) in terms of efficiency and turnaround time for analyses. 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 in the same manner on the same plate for improved accuracy and precision of quantification, various selective detection techniques, and sequential in situ spectral recording for positive results are some of its key features. Additionally, HPTLC enables unlimited storage of the full sample on the layer, minimising exposure risks and drastically reducing disposal problems with toxic organic effluents, hence lowering risks of environmental pollution. Given these benefits, HPTLC-based techniques are becoming important tools for regular investigation.

**Introduction**

A more sophisticated and automated variation of thin-layer chromatography is known as high-performance thin-layer chromatography (HPTLC). This contemporary instrumental method works well for both qualitative and quantitative analytical needs. 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. Ultra-high purity requirements or low UV transparency do not place restrictions on the choice of solvent for the mobile phase. You can utilise corrosive and UV-absorbing mobile phases.
6. A wide range of stationary phases with unique selectivity for mixture components is available. 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 sample fractions are maintained on the TLC/HPTLC plate, 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 picogram (pg) range.
15. There are numerous general and specialised detection methods available.
16. Samples and standards are chromatographed and quantified under the same experimental conditions on a single TLC/HPTLC plate, resulting in high quantification accuracy and precision. (Patel et al., 2010)

**HPTLC Methodology**

The analytical goal, which may involve the quantitative or qualitative identification of components, the separation of two components or multicomponent mixtures, or the optimisation of analysis time, must be determined before HPTLC is started. 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. 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. Modification of the stationary phase, mobile phase, or use of pre- or post-chromatographic derivatization methods are some examples.

The stationary phase, which is the immobile component of the chromatographic system, can be changed to enhance 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 a stationary phase that is more appropriate for them.

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 to improve the sensitivity and selectivity of the detection method by optimising the mobile phase conditions.

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)**

|  |  |  |
| --- | --- | --- |
| Parameters | HPTLC | TLC |
|  Mean particle size | 5–6 mm | 10–12 mm |
| Technique | Automated/instrumental |  Manual |
| Layer thickness | 100 mm | 250 mm |
| Plate height | 12 mm |  30 mm |
| Efficiency | High due to smaller particle size generated | Less |
| Analysis time | 3–5 cm shorter migration distance and significantly less time is spent on analysis |  Slower |
| Separations | 3–5 cm | 10–15 cm |
| Solid support | a variety of stationary phases are available, including silica gel for normal phase and C8, C18 for reversed-phase modes. | Silica gel, Kiesulguhr and 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 |  1–1.5 mm | 3–6 mm |
| Sample volume | 0.1–0.5 ml | 1–5 ml |
| Separated spot’s diameter | 2–5 mm | 6–15 mm |
| Sample tracks per plate | <36 (72) | <10 |
| Scanning | Utilising a UV/visible/fluorescence scanner, a more sophisticated type of densitometer, allows for qualitative and quantitative scanning of the complete chromatogram. | Not possible |
| Detection limits (absorption) |  100–500 pg | 1–5 ng |
|  Separation time | 3–20 min | 20–200 min |

**Stationary Phase**

High-Performance Thin Layer Chromatography, or HPTLC, is the most sophisticated version of current TLC methods. It makes 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 cm.

HPTLC plates offer enhanced resolution, improved detection sensitivity, and superior in situ quantification capabilities. They find extensive application in the industrial pharmaceutical sector for densitometric quantitative analysis.

Over 90% of documented cases involving pharmaceutical and drug analysis use normal phase adsorption TLC using silica gel plates and less polar mobile phases like chloroform-methanol. Additionally, silica gel phases with phenyl chemical modifications, hydrocarbon-impregnated silica gel plates, and lipophilic C-18, C-8, and C-2 are used in reversed-phase TLC. A more polar aqueous mobile phase, like methanol-water or dioxane-water, is used in reversed-phase TLC. Aluminium oxide, magnesium silicate, magnesium oxide, polyamide, cellulose, kieselguhr, ion exchangers, and polar modified silica gel layers with bonded amino, cyano, diol, and thiol groups are some of the precoated layers that are available for TLC.

Specialised chiral layers are used for optical isomer separations. These include cellulose with mobile phases supplemented with chiral selectors like cyclodextrins or C-18 modified silica gel plates impregnated with a Cu (II) salt and an optically active enantiomerically pure hydroxyproline derivative. Other examples include silica layers impregnated with a chiral selector like brucine. The separation of amino acids and their derivatives is the main application of these techniques (Sherma 2007).

**Layer Prewashing**

Plates are normally handled solely at their upper edge to avoid contamination. 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 20 cm x 10 cm twin-trough chamber (TTC) usually uses 20 cc of methanol per trough. As an alternative, the method's mobile phase or a mixture of methanol and ethyl acetate can be employed as a prewashing solvent. It is possible to grow up to two 20 cm x 10 cm or four 10 cm x 10 cm plates back-to-back in each trough of the TTC. Following development, the plate must be removed and dried for 20 minutes at 120°C 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 provides protection from fumes and dust.

**Table 1.2 Sample application parameters on HPTLC plate**

|  |  |
| --- | --- |
| Parameter | HPTLC |
| TLC's distance from the plate's lower edge | 8 mm |
| Measurement from the plate's lower border to the centre of the horizontal development chamber (HDC) | 5 mm |
| x-position of first track | 15mm |
| Maximum diameter of application spot | 5mm |
| Minimum space between bands/spots | 2mm |
| Band length | 8mm |
| Maximum number of tracks on a 20 10 cm plate | 16 |
| Maximum number of tracks on a 10 10 cm plate | 7 |

**Mobile Phase**

The adsorbent substance employed as the stationary phase and the physical and chemical properties of the analyte are just a couple of the variables that affect the choice of mobile phase for chromatographic analysis. Based on the various selectivity features of the mobile-phase systems, many mobile-phase systems are used. Common mobile-phase systems used in normal-phase thin-layer chromatography (TLC) include diethyl ether, methylene chloride, and chloroform, either alone or in combination with hexane as a strength-adjusting solvent. The mobile phase for reversed-phase TLC commonly consists of water combined with methanol, acetonitrile, and tetrahydrofuran, with the inclusion of a strength-adjusting solvent.

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When using ion pairing on C-18 layers, the mobile phase may consist of a mixture of methanol and 0.1 M acetate buffer (pH 3.5) with an additional 25 mM sodium pentanesulfonate (15.5:4.5). It is essential to undertake independent volumetric measurements of the mobile-phase components using the appropriate volumetric glassware. 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.

**Sample Preparation and Application**

To successfully perform a High-Performance Thin-Layer Chromatography (HPTLC) separation, samples must be properly prepared. Pharmaceutical dosage forms can be dissolved in a suitable solvent that completely solubilizes the analyte while leaving excipients or other undesirable components undissolved when the analyte concentration is high enough. A test solution produced by this procedure can be used as a spot for HPTLC analysis right away. Sample preparation for HPTLC is comparatively easier than for other chromatographic methods. However, multiple more stages, including as grinding, sonication, filtration, extraction, centrifugation, and concentration techniques, can be required if the analyte concentration in the sample is low.

It is frequently possible to use less refined samples because HPTLC layers are not frequently reused. Nonpolar and volatile solvents like methanol, ethanol, or chloroform should be used to dissolve the material. For sample application, automated equipment is easily available, especially for quantitative HPTLC. It is advised to use the spray-on method with the Automatic TLC Sampler (ATS) 4 or Linomat 5. The Nanomat or ATS 4 can be utilised for spot application through touch. The sample should be dissolved using this procedure in the solvent with the smallest appropriate solvent concentration. Application of the sample in the form of thin bands is preferable to achieve the best resolution and sensitivity in a particular chromatographic separation. However, it is essential to limit the sample band's spreading during application. The sample is typically contained in a syringe, which a motor empties while being electronically controlled in terms of delivery volume and speed (refer to Table 1.2 for more details).

**Development of Chromatogram**

Although the creation of a chromatogram is an essential phase in the TLC process, some vital factors are frequently ignored. The vapour phase, which depends on the kind, size, and saturation level of the chamber employed during development, affects the separations made when performing HPTLC. Controlling the interactions between these three phases, as well as variables like temperature and relative humidity, is crucial for reliable and repeatable TLC separations. Usually, horizontal development chambers, twin-trough chambers, or flat-bottom chambers are used to generate HPTLC plates.

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

1. Prepare the necessary amount of mobile phase first. Place an appropriately sized piece of filter paper into the rear trough of the TLC chamber.
2. Carefully pour the mobile phase into the chamber, making sure the filter paper is well-moisturized and clings to the TLC chamber's back wall.
3. Allow the solvent volume in both troughs to equalise by tilting the TLC chamber to the side at a 45-degree angle.
4. After replacing the lid, place the chamber on the bench and give it 20 minutes to equilibrate.
5. Use a pencil to mark the right edge of the TLC plate with the necessary development distance (for example, 70 mm from the lower edge of the plate).
6. Sliding the lid to the side, insert the TLC plate into the front trough with the layer and filter paper facing one another and the back of the plate resting on the TLC chamber's front wall.
7. Replace the cover, then continue to develop the plate until the solvent front has travelled the designated distance.
8. Remove the plate from the chamber, align the chromatography direction, and dry it vertically in a stream of cold air for 5 minutes.
9. Discard the leftover mobile phase and filter paper after each development.
10. After each development, throw away the filter paper and remaining mobile phase.
11. **Detection**

Different zones can be seen on the layer after the mobile phase has been heated off the formed plate. These zones can be recognised by their inherent colour, fluorescence, quenching of fluorescence, or by the emergence of coloured, UV-absorbing, or fluorescent zones as a result of reagent reaction (a process known as postchromatographic derivatization).

Cabinets with short-wave (254 nm) and long-wave (366 nm) UV lamps are used 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 ability to use a variety of methods for zone detection and identification is one of the main benefits of offline thin-layer chromatography (TLC).

One or more chromogenic, fluorogenic, or biological detection techniques may then be used once the generated layer has been evaluated under both long- and short-wave UV light. 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.

**Derivatization**

Derivatization is typically required to aid in the visualisation of the target analytes. Derivatization can be done in two ways: by immersion or by spraying the plates with the proper reagent. Immersion-based derivatization is the recommended method for improved reproducibility. 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.

**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. Allow any extra reagent to drip off the plate, and then carefully clean the plate's back with a paper towel. Remove the plate from the holder, then dry it with a spray of cold air while positioning it vertically in the chromatographic direction.

**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, use cold air.

**Heating**

1. Turn on the plate heater and adjust the temperature.
2. Carefully place the plate on the heating surface after the temperature has stabilised.
3. Permit the plate to stay on the heater for the allotted amount of time.
4. Carefully remove the heated plate from the heater once the allotted amount of time has passed.

**Quantification**

A chromatogram spectrophotometer, also known as a densitometer or scanner, is used to quantify the sample and reference zones while doing High-Performance Thin Layer Chromatography (HPTLC) quantitative tests in situ in contemporary practise. This gadget uses a rectangular slit-shaped fixed sample light beam. Typically, the TLC Scanner 3 instrument and winCATS software for data analysis are used to complete the quantitative assessment. 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 for stability testing and dissolution profiles, single level calibration is very suitable. The initial sample volume and dilution parameters are considered in the calculation to calculate the analyte concentration in a sample.

**Documentation**

Utilising a digital documentation system, each individual plate is subjected to documentation using three different forms of light: white light, UV light at 254 nm, and UV light at 366 nm. 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.

**Validation of Method**

Since the analyst won't be able to determine whether the produced technique and its performance parameters are acceptable until validation has been carried out, the process of validating an analytical method cannot be isolated from its development. Any analytical procedure's adequacy for the analysis for which it is intended must be based on objective validation data. Because it can ensure that the planned method is appropriate to be used on a certain system, validation is a crucial step in evaluating the dependability and reproducibility of the method. Various laws and standards require validation in analytical labs, which is essential. These include current good manufacturing practises (cGMP), good laboratory practises (GLP), and good clinical practises (GCP), as well as the International Conference on Harmonisation (ICH). The United States Pharmacopoeia (USP), Food and Drug Administration (FDA), Environmental Protection Agency (EPA), and other quality and accreditation standards like the International Standards Organisation (ISO) 9000 series, ISO 17025, the European Norm (EN 45001), and others impose additional regulatory requirements. The dependability of analytical data relies crucially on three factors: the trustworthiness of the instruments employed, the soundness of the methods utilized, and the adequate training of the analysts involved. To ensure reliability, various validation parameters are typically monitored. These parameters include selectivity, stability before, during, and after development, linearity of the calibration graph, the range of levels over which the analyte can be quantified, limits of detection for accurate and precise quantification, accuracy (indicating systematic errors), precision (indicating random errors), sensitivity (the ability to measure small concentration variations), and ruggedness (the outcomes when the method is used by different analgesics (Koll et al. 2003; Reich et al. 2008; Patel et al. 2009; Patel et al. 2010).

**Specificity**

In order to discover any potential interferences from formulation constituents, the sample solutions are analysed to determine the specificity of the devised approach. By contrasting the sample spot's retardation factor (Rf) values with those of the standard, the validity of the sample spot is verified.

**Sensitivity**

Based on the limits of detection (LOD) and quantification (LOQ), the method's sensitivity is assessed. 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 until the average responses are around 3–10 times the standard deviation (SD) of the responses from six repeat measurements in order to experimentally validate the LOD and LOQ.

**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.

**Precision**

The examination of precision is made possible by measuring both intraday and interday precisions. To assess the intraday precision, samples of the analyte at low, medium, and high concentrations are examined using calibration curve solutions. Measuring both intraday and interday precisions enables the analysis of precision. Using calibration curve solutions, samples of the analyte at low, medium, and high concentrations are evaluated to determine the intraday precision.

**Repeatability**

By analysing data from analyses on different analyte concentrations in the low, medium, and high ranges of the calibration curve, the repeatability of peak area measurements is assessed. 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.

**Retardation Factor**

The retardation factor (Rf) quantifies the extent of separation resulting from the movement of the solvent through the sorbent layer, as depicted in the given formula. Its value relies on the development time and the velocity coefficient or the velocity of the solvent front.

 Rf = Migration distance of substance

 Migration distance of solvent front from origin

**Peak Purity**

Peak purity is determined by comparing the spectra at three specific locations: the peak's start (s), peak's peakiest point (m), and peak's peakiest point (e). In the purity test, the peak's maximum spectrum is cross-referenced with the spectrum recorded during the peak's starting slope. 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 acceptance criteria for evaluation of validation experiments (Ferenczi-Fodor et al. 2001; Patel et al. 2010)**

|  |  |  |
| --- | --- | --- |
| Characteristics – parameter | Impurity testingAcceptance criteria | Assay |
| Residual plot | No trend | No trend |
| RSD residuals |  | <1.5% |
|  | Impurity level <0.5% | < 10% |
|  | Impurity level <0.5% | < 5% |
| Y-axis intercept | <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 | From limit of quantification to 120% of the specified limit of impurity | 80–120% |

**Conclusion**

High-Performance Thin-Layer Chromatography (HPTLC) stands as an 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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