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Review Article

Analytical Method Development and Validation by HPTLC

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ABSTRACT

High Performance Thin Layer Chromatography (HPTLC) is an advanced form of Thin Layer Chromatography (TLC), used for both qualitative and quantitative analysis of compounds. It offers better separation, sensitivity, and automation compared to traditional TLC. HPTLC works on the principle of differential migration, where substances are separated based on their interaction with the stationary phase (usually silica gel) and a chosen mobile phase (solvent system). Key components of HPTLC instrumentation include an auto-sampler, developing chamber, digital imaging system, and a TLC scanner, all of which improve accuracy and efficiency. The method development process includes steps such as sample preparation, selection of stationary and mobile phases, plate pre-washing, sample application, chromatogram development, and detection. Detection is done using UV light or chemical derivatization, followed by quantification using a densitometer. Validation of the HPTLC method ensures reliability and includes parameters such as linearity, precision, accuracy, specificity, sensitivity, robustness, and limits of detection and quantification. Overall, HPTLC is a powerful tool widely used in pharmaceutical, clinical, and herbal analysis due to its high accuracy, flexibility, and efficiency.

INTRODUCTION

HPTLC is an effective analytical method that is equally suitable for qualitative and quantitative analytical tasks [1,2] High performance thin layer chromatography [HPTLC] is a more complex and automated version of thin layer chromatography [TLC] with involved and enhanced detection limits and separation efficiency. It is sometimes referred to as flatbed chromatography, planer chromatography, or high-pressure thin layer chromatography [3]Since its inception in the 1970s, a refined version of conventional TIC has undergone sustained development [4]. Separation can occur due to adsorption, partition, or both, depending on the type of adsorbents used on the plates and the development solvent [5]. Since the operation parameters can only be accepted if the performance requirement is met, method development and validation cannot be separated.

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Because it can verify that the intended results are achieved, validation is an essential step in determining the method's reliability and reproducibility.[6]

PRINCIPLE

The fundamental principle of high-performance thin-layer chromatography is the differential migration of compounds between a stationary and mobile phase.[7]Complex physiochemical interactions, such as adsorption, partition, and capillary action, contribute to the separation efficiency as analytes migrate.[8] The fundamental unit of separation in HPTLC is adsorption, which is based on the same physical principles as in TLC (adsorption chromatography).[9]A modified sorbent material, typically silica gel, with precisely controlled particle size and pore Dimension forms the flat surface on which the separation takes place.[10]Capillary action allows solvent from the mobile phase to flow through. The components move toward the adsorbent in accordance with their affinities. The component with more affinity towards the stationary phase travels slower. The component that has a lower affinity for the stationary phase moves more quickly.[11]

PHASES[12]

STATIONARY PHASE

The interaction between the column and the target molecule is the stationary phase. The HPTLC plate set has smooth layers because the small particles are evenly distributed. The HPTLC plates are about 10x10 or 10x20 cm in size. In a typical TLC, silica gel and a less polar mobile phase. The majority of the compounds are subjected to analysis with methods like chloroform-methanol. Chemically modified lipophilic silica gel phases are combined with methanol-water and other more polar aqueous mobile phases in reversed-phase TLC. The detection's sensitivity and resolution are enhanced by these plates. In addition to the biochemical and clinical sciences, the pharmaceutical industry is beginning to use them for a number of quantitative drug analyses.

MOBILE PHASES

In this phase, the solvent dissolves the target molecule. Thin layer chromatography's mobile phase is determined by the characteristics of the analyte. The selection of the mobile phase is primarily determined by the analyte's physical and chemical characteristics as well as the makeup of the stationary phase. The suggested ether, methylene chloride, and chloroform are the components of the mobile phase of TLC. They can be used alone or in conjunction with hexane. If the device use reversed-phase TLC. Tetrahydrofuran, acetonitrile, or even methanol diluted in water can be used to adjust the strength

1) HPTLC has a high level of detection sensitivity and resolution.
2) High separation efficiency of components.
3) Can detect trace amounts of compounds.
4) Visualization can be performed without staining.
5) Can analyze multiple samples simultaneously.
6) High resolution of complex mixtures.
7) Only minimal sample preparation is necessary.
8) Rapid analysis compared to traditional chromatography.
9) Quantitative and qualitative analysis can be performed.
10) Use of reagents and solvents sparingly

DISDVANTAGES OF HPTLC

1) HPTLC is more expensive than conventional TLC.

2) Requires specialized supplies and equipment.
3) More expensive HPTLC plates
4) Solvents of high purity are required.
5) 5)More complex stationary phase.
6) 6)Testing and quality control raise costs.
7) 7)Skilled technicians are required for optimal results.
8) Equipment calibration and maintenance raise costs.
9) Tools for data analysis and cost-effective software are required.
10) 10)The need for reference materials and standards of the highest quality
11) 11)The cost of technicians' education and training.

INSTRUMENTATION OF HPTLC

AUTO SAMPLER

Using a capillary tube, you would manually locate the sample on a silica plate. However, an HPTLC auto-sampler is a precise instrument that uses nitrogen gas to apply a predetermined amount of sample. Additionally, data regarding the quantity of sample to be applied as well as the number, width, and position of the samples' bands are fed into a computer system that is connected to the autosampler.It is important to keep in mind that, in contrast to TLC, you need to filter the sample with a syringe filter before applying it to the TLC plate. If you don't, the undissolved sample or precipitate might clog the HPTLC syringe,(which could cost you a pretty penny)[15]

Fig No-2 AUTO SAMPLER[16]

DEVELOPING CHAMBER

Usually, a rectangular glass chamber is used to develop HPTLC plates, similar to TLC. The chamber is saturated for about 20–30 minutes by adding the solvent and covering it properly. A filter paper soaked in the solvent system can also be placed inside to help the solvent vapors spread evenly. After saturation, the plate is placed vertically in the chamber, making sure that the sample spots remain above the solvent level. There are two main types of vertical developing chambers – the flat bottom chamber and the twin trough chamber, which has a divider at the base separating it into two parts. The twin trough chamber requires less solvent compared to the flat bottom one. Experts who use HPTLC also recommend using an Automatic Developing Chamber (ADC), where the plate is placed in a pre-saturated chamber, conditioned for a certain period, and then automatically dipped in the solvent. When the solvent front reaches the desired height, the plate is automatically removed. This automation avoids human interference, giving more accurate and reliable results.[15]

FIG N0-3 DEVELOPING CHAMBER[17]

DIGITAL CAMERA FOR PHOTO DOCUMENTATION

Similar to TLC, HPTLC plates are typically developed in a rectangular glass chamber. The older UV cabinets are being replaced by UV cabinets with improved design and UV cabinets that make it possible to install a digital camera and take a picture of the plateIn order to identify plant extracts from botanical reference materials (BRM), identify adulterants or replacement, and study Formulation, HPTLC is currently a fundamental prerequisite for any laboratory engaged in herbal analysis.According to forensic analysts, TLC for the chemical inspection and some initial points[18]

FIG NO 4- DIGITAL CAMERA FOR PHOTO DOCUMENTATION[19]

TLC CAMERA

The TLC Scanner 4 is a scanning densitometer. It determines how well substances are separated when reflected in absorption or fluorescence modes. The produced densitometric data may be quantitatively evaluated thanks to the TLC Scanner 4, which is controlled by vision CATS software, It is possible to choose one or more wavelengths for scanning densitometry from the 190-900 nm range of light. Consequently, detection can be adjusted to match the analyte's ideal specificity and sensitivity to its spectral characteristics. You will select a multiwavelength scan (190 to 900) to choose the simplest wavelength for your molecule. When scanning it for the first time, enter the number nm). Peaks at the simplest wavelength will be sharper than at other wavelengths. You will choose that particular wavelength each time you scan your plate after you have determined which wavelength is best for your compound. After that, the amount of your chemical can be calculated with just a few mouse clicks. The compound's is directly proportionate to its height. By selecting the height, which generates data for each peak within that Rf value, you can display the information (areas) or the peaks (3D view) separately or as an overlay graphic. Additionally, you will even give the peaks names of substance[18]

Figure no 5- TLC scanner [20]

METHOD DEVELOPMENT

STEPS INVOLVED

SAMPLE PREPARATION

A highly concentrated solution is needed because only a small amount of the sample is applied. In normal phase chromatography, where silica gel pre-coated plates are used, the solvents must be volatile or non-polar. In reversed phase chromatography, the sample is usually mixed with polar solvents. The solvents commonly used for sample preparation include ammonia, methanol, chloroform, and ethanoic acid. The more non-polar a compound is, the faster it will elute compared to a polar compound.[21]

SELECTION OF STATIONARY PHASE

The type of compounds that will be separated should guide the selection of the stationary phase during method development.[22] In order to select the stationary phase, the plates are typically washed with methanol and oven-dried to remove excess solvent.[23] HPTLC uses smaller plates with a significantly shorter analysis time (7–20 minutes) and development distance (typically 6 cm)[24]. Modified silica gel plates offer enhanced selectivity for specific applications, making the choice of stationary phase essential.[25]

LAYER PREWASHING

Ascending, dipping, and continuous are all common prewashing techniques. [25]The plates need to be cleaned properly in order to get rid of water vapour or other volatile contaminants. [26]Under white and ultraviolet (UV) light, all plates should be examined for any potential damage or impurities on the layer. Usually, plates are used without pre-treatment unless impurities appear during chromatography due to plate contamination. However, prewashing the plates is recommended to enhance the reproducibility and reliability of quantitative analysis. For prewashing, plates are generally developed with methanol, but mixtures of methanol and ethyl acetate, or even the mobile phase used in the method, can also be applied. After being prewashed, the plates should be dried in a clean drying oven at 120°C for 20 minutes before being equilibrated with the laboratory environment (temperature, relative humidity) in a container that keeps out dust and fumes. [27]

MOBILE PHASE SELECTION AND OPTIMIZATION

The mobile phase is selected based on the physical and chemical properties of the analytes and the type of adsorbent material used as the stationary phase.[28]] The elution power of the mobile phase depends on a property known as eluent strength, which is related to the polarity of the mobile phase components.[29] The mobile phase is typically optimized in a methodical manner, with the initial screening of solvents and subsequent compositional fine-tuning [30] The more non-polar a compound is, the faster it will elute, while the more polar a compound is, the slower it will elute.[31]

SAMPLE APPLI CATION

For quantitative HPTLC, there are automated instruments available for applying samples. The spray-on methods are commonly used with devices like the Linomat 5 or Automatic TLC Sampler (ATS) 4. The sample syringe is typically operated by a motor, and both the volume and delivery speed are electronically controlled. A stream of inert gas like nitrogen is directed around the syringe tip to atomize the sample and produce a band on the TLC/HPTLC plate when either the syringe or the plate moves linearly. Applying samples in bands helps improve separation and enhances densitometric detection. The highest resolution and sensitivity are obtained when the sample is applied as narrow bands. Although circular spots have some disadvantages, the “line” or “band” application offers several advantages.[18]

CHROMATOGRAM DEVELOPMENT

Important parameters are frequently overlooked in the HPTLC process, despite the fact that chromatogram development is the most crucial step. HPTLC plates are developed in twin-trough chambers or horizontal development chambers. In most cases, saturated twin-trough chambers with filter paper offer the highest level of reproducibility. The twin-trough design also helps avoid issues related to solvent vapor preloading and humidity.[32]

PLATE LABELLING

For proper identification, the plates should be clearly labeled in accordance with GMP/GLP guidelines. Typically, a soft pencil or fluorescent pen is used to mark the number in the upper right corner. The sequential plate number, the date of the analysis, and the project number ought to be mentioned on the label. Additionally, Merck offers laser-coded HPTLC silica plates with unique code numbers to support documentation for GLP-conducted analyses.[27]

DERIVATIZATION

Derivatization can be defined as a procedural technique that mainly modifies the functionality of an analyte to facilitate chromatographic separation. It can be carried out either by immersing the plates or by spraying them with an appropriate reagent. Immersion is considered to be the most effective derivatization technique for increasing reproducibility.[33]

DETECTION

Detection under UV light is the first step and is non-destructive. Spots of fluorescent compounds can be observed at 254 nm, which is a short wavelength. Compounds that do not absorb UV can be visualized using a 0.1% iodine solution. Derivatization with a visualizing agent is required if a single component does not respond to UV. The process of quenching fluorescence caused by UV light (200–400 nm) improves the sorbent layer's ability to detect separated compounds. Visualization at UV 254 nm (F254) is described as phosphorescence quenching. After the excitation source is removed in this instance, the fluorescence only lasts for a very brief time—more than ten seconds. Under 254 nm, you should be able to find essential oil compounds like anthraglycosides, coumarins, flavonoids, and propyl phenols. You should also find some alkaloids like indole, isoquinoline, and quinoline types. [34]

DOCUMENTATION

Older UV cabinets are gradually being replaced by more advanced models that make it possible to install a digital camera to capture plate images. Despite not fully complying with GLP, this device is popular in small laboratories. Any lab involved in herbal analysis now considers HPTLC essential for identifying plant extracts by comparing them with extracts from Botanical Reference Materials (BRM) to detect adulterants or substitutes, conduct formulation studies, and more. Forensic analysts often start with TLC for chemical analysis and a microscope for physical examination. When developing a HPTLC method, this step is very important. [18]

QUANTITATION

Generally, quantitative evaluation is carried out by measuring the zones of samples and standards using a densitometer or scanner with a fixed light beam shaped as a rectangular slit. The chromatogram can be scanned in either reflectance or transmittance mode, using absorbance or fluorescence detection, with scanning speeds up to 100 mm/s. Linear and nonlinear regression equations can be used with calibration at single and multiple levels based on the scanned spectra. Scanning is performed using two methods: Slit Scanning and Video Scanning.[35]

METHOD VALIDATION[36]

There are Various Parameter which are Follows

1. LINEARITY AND CALIBRATION CURVE

Five replicate measurements were taken at each concentration to evaluate the linear relationship between peak area and drug concentration over the concentration range, expressed in ng per band. All the solutions were applied, and the chromatograms were recorded at 255 nm.

2. PRECISION

Repeatability and intermediate precision tests were used to assess the developed method's precision. Sample application and peak area measurement were assessed by performing six replicate measurements of an equivalent band using the sample solution. The repeatability of sample application and peak area measurement was expressed as relative standard deviation (%RSD) and was found to be very low.

3. LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION(LOQ)

Using the formulas listed below, the developed method's limits of detection and quantification were calculated using the standard deviation of the y-intercepts and the slope of the medicine's calibration curves. In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank methanol was spotted three times, and the sample was run within the solvent system. Both the detection and quantification limits were determined. LOD was calculated as three times the background level, and LOQ was calculated as ten times the background level. By diluting known drug concentrations until the measured responses were approximately 3–10 times the standard deviation (SD) of the responses from six replicate determinations, LOD and LOQ were experimentally verified.

$$\text{Limit of detection} = 3\alpha/s,$$

$$\text{Limit of quantification} = 10\alpha/s$$

4. SPECIFICITY

The specificity of the developed method was confirmed by analyzing the sample solutions and marketed tablets to check for any interference from the formulation ingredients. The drug spot in the sample was verified by comparing its retardation factor (Rf) value with that of the standard.

5. SENSITIVITY

The limits of detection (LOD) and quantification (LOQ) were used to determine the developed method's sensitivity. The noise was measured by scanning a blank spot (methanol) six times. The LOD and LOQ were determined by applying a series of drug solution concentrations to the plate and analyzing the results.

6. ACCURACY

The accuracy of the method was evaluated by performing recovery studies at three levels. The standard drug was added to the pre-analyzed MME formulations, solutions, and marketed tablets in three different amounts for recovery experiments—80 percent, 100 percent, and 120 percent—and the resulting mixtures were reanalyzed six times.

7. ROBUSTNESS

Small modifications to the mobile phase's volume, composition, chamber saturation time, and solvent migration distance were used to assess the method's robustness. The effects on the results were examined, and the robustness was determined in triplicate at a specified level.

CONCLUSION

HPTLC is a reliable and sensitive method for both qualitative and quantitative analysis of many compounds. It uses advanced tools like auto-samplers, scanners, and digital systems for better results than regular TLC. The process includes sample prep, choosing phases, derivatization, detection, and measurement. It gives fast, accurate, and repeatable results. HPTLC is widely used in pharma, clinical, and forensic fields, and method validation ensures its accuracy, precision, and reliability.

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