High Performance Thin Layer Chromatography (HPTLC)

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INTRODUCTION:

The science of analytical chemistry can be described in simplified terms as the process of obtaining knowledge of a sample by chemical analysis of some kind. The sample under investigation may consist of any solid, liquid or gaseous compound and the result of the analysis is data of some kind that is related to the initial question raised about the sample. From the data obtained in the analysis some knowledge about the sample can be extracted. This knowledge may be either qualitative or quantitative (or both). Examples of qualitative information are types of atoms, molecules, functional groups or some other qualitative measure, while the quantitative information provides numerical information such as the content of different compounds in the sample. Nowadays an analytical chemical analysis generally includes some sort of analytical instrument that performs the actual analysis, while the data processing and instrument control are taken care of by software run on a computer. Hence it is no exaggeration to say that analytical chemistry has become computerised. The shape of the data of analytical chemical analyses has, moreover, changed . From a single sample it is now possible after a very short period of analysis to obtain enormous amounts of data. By means of techniques like ultraviolet-visible (UV-Vis) spectroscopy, fluorescence spectroscopy, infrared (IR) spectroscopy, near infrared (NIR) spectroscopy, Raman spectroscopy, mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR), High performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) large amounts of data on a sample can be collected in a short period of time .

Chemical analysis is an essential component in allowing a laboratory to ensure routine acceptable performance of analytical methods. Despite the considerable amount of important published work on this subject, diversity still prevails in the employed methodologies because validation of an analytical method depends on the specific purpose of that method [4]. This can lead to difficulties in validation approaches and the interpretation of results. Aiming to assist in the planning of validation methods, we discuss relevant approaches of various parameters in quantitative high-performance thin layer liquid chromatographic methods and validation fields in pharmaceutical analysis. Moreover, this article provides full review on HPTLC method development that should be useful as an introduction to analytical validation for practical applications in academic research or the industrial sector.

AUTOMATION OF HPTLC

Modern TLC is widely known and practical as HPTLC, which can only be performed on precoated layers, using instrumentation and mainly for the purpose of quantification. Hence, here the terminology TLC and HPTLC is used interchangeably. To teach the principal of chromatography, almost all over the world, TLC is used. The primary reasons for this choice are visibility of the sample during chromatography, simplicity to perform, and ultra-low-cost apparatus for demonstration. A popular approach for improving resolution under capillary flow-controlled conditions is to use multiple developments. Either one-dimensional or two-dimensional separations are possible in planar chromatography. Mobile-phase velocity can also be controlled by external means, such as in forced-flow development HPTLC are the fastest chromatography method, since chromatography of samples is done in parallel. Being offline i.e., each step of the procedure is performed independently, makes TLC/HPTLC is not only faster but flexible enough for one HPTLC System to analyze different samples in parallel. Consumption of stationary and mobile phase is directly proportional to the number of samples being analyzed

KEY FEATURES AND SEPARATION EFFICIENCY:

Cost per analysis is very low. One 20x10 cm plate can accept about 20 samples and requires 15 ml of mobile phase. Disposable stationary phase in TLC/HPTLC has two distinct advantages in sample preparation. One it is possible to do sample cleanup on the plate itself. The other is that sample cleanup may not be that critical as residue left behind is inconsequential.

Fatty matrix can be cleaned up on the plate itself, after sample application. Investment in equipment is another feature of TLC/HPTLC where it scores. One can start with a simple basic Setup and invest in phases to an ultra-sophisticated fully automatic HPTLC gradient System with multiple detectors. Visibility of the sample throughout the chromatographic analysis i.e., after sample application and chromatograph development, in situ derivatization is unique to HPTLC. Post-chromatography derivatization (PCD) is very simple and routinely possible in any lab. After recording data, a plate can be derivatized to get additional information. PCD is done

for several reasons viz. to detect compounds with a specific functional group or to lower detection limits by up to several orders of magnitude of target fractions or for universal detection of all organic compounds present or to visualize the sample by our eyes. Although Silica gel is by far the most widely used adsorbent (stationary phase), many other adsorbents have been used as the separation medium e.g., reverse phases, bonded phases, alumina, Kieselguhr, etc. Most Solvents can be used in the mobile phase, as the layer is disposable. Gas phase too plays an important role in TLC/HPTLC in the developing chamber's vapor saturation, its pH, and humidity in the developing chamber. TLC and HPTLC can be used for qualitative, semi-quantitative, and quantitative analysis. It can also be used for the identification of industrial fractions after chromatographic separations as well for the identification of herbal extracts, complex mixtures by "HPTLC fingerprint".

Most labs use TLC/HPTLC for impurity analysis, assay, or comparison with similar samples, screening of unknown samples or of a large number of samples. Quality control, analytical R&D, process monitoring, and environmental labs find TLC/HPTLC as a useful tool for everyday analysis.Chromatogram development in TLC/HPTLC can be done with the plate in vertical or horizontal position. It can also be done by linear, circular or anti-circular movement of the mobile phase. Development can also be done in one dimension, with one mobile phase (isocratic) or repeatedly in the same direction with differing mobile phases (gradient). 2D chromatography is very useful for high resolution separations using a different mobile phase in each direction. Chromatographic stability of samples can be studied by 2D technique. However, isocratic linear development in vertical mode is practiced more than 95% of the time

STEPS INVOLVED IN HPTLC:

Selection of chromatographic layer:

- Precoated plates - different support materials - different Sorbents available
- 80% of analysis - silica gel GF · Basic substances, alkaloids and steroids - Aluminium oxide Amino acids, dipeptides, sugars and alkaloids - cellulose
- Non-polar substances, fatty acids, carotenoids, cholesterol
  - RP2, RP8 and RP18
- Preservatives, barbiturates, analgesic and phenothiazine- Hybrid plates-RPWF254s.

Sample and Standard Preparation

- To avoid interference from impurities and water vapours.
- Low signal to noise ratio –Straight base line- Improvement of LOD.
- Solvents used are Methanol, Chloroform: Methanol (1:1), Ethyl acetate: Methanol(1:1), Chloroform: Methanol:Ammonia (90:10:1), Methylene chloride : Methanol (1:1), 1% Ammonia or 1% Acetic acid.

Dry the plates and store in dust free atmosphere

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 Aluminium sheets should be kept in between two glass plates and placing in oven at 110- 120ºC for 15 minutes

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

Selection of mobile phase

- Trial and error.
- One's own experience and Literature based.

Normal phase

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

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

Reversed phase

- Stationary phase is non-polar.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. Multi component mobile phase once used not recommended for further use and solvent Composition is expressed by volumes (v/v) and sum of volumes is usually 100 Twin trough chambers are used only 10 -15 ml of mobile phase is required.

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 of solvent vapours - less solvent for the sample to travel- lower R_f values

Chromatographic development and drying:

- After development, remove the plate and mobile phase is removed from the plate to avoid contamination of lab atmosphere.
- Dry in vacuum desiccators; avoid hair drier, essential oil components may evaporate.
- Chamber Saturation, 20 min in a chamber lined with filter paper on three sides.
- Chamber type- twin-trough chamber.
- Grease for sealing- not to be used.
- Opening the lid for plate insertion- Slide the lid. Do not lift it.
- Layer saturation- 5 min (keep an aliquot of the mobile phase in one trough.
- After 15 min of chamber saturation, keep the plate in the second trough for 5 min.
- Layer facing the chamber, not the wall.
- Development distance- 70 mm.
- Mobile-phase front detection- by CCD.

Derivatization:

- Scan speed – 20 mm/s
- Center of scan beam and of sample band should overlap.
- Always record spectra of all samples, except when not required in method.
- By immersion technique.
- Dip speed.
- Dip time: 1 s

Auto Spray Loading:

The main requirement of sample application are precise sample volumes, precise positioning for accurate volumes and sharpest possible “bands” by spray-on method using an inert gas.Circular spots have numerous disadvantages while “line” or “band”

application is advantageous

Detection and visualization:

- Detection under UV light is first choice-non-destructive and spots of fluorescent compounds can be seen at 254 nm (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

Quantification:

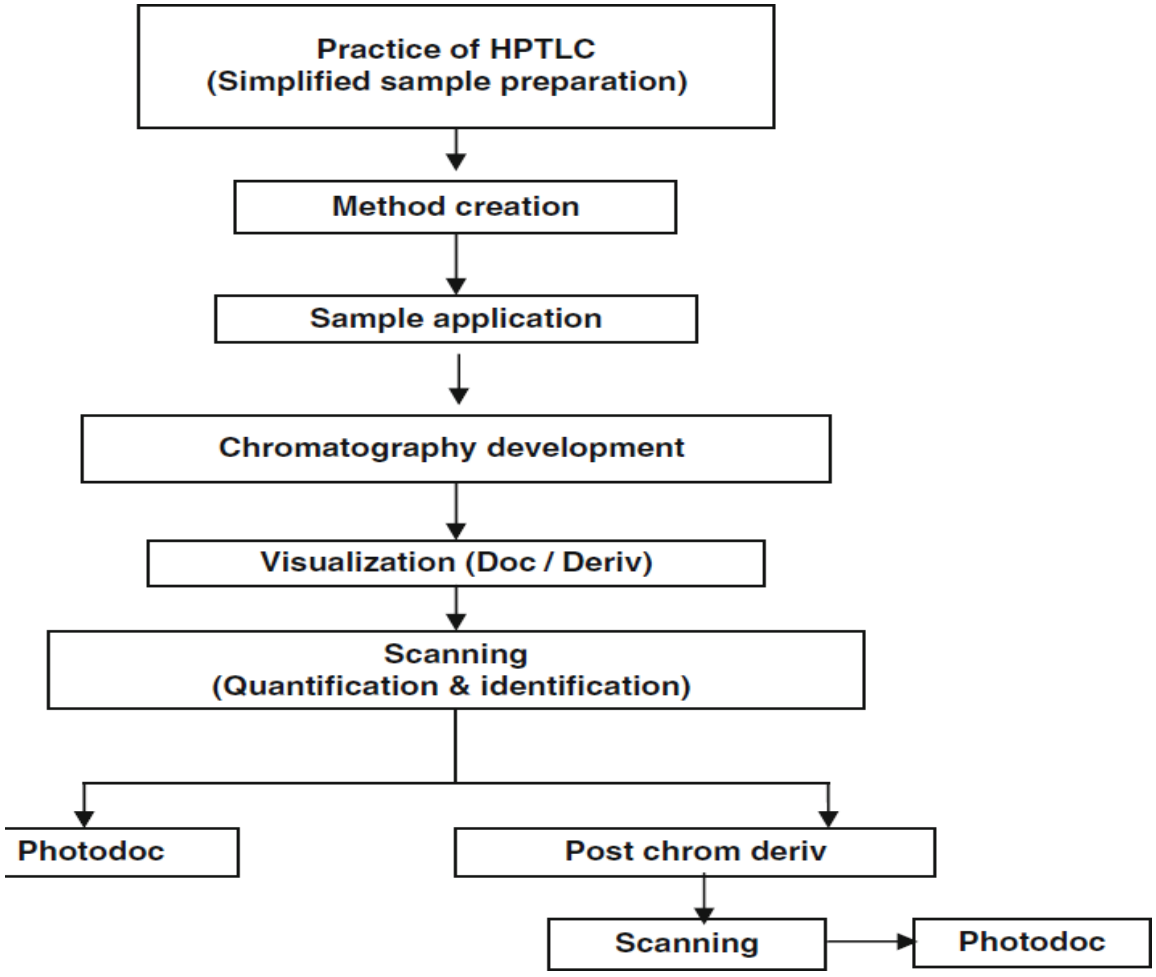
- Sample and standard should be chromatographer on same plate-after development chromatogram is scanned TLC scanner III scan the chromatogram in reflectance or in transmittance mode by absorbance or by fluorescent mode.
- Scanning speed is selectable up to 100 mm/s - spectra recording is fast 36 tracks with up to 100 peak windows can be evaluated. Calibration of single and multiple levels with linear or non-linear regressions are possible (Figure 1).

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.

Digital Camera-Based Image Documentation

UV Cabinets are now being replaced with improved design UV Cabinets which allow digital camera to be fixed for recording images of the plate. Small labs prefer this device although it does not conform to GLP. Today, HPTLC is a primary requirement for any laboratory involved in herbal analysis for establishing the identity of plant extracts by comparison with Botanical Reference Material (BRM) extracts to detect substitutes or adulterants, studies of formulations, etc. Forensic analysts have long ago stated that their starting points are a microscope for physical inspection and TLC for chemical inspection



Software-Induced Scanning

An “Entry Level” HPTLC system is already very advanced and can do most of the routine jobs. It can scan for quantification in absorbance and fluorescence modes and record UV–Vis absorbance spectra, in situ. Depending on end-user requirements, gradient chamber and/or a photo documentation device and a bioluminescence detector may be added or a fully automatic system could be procured. Hyphenation with MS or IR or NMR can be achieved with a suitable commercially available interface. A recently available device interfaces HPTLC with MS. This interface enables the extraction of the chosen fraction from the layer and feeds it directly into the MS. This opens great new possibilities for an analytical lab. The analysis output from LC–MS can be greatly increased, when compiled to TLC/HPTLC. Any specified fraction from a plate can be analyzed. Other fractions present can be ignored. Optimization of MS parameters for a particular molecule can be optimized using TLC (Table 1). LC–MS and TLC–MS are complimentary techniques .

Validation of Method:

Validation is an important step in determining the reliability and reproducibility of the method because it is able to confirm that the intended method is suitable to be conducted on a particular system. The necessity for validation in analytical laboratories is derived from regulations such as International Conference on Harmonization (ICH) and current good manufacturing practices (cGMP), good laboratory practices (GLP), and the good clinical practices (GCP). Other regulatory requirements are found in quality and accreditation standards such as the International Standards Organization (ISO) 9000 series, ISO 17025, the European Norm (EN45001), United States Pharmacopoeia (USP), Food and Drug Administration (FDA), and Environmental Protection Agency (EPA). The reliability of analytical data is critically dependent on three factors, namely, the reliability of the instruments, the validity of the methods, and the proper training of the analysts.

Specificity

The specificity of the developed method is established by analyzing the sample solutions in relation to interferences from formulation ingredients. The spot for the sample is confirmed by comparing retardation factor (R_f) values of the spot with that of the standard .

Sensitivity

Sensitivity of the method is determined with respect to limit of detection (LOD) and limit of quantification (LOQ). Noise can be determined by scanning blank spot (solvent) six times. Series of concentrations of drug solutions are applied on plate and analyzed to determine LOD and LOQ. LOD is calculated as three times the noise level, and LOQ is calculated as ten times the noise level. LOD and LOQ are experimentally verified by diluting the known concentrations of sample until the average responses are approximately 3–10 times the standard deviation (SD) of the responses for six replicate determinations

Linearity and Calibration Curve:

Linearity of the method is evaluated by constructing calibration curves at different concentration levels. Calibration curve is plotted over a different concentration range of analyte. The calibration curve is developed by plotting peak area vs. concentrations with the help of the win-CATS software.

Accuracy

Accuracy of the method is evaluated by carrying the recovery study at three levels. Recovery experiments are performed by adding three different amounts of standard drug, i.e. 80, 100, and 120% of the drug, to the preanalyzed formulations, and the resultant is reanalyzed six times.

Table 1: Features of HPLC and HPTLC

Features	HPLC	HPTLC
Stationary phase	Liquid/solid	Solid
Mobile phase	Liquid	Liquid
Conditioning phase	None	Gas
Results	By detector	Detector +eyes
Analysis in parallel	No, Only one at a time	Yes, Upto 100 samples
High pressure required	Yes	No
Time per analysis	2-60 min	1-3 min
Data taken from chromatography	Little to very high	High to Very high
Fraction collection/micro preparative chromatography	Requires preparation scale chromatography and fractional collector	Simple, no special requirement
Sensitivity	High to ultra high	Moderate to ultrahigh
Fluorescence Data	Possible, optional	Possible ,built in
Abs. spectra for identification	Yes (PDA)	Yes

Detector	UVfluorescence, electrochemical light detector MS etc	Uv-visible, bioluminescence, MS
Post chromatographic derivatization	Limited possibilities cumbersome	Simple,possible for every sample,gives additional information
Sample clean up	Through column reusable	Not so important layer disposable
Chromatographic fingerprint	Yes ,but limited	Yes, comprehensive
Cost per analysis, analyst skill	High	low
chromatographic image	No	Yes at 245,366nm and visible.

Precision

Precision is evaluated in terms of intraday and interday precisions. Intraday precision is determined by analyzing sample

solutions of analyte from formulations at three levels covering low, medium, and higher concentrations of calibration curve for five times on the same day. Interlay precision is determined by analyzing sample solutions of analyte at three levels covering low, medium, and higher concentrations over a period of 7 days. The peak areas obtained are used to calculate mean and % RSD (relative SD) values

Table 2: Analytical procedure and required validation characteristics

analytical procedure	Identification	Assay / in-vitro release study/ dissolution study/content/ potency	Testing for impurities	
Characteristics			Quantitative	Limit test
Linearity	-	+	+	-
Range	-	+	+	-
Specificity	+	+	+	+
Accuracy	-	+	+	-
Precision Repeatability	- -	+ +	+ +	- -
Intermediate precision	-	+	+	+
LOD	-	-	-	+
LOQ	-	-	+	-

Repeatability:

Repeatability of measurement of peak area is determined by analyzing different amount of analyte covering low, medium, and higher ranges of the calibration curve seven times without changing the position of plate. Repeatability of sample application is assessed by spotting samples covering similar range of calibration curve seven times and analyzing them once.

Retardation Factor

Retardation factor (R_f) is defined as the amount of separation due to the solvent migration through the sorbent layer as shown in the formula. It depends on time of development and velocity coefficient or solvent front velocity

Peak Purity

The purity of the peak is determined by comparing the spectra at three different levels: peak start (s), peak maximum (m), and peak end (e). During the purity test, the spectrum taken at the first-peak slope is correlated with the spectrum of peak maximum [r (s, m)] and the correlation of the spectra taken at the peak maximum with the one from the down slope or peak end [r (m, e)] which is used as a reference spectra for statistical calculation. An error probability of 1% only is rejected if the test value is greater than or equal to 2.576 as a

reference spectra for statistical calculation .

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