International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 10 (2): March-April 2020

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Pharmaceutical Sciences **Review Article……!!!**

Received: 12-03-2020; Revised: 30-03-2020; Accepted: 02-04-2020

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY: A REVIEW

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Keywords:

Chromatographic techniques, HPTLC, Quantitative, Validation

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ABSTRACT

HPTLC is the most versatile technique and is known for uniformity, purity profile, assay values and precision and accuracy of results. It can handle several samples of even divergent nature and composition. HPTLC is a modern analytical separation method with extensive versatility, although already much utilized, is still with great potential for future development in research and development. It is accepted as a time-saving and most economical machine practically with minimum trouble shootings. It speeds up analysis work which is usually not possible with other parallel chromatographic appears to hold considerable promise for the analysts who previously have had reservation towards the use of planar chromatography in Hyphenation with mass/infra red/laser spectroscopy etc. Opens a new dimension which makes it the most prestigious among the analytical chemists in the present perspective. In this article, we present the practical view of the concepts of various parameters in the validation fields of quantitative high-performance thin layer chromatography analytical method in pharmaceutical analysis validation, whether they are for an academic or an industrial researcher

INTRODUCTION

1. **Analytical chemistry:** The science of analytical chemistry can be 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 and 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). Example of qualitative information are types of atoms, molecules, functional group and some other qualitative measures, while the quantitative information provides numerical information such as the content of different compound 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 the taken care of by software run on a computer. Hence is no exaggeration to say that analytical chemistry has become computerised. The shape of the data of analytical chemical analyses has, moreover, changed. Frome a single sample it is now possible after a very short period of analysis to obtained enormous amount of data. By means of techniques like ultraviolet-visible (UV- Vis) spectroscopy, fluorescence spectroscopy, infracted (IR) spectroscopy, near infracted (NIR) spectroscopy, Raman spectroscopy, Mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR), High Performance Liquid Chromatography (HPLC) and High Performance Thin Layer Chromatography (HPTLC) large amount 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 a analytical methods depends on a specific purpose of that method. 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 academic researcher the industrial sector.

2. 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 principle 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 dem0stration. A popular approaches for improving resolution under capillary flow-controlled conditions is to use multiple developments. Either one-dimensional or two-dimensional separation 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 sample 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 sample in parallel. Consumption of stationary and mobile phase is directly proportional to the number of samples being analyzed.

3. Key features and separation efficiency

Cost per analysis is very low. Low 20 x 10 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 samples 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 cleanup up o 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 phase 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 chromatographic development, in situ derivatization is unique to HPTLC. Post-chromatography derivatization (PCD) is very simple and routinely possible in any laboratory. After recording data, a plate can be derivatized to get additional information. PCD is done for several reasons 1. To detect compound with a specific functional group. 2. to lower detection of all organic compounds present. 3. To visualize the sample by our eyes.

Although Silica gel is by far the most widely used adsorbent (stationary phase), many other absorbents have been used as the separation medium e.g., reverse phases, bounded 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 vapors saturation, its pH, and humidity in the developing chamber. TLC/HPTLC can be used for qualitative, semi-quantitative, and quantitative analysis. It can also be used for the identification of industrial fraction after chromatographic separations as well for the identification of herbal extracts 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. Quantity control, analytical R&D, process monitoring, and environmental labs find TLC/HPTLC as a useful too 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 different 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.

4. Steps involved in HPTLC

4.1 Selection of chromatographic layer

Precoated plates

Different support materials

Different Sorbents available

Silica gel GF: Basic substances, alkaloids and steroids

Aluminium oxide: Amino acids, dipeptides, sugars alkaloids

Cellulose: Non – polar substances, fatty acids, carotenoids, cholesterol

4.2 Sample and Standard Preparation

Solvents used are Methanol, Chloroform: Methanol (1:1), Ethyl acetate: Methanol (1:1), Chloroform: Methanol: Ammonia or 1% Acetic acid.

Dry the plates and store in dust free atmosphere.

4.3 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 – 120 (0c) 30 min

Prior to Aluminium sheets should be kept in between two glass plates and placing in oven at 110 – 120 (0c) for 15 minutes.

4.4 Application of sample and standard

Usual concentration range is 0.1 – 1 ug / ul above this causes poor separation.

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

Base wise application better separation high response to densitometer

4.4.1 Selection of mobile phase

Trial and error

One's own experience and Literature based.

4.4.2 Normal phase

Stationary phase is polar, Mobile phase is polar Non-polar compounds eluted first because of lower affinity with stationary phase.

4.4.3 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.

4.4.4 Pre-conditioning (Chamber Saturation)

Un-saturated chamber causes high Rf values. Saturated chamber by lining with filter paper for 30 minutes prior to development; uniform distribution of solvent vapours – less solvent for the sample to travel lower Rf values.

4.5 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 plates in the second trough for 5 min. Layer facing the chamber, not the wall. Development distance-70 mm. Mobile-phase front detection- b y CCD.

4.6 Derivatization

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

4.7 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.

4.8 Detection and visualization

Detection under UV light is first choice-non-destruction 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.

4.9 Quantification

Sample standard should be chromatographer on some plate-after development is scanned TLC scanner 3 scan the chromatogram in reflectance or in transmittance mode by absorbance or by fluorescent mode. Scanning speed 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

4.10 Scanning Densitometry

Scanning Densitometry allows measuring the absorption and/or florescence of underivatized, derivative substance at wavelength between 200 and 800 nm. Up31 wavelength can be evaluated and spectra of any peak can be recorded. Biological tests can be performed directly on the HPTLC plate.

4.11 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 plat extracts by comparison with Botanical Reference Material (BMR) 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.

4.12 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 HPLTC 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 complied to TLC/HPTLC. Any specified fraction from a plate can be analyzed. Other fractions present can be present ignored. Optimization of MS parameters for a particular molecule can be optimized using TLC. LC-MS and TLC-MS are complimentary techniques.

4.13 Validation of Method

Validation is an important step in determining the reliability and reproducibility of the method because it is able to conform 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 practices (cGMP), good laboratory practices (GLP), and the good clinical practices (GCP). Other regulatory requirements are found in quantity and accreditation standards such as The International Standards Organization (ISO) 9000 series, ISO 17025, the European Norn (EN 45001), 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.

4.14 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 samples is confirmed by comparing retarding factor (Rf) values of the spot with that of the standard.

4.15 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 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.

4.16 Linearity and Calibration Curve

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

4.17 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 standards 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 timr	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 Require preparation scale	High to very high Sample, no special
Fraction collection/micro preparative	chromatography and fractional collector	requirements
chromatography	High to ultra high	Moderate ultrahigh

Sensitivity	Possible, optional	Possible, built in
Post derivatization	Limited	Simple, possible
Sample clean up	Through column reusable	Disposable
Chromatographic fingerprint	Yes, but limited	Yes, comprehensive
Cost per analysis, analyst skill	High	Low
Chromatographic image	No	Yes at 245,366 nm

4.18 Precision

Precision is evaluated in terms of intraday and interday precision. Intraday precision is determined by analyzing sample solution of analyte from formulations at three levels covering low, medium, and higher concentrations of calibrations 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 Characteristics	Identification	Assay /in vitro release study / dissolution study / Content / Potency	Testing for impurities	
			Quantitative	Limit Test
Linearity	-	+	+	-
Range	-	+	+	-
Specificity	+	+	+	+
Accuracy	-	+	+	-
Precision	-	+	+	-
Repeatability	-	+	+	-
Intermediate precision	-	+	+	+
LOD	-	-	-	+
LOQ	-	-	+	-

+ Characteristics normally evaluated - Characteristics normally not evaluated.

4.19 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 a position of plate. Repeatability of sample application is assessed by spotting samples covering similar range of calibration curve seven times and analyzing them once.

4.20 Robustness

By introducing small changes in mobile-phase composition, its volume, chamber saturation time, and slight change in the solvent migration distance, the effects on the results are examined. Robustness of the method is determined in triplicate and the mean and % RSD of

peak area are calculated. Parameters that are affected by the changes in chromatographic conditions are retardation factor (Rf) and peak purity.

4.21 Retardation Factor

Retardation factor (Rf) 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.

4.22 Peak Purity

The purity of the peak is determined by comparing the spectra at three different level: 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 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 (13-15).

CONCLUSION:

HPTLC method is having active application in qualitative and quantitative analysis of a wide range of compounds. Such as herbal and botanical dietary supplements, nutraceuticals, traditional western medicines, traditional Chinese medicines and Ayurvedic (indian) medicines. It is used in assaying radiochemical purity of radiopharmaceuticals; the determination of the pigments that a plant contains; detection of pesticides or insecticides in food; analyzing the dye composition of fibres in forensics, identifying compounds present in a given substance; to check starting raw materials (plant extracts, extracts of animal original, fermentation mixtures), intermediates (crude products, reaction mixtures, mother liquors, and secondary products0, pharmaceutical raw materials (identification, purity testing, assay, separation of closely related compounds, stability testing), and formulated products (identification, purity testing, assay, stability testing under storage and stress, content uniformity test, dissolution test); and for the detection and identification of drugs and their metabolites in biological media such as urine, plasma, or gastric fluid (pharmacological, toxicological, pharmacokinetic, metabolic, bioequivalence, forensic, and compliance and pharmacodynamic studies).

Validation requirements for HPTLC procedures are highly diversified, depending upon the actual type of analysis, as HPTLC can be used in wide range of application. This technique

has most successfully been used in the fields of education, forensic science, herbal analysis, organic synthesis, and foods, to name a few. The introduction of TLC-MS interface will lead to a paradigm shift in the role of TLC-HPTLC in all labs. Recent improvements in the automatic development chamber will also lead to reproducible results independent of the laboratory's environment.

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