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Rakesh S. Shivatare
R&D, Ari Healthcare Pvt. Ltd., International Biotech Park, MIDC Phase II, Hinjewadi, Pune, India

Dheeraj H. Nagore
R&D, Ari Healthcare Pvt. Ltd., International Biotech Park, MIDC Phase II, Hinjewadi, Pune, India

Sanjay U. Nipanikar
R&D, Ari Healthcare Pvt. Ltd., International Biotech Park, MIDC Phase II, Hinjewadi, Pune, India

Correspondence:
Dheeraj H. Nagore
R&D, Ari Healthcare Pvt. Ltd., Unit no-4, International Biotech Park, BTS-2, Chrysalis Enclave, 4th floor, Plot No 2A, MIDC Phase II, Hinjewadi, Pune, India
Tel: +91-8550993932
E-mail:
dheeraj.nagore@ariheathcare.in

'HPTLC' an important tool in standardization of herbal medical product: A review

Rakesh S. Shivatare, Dheeraj H. Nagore, Sanjay U. Nipanikar*

Abstract

The analysis and quality control of herbal medicines are moving a step ahead towards an integrative and comprehensive direction, in order to tackle the complex nature of herbal medicines. High-performance thin layer chromatography (HPTLC) is one of the sophisticated instrumental techniques for qualitative and quantitative analysis of the herbs and herbal drugs. This article emphasize on HPTLC based analytical method development and evaluation of validation characteristics.

Keywords: High-performance thin layer chromatography (HPTLC), Herbal drugs, Analytical method.

Introduction

In the present era, universal trend has been shifted from synthetic to herbal medicine i.e. 'Return to Nature'.[1] Ayurveda is a time-tested, trusted worldwide plant based system of medicines[2] which is developed through daily life experiences with the mutual relationship between mankind and nature.[3] As per WHO, there are three kinds of herbal medicines: raw plant material, processed plant material and medicinal herbal products.[4] Herbal medicines are complex chemical mixtures obtained from a plant which is widely used in health-care in both developed and developing countries.[5] It is no wonder that the world's one-fourth population is using traditional medicines for the treatment of various ailments.[6] However, one of the impediments in the acceptance of the Ayurvedic or Herbal medicines is the lack of standard quality control profiles.[7] Due to the complex nature and inherent variability of the chemical constituents of the plant based drugs, it is difficult to establish quality control parameter.[8] Quality assurance of herbal medicine is an important factor and basic requirement for herbal drug industry and other drug development organization.[9] There are several problems which influence the quality of herbal drugs.

- ✓ Variable sources of the raw material.
- ✓ The chemical constituents of herbs and herbal products may vary depending on stage of collection, parts of the plant collected, harvest seasons, plant origins (regional status), drying processes and other factors.[10]
- ✓ Extracts are usually mixtures of many constituents.
- ✓ The active principle(s) is (are), in most cases unknown.
- ✓ Selective analytical methods or reference compounds may not be available commercially.[11-13]

All pharmacopoeias set standards for the quality, purity, strength, and consistency of these products–critical to the public health. USP–NF contains approximately 4500 monographs for drug substances, dosage forms, excipients and other therapeutics. Today, USP proposes the first 23 ingredients to be included in the new Herbal Medicines Compendium (HMC).[14] The IP 2007, which was made effective from last July, has already over 1600 monographs.[15] The British Pharmacopoeia 2012 contains approximately 3375 monographs for substances, preparations and articles used in the practice of medicine.[16]

Standardization is an important step for the establishment of a consistent biological activity, a consistent chemical profile, or simply a quality assurance program for production and USP manufacturing of an herbal drug.[17] It is the process of developing and agreeing upon technical standards. Specific standards are worked out by experimentation and observations, which would lead to the process of prescribing a set of characteristics exhibited by the particular herbal medicine. Hence standardization is a tool in the quality control process.[18]

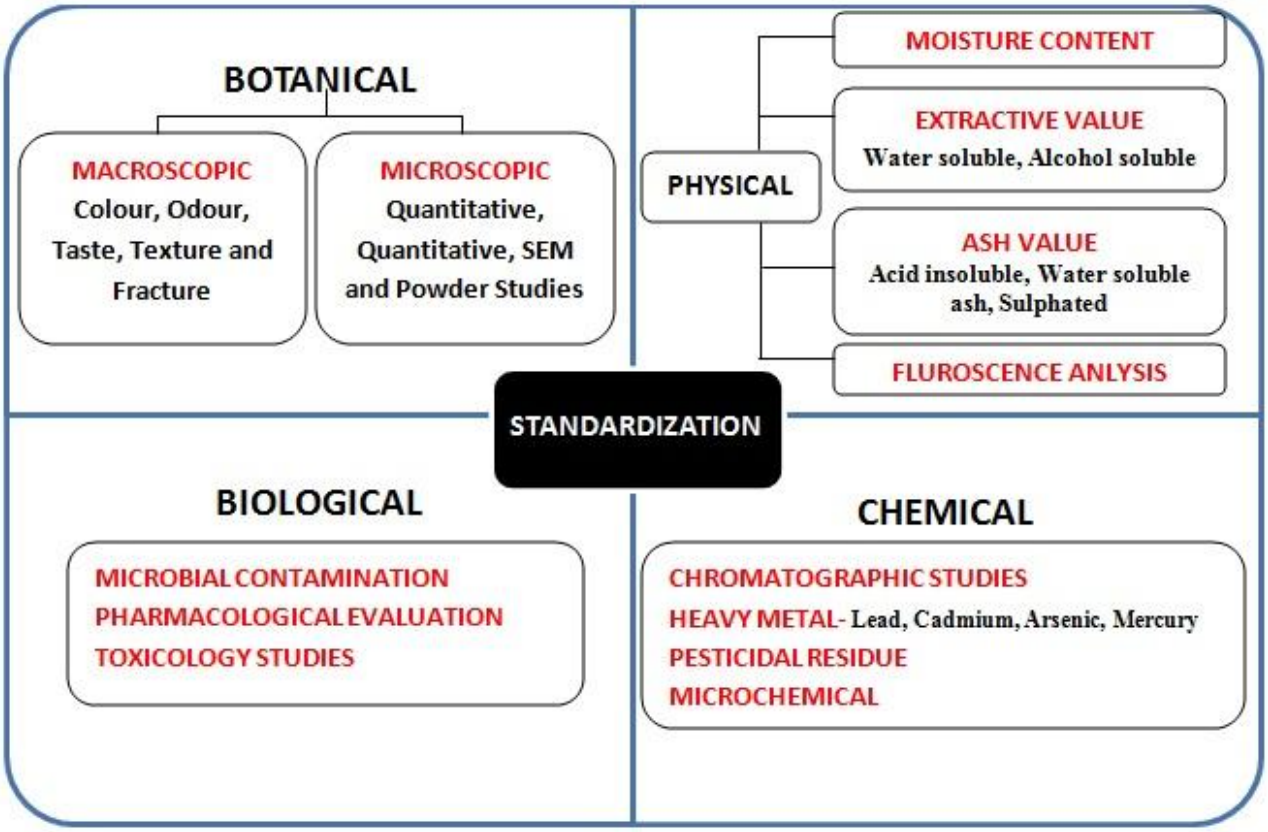


Figure 1: A schematic representation of standardization of herbal drugs

HPTLC is a modern adaptation of TLC with better and advanced separation efficiency and detection limits. The table 1 compares HPTLC and TLC.[19-28]

Table 1: Difference between TLC and HPTLC

PARAMETER	TLC	HPTLC
Technique	Manual	Instrumental
Efficiency	Less	High (Due to smaller particle size)
Layer	Lab Made/ Pre-Coated	Pre-coated
Mean particle size	10-12 um	5-6 um
Layer Thickness	250 um	100 um
Plate Height	30 um	12 um
Solid Support	Silica Gel, Alumina, Kiesulguhr	Silica Gel- Normal Phase C8 and C18- Reverse phase
Sample Spotting	Manual Spotting (Capillary/	Auto sampler (Syringe)

	Pipette)	
Sample Volume	1-5 ul	0.1-0.5 ul
Shape of Sample	Circular (2-4 nm Dia)	Rectangular (6 mm L × 1mm W)
Separation	10-15 cm	3-5 cm
Separation Time	20-200 Min	3-20 Min
Sample tracks per plate	≤ 10	≤ 36 (72)
Detection Limits (Absorption)	1-5 pg	100-500 pg
Detection limits (Fluorescence)	50-100 pg	5-10 pg
PC connectivity, Method Storage	No	Yes
Validation, Quantitative Analysis, Spectrum Analysis	No	Yes
Analysis Time	Slower	Shortage Migration Distance and the analysis time is greatly reduced
Wavelength Range	254 or 366 nm, Visible	190 or 800 nm, Monochromatic
Scanning	Not possible	Use of UV/ visible/ fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and scanner is an advanced type of Densitometer

There are several advantages of using HPTLC for the analysis of compounds as compared to other techniques, like HPLC, spectrophotometry, titrimetry, etc.[26] Some of the advantages of HPTLC are:

- ✓ Ability to analyze crude samples containing multi-components.
- ✓ The separation process is easy to follow especially with colored compounds.
- ✓ Several samples can be separated parallel to each other on the same plate resulting in a high output, time saving, and a rapid low-cost analysis.
- ✓ Choice of solvents for the HPTLC development is wide as the mobile phases are fully evaporated before the detection step.
- ✓ Two-dimensional separations are easy to perform. Stability during chromatography should be tested using two-dimensional development.[28]
- ✓ Specific and sensitive colour reagents can be used to detect separated spots (Dragendroff reagent/Kedde reagent).[29]
- ✓ HPTLC can combine and consequently be used for different modes of evaluation, allowing identification of compounds having different light-absorption characteristics or different colours.
- ✓ Contact detection allows radiolabelled compounds to be monitored and microbial activity in spots to be assessed.
- ✓ HPTLC method may help to minimizes exposure risk of toxic organic effluents and significantly reduces its disposal problems, consequently, reducing environment pollution.[21-31]

Classification of HPTLC

HPTLC techniques may be classified into four classes i.e. Classical, High performance, Ultra and Preparative thin-layer chromatography. They differ with classical TLC in the particle size distribution and thickness of the sorbent layers. The mean particles sizes are 12, 5, 25 μm for classical, high-performance and preparative thin-layer chromatography, respectively, whereas Ultra thin layer chromatography does not have particles but a monolithic layer with 1–2 um macropores.[32] Another difference is the thickness of the sorbent layers which is 250 um, 200 um, 10 um and 0.5–2 mm, for classical, high-performance, ultra-thin and preparative sorbent layers, respectively.[21]

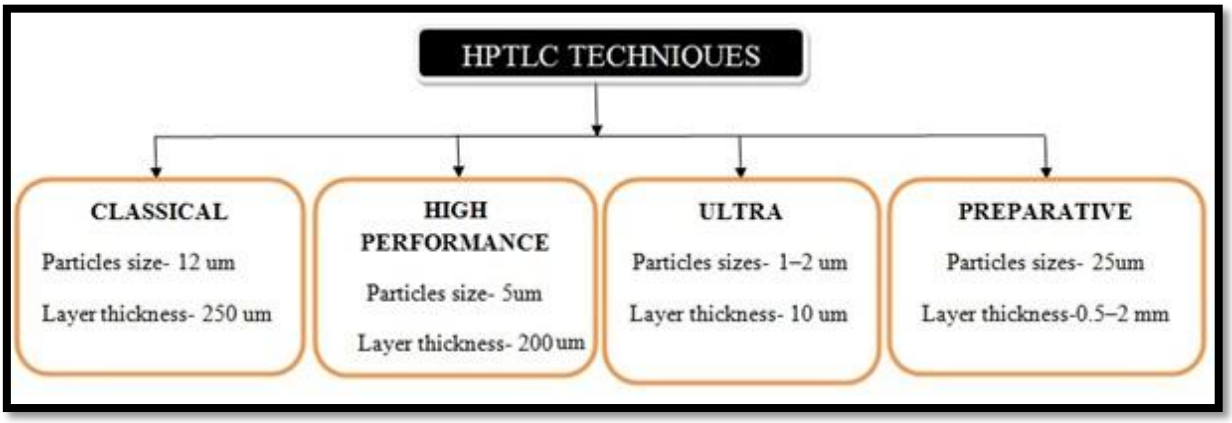


Figure 2: Classification of HPTLC techniques

Common Methodology for HPTLC Analysis

Method development in thin-layer (planar) chromatography is one of the most significant steps for a qualitative and quantitative analysis. During establishing a new analytical procedure, always starts with wide literature survey[33] i.e. primary information about the physicochemical characteristics of sample and nature of the sample (structure, polarity, volatility, stability and solubility). It involves considerable trial and error procedures.[34] General steps involved in HPTLC method developments are as follow: [23, 35]

Basic Steps:

- Selection of the stationary phase
- Mobile phase selection and optimization
- Sample Preparation and Application
- Chromatogram Development (separation)
- Detection

Quantitation:

HPTLC method validation for pharmaceutical analysis:

- Specificity
- Linearity
- Range
- Accuracy
- Precision
- Detection Limit, Quantitation Limit
- Robustness

Basic Steps:

Selection of the stationary phase-

During method development, stationary phase selection should be based on the type of compounds to be separated.[36] HPTLC uses smaller plates (10*10 or 10*20 cm) with significantly decreased development distance (typically 6 cm) and analysis time (7–20 min). HPTLC plates provide improved resolution, higher detection sensitivity, and improved in situ quantification and are used for industrial pharmaceutical densitometric quantitative analysis.[31, 37]

Mobile phase selection and optimization-

The selection of mobile phase is based on adsorbent material used as stationary phase and physical and chemical properties of analyte. [28, 29] The Table 2 gives the details of mobile phase generally used in detection of some chemical compounds.

Table 2: Generally used Mobile phase in detection of some chemical compounds

SN	Chemical Compounds	Mobile Phase
1	Polar Compounds Anthraglycosides, Arbutin, Alkaloids, Cardiac Glycosides, Bitter Principles, Flavonoids, Saponin	Ethyl Acetate: Methanol: Water [100:13.5:10]
2	Lipophilic Compounds	Toluene: Ethyl Acetate [93:7]

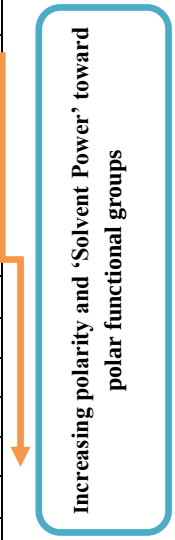
	Essential oils, Terpenes, Coumarin, Napthoquinons, Velpotriate	
3	Alkaloids	Toluene: Ethyl Acetate: Diethyl Amine [70:20:10]
4	Flavonoids	Ethyl Acetate: Formic Acid: Glacial Acetic Acid: Water [100:11:11:26]
5	Saponin	Chloroform: Glacial Acetic Acid: Methanol: Water [64:32:12:8]
6	Coumarin	Diethyl Ether: Toluene [1:1] Saturated with 10% Acetic Acid
7	Bitter Drug	Ethyl Acetate: Methanol: Water [77:15:8]
8	Cardiac Glycosides	Ethyl Acetate: Methanol: Water [100:13.5:10] OR [81:11:8]
9	Essential Oil	Toluene: Ethyl Acetate [93:7]
10	Lignans	Chloroform: Methanol: Water [70:30:4] Chloroform: Methanol [90:10] Toluene: Ethyl Acetate [70:30]
11	Pigments	Ethyl Acetate: Formic Acid: Glacial Acetic Acid: Water [100:11:11:26]
12	Pungent Testing	Toluene: Ethyl Acetate [70:30]
13	Terpenes	Chloroform: Methanol: Water [65:25:4]
14	Triterpenes	Ethyl Acetate: Toluene: Formic Acid [50:50:15] Toluene: Chloroform: Ethanol [40:40:10]

Sample Preparation and Application-

A good solvent system is one that moves all components of the mixture off the baseline, but does not put anything on the solvent front. The peaks of interest should be resolved between Rf 0.15 and 0.85. The elution power of the mobile phase depends on a property called eluent strength which is related to the polarity of the mobile phase components.[23] The more nonpolar the compound, the faster it will elute (or the less time it will remain on the stationary phase) and the more polar the compound the slower it will elute (or more time on the stationary phase). The following chart is helpful in predicting the order of elution.[21, 28]

Table 3: Common Mobile Phases listed by Increasing Polarity

S. No	Solvent	Eluent Strength	
1	N- Pentane	0.00	
2	Hexane	0.01	
3	Cyclohexane	0.04	
4	Carbon tetrachloride	0.18	
5	Toluene	0.29	
6	Chloroform	0.40	
7	Methylene Chloride	0.42	
8	Tetrahydrofuran	0.45	
9	Acetone	0.56	
10	Ethyl Acetate	0.58	
11	Aniline	0.62	
12	Acetonitrile	0.65	
13	Ethanol	0.88	
14	Methanol	0.95	
15	Acetic Acid	Large	



Pharmaceutical preparation with sufficiently high concentration of analyte is simply dissolved in a suitable solvent that will completely solubilize the analyte and leave excipients undissolved to yield a test solution that can be directly applied on HPTLC plate.[34, 38] It is a fact that application of the sample is the most critical step to obtain good resolution for quantification in HPTLC21.Sample application technique depends on factors such as the type of sample matrix, workload and time constraints.[39,23]

Chromatogram Development (separation)-

Although chromatogram development is the most crucial step in the HTLC procedure, important parameters are generally overlooked.[28] HPTLC plates are developed in twin-trough chambers, or horizontal-development chambers. In general, saturated twin-trough chambers fitted with filter paper offer the best reproducibility. Twin-through chamber avoids solvent vapor preloading and humidity.[40-42]

Detection-

Detection of separated compounds on the sorbent layers is enhanced by quenching of fluorescence due to UV light (ranged normally at 200-400 nm). This process is commonly called Fluorescence quenching.

Visualization at UV 254 nm

F254 should be described as phosphorescence quenching. In this instance the fluorescence remains for a short period after the source of excitation is removed. It is very short lived, but longer than 10 seconds.[42] F254 fluorescent indicator is excited with UV wavelength at 254 nm and emits green fluorescence.[43] Compounds that absorb radiation at 254 nm reduce this emission on the layer, and a dark violet spot on a green background is observed where the compound zones are located.[23, 44] This quenching is caused by all compounds with conjugated double bonds. Anthraglycosides, coumarins, flavonoids, propylphenols in essential oils, some alkaloid type such as indole, isoquinoline and quinoline alkaloids etc. should be detected under 254 nm. [29, 45]

Visualization at UV 366 nm

F 366 should be described as Fluorescence quenching. In this instance the fluorescence does not remains after the source of excitation is removed.[42] This quenching is shown by all anthraglycosides, coumarins, flavonoids, Phenolcarboxylic acids, some alkaloid types (Rauwolfia, Ipecacuanha alkaloids).[29]

Visualization at white light

Zone containing separated compounds can be detected by viewing their natural color in daylight (White light).[37]

Derivatisation

Derivatization can be defined as a procedural technique that primarily modifies an analyte's functionality in order to enable chromatographic separations. Derivatization can be performed either by immersing the plates or by spraying the plates with a suitable reagent (Table 4). [28,29,46,47] For better reproducibility, immersion is the preferred derivatization technique.

Table 4: List of common derivatization reagents

SN	Colour Reagent	Chemical Compounds	Colour
1	Dragendroff Reagent It forms complex reaction with some nitrogen containing compounds	Alkaloids	Red-brown Zone (vis)
2	Natural products- Polyethylene Glycol reagent i.e. Diphenylboric acid -2-aminoethyl ester forms complexes with 3-hydroxyflavones via condensation reaction	Flavonoids	Intense yellow, Orange and Green Fluorescent zones in UV 366 nm
3	Vanillin Sulphuric Acid OR Anisaldehyde Sulphuric Acid	Bitter Principle	Red-brown, Yellow-brown, Dark green Zone (vis)
		Saponin	Coloured zones (vis)
		Essential Oil	Blue, brown or red zones (vis)

4	10 % Ethanolic KOH	Anthraquinones (Emodin, Rhein)	Red zones (vis) Red Fluorescence (UV 366 nm)
		Anthrones (Aloin, Cascarosides)	Yellow zones (vis) Yellow Fluorescence (UV 366 nm)
		Coumarins, Scopoletin, Umbelliferone	Bright blue Fluorescent zone (UV 366 nm)
5	Ninhydrin Reagent	Amino acids, peptides, amines and amino-sugars	Yellow, brown to pink and violet (vis)
6	Iodine It produce iodine reaction possibly result in an oxidative products	Indole, quinoline derivative, thiols and all organic compounds	Dark zone (UV 254)

Quantitation:

Generally quantitative evaluation is performed by measuring the zones of samples and standards using a densitometer or scanner with a fixed sample light beam in the form of a rectangular slit. The chromatogram can be scanned in reflectance or in transmittance mode by absorbance or by fluorescent mode; scanning speed is used up to 100 mm/s.[23,28] Due to scanning spectra calibration of single and multiple levels of linearity, linear and nonlinear regression equations are possible. Scanning has been done by two methods i.e. Slit Scanning and Video Scanning.[21]

Slit Scanning-

Slit-scanning densitometry is now relatively mature and although limited to absorption and fluorescence detection in the UV–visible range. It consists of fiber optic bundle for illumination of sample zones and collection of reflected light (or fluorescence). Photodiode-array detector is used for simultaneous length detection and spectral recording.[28]

Video Scanning-

Video densitometry is fast and simultaneous data acquisition from the whole plate. In which optical scanning takes place electronically, using a computer with video digitizer, light source, monochromators, and appropriate optics to illuminate the plate and focus the image onto a charge-coupled device (CCD) video camera. It is also useful in two-dimensional separations for thin-layer chromatography with image analysis.[28]

HPTLC method validation for pharmaceutical analysis:

Specificity-

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. It includes Identification, Purity tests and Assy.[48]

The specificity of the method was ascertained by analyzing standard drug and sample. The peak purity of chemical compound was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M), and peak end (E) positions of the spot.[28]

Linearity-

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.[48] The linearity is usually documented as the ordinary least squares (OLS) curve or simply as linear regression curve of the measured responses (peak area or height) as a function of increasing analyte concentrations.[49]

Range-

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.[48] In the Pharmaceutical Industry usually a range from 80 to 120% of the target concentrations was tested.[17]

Accuracy-

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.[48] For bio-analytical method, accuracy should be performed for at least three quality control (QC) samples (low, medium, and high) in triplicate, and the accuracy was expressed as % recovery.[50]

Precision-

Precision of the analytical method can be divided into three categories, i.e., repeatability, intermediate precision, and reproducibility. Repeatability, or intraassay within-day precision, is determined when the analysis is done in one laboratory by one analyst with same conditions (equipment, TLC plate, reagents) and performed within 1 day work. Intermediate precision is obtained when the analysis was performed within a laboratory by different analysts, equipment, reagents, and plates over a number of days or weeks. Reproducibility represents the precision obtained from some laboratories with the aim to verify whether the method can yield the same results in different laboratories.[48, 51]

For bioanalytical method, it is recommended to test the precision using a minimum of five determinations per concentration. A minimum of three levels of concentrations in the expected range is recommended; RSD is not permitted more than 15%, and at the maximum limit, lower concentration RSD of 20% is acceptable. [52, 53]

Detection Limit, Quantitation Limit-

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit (QL) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy48. Generally, QL can be estimated as 2–3 times of DL. DL and QL for instrumental (chromatographic) analytical methods can be defined in terms of the signal-to-noise ratio (2:1–3:1 for DL and 10:1 for QL) or in terms of the ratio of the standard deviation of the blank response, the residual standard deviation of the calibration line, or the standard deviation of intercept (s) and slope (S) can be used. [54, 55]

Robustness-

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.[48] Some important parameters for testing of the robustness evaluation for HPTLC methods are mobile phase composition, pH of the mobile phase, temperature, development distance, spot shape, spot size, batch of the plates, volume of samples, drying condition (temperature, time) and condition of spot visualization (spraying reagents, dipping reagents, UV detection).[56]

HPTLC vs. HPLC

High-performance thin-layer chromatography (HPTLC) is still increasingly finding its way in pharmaceutical analysis in some parts of the world. With the advancements in the stationary phases and the introduction of densitometers as detection equipment, the technique achieves for given applications a precision and trueness comparable to high-performance liquid chromatography (HPLC). Basic differences between HPLC and HPTLC are given in following table 5.[28, 57, 58]

Table 5: Differences between HPLC and HPTLC

Parameters	HPLC	HPTLC
Type	Reverse Phase Chromagraphy	Straight Phase Chromagraphy
Stationary phase	Liquid	Solid
Conditioning phase	None	Gas
Separation by	Partition	Adsorption
Results	By machine	By machine + eyes
Analysis	On - line	Off - line
Resolution	Very high	Moderate to high
Chromatography System	Closed	Open
Separating medium	Tubular column	Planar layer (plate)
Strongly Retarded Fractions Seen As	Broad peaks	Sharp Peaks
Analysis in parallel	No. Only 1 at a time	Yes. Upto 100 samples.
High temp. / pressure	High pressure	None
Time per sample	2- 60 min	1-30 min
Data obtained from chromatography	Limited to very high	High to very high

Post chromatography derivatisation	Limited possibilities. Cumbersome.	Simple. Possible for every sample. Gives additional information
Fraction collection / micro preparative chromatography	Requires prep. scale chromatograph & fraction collector	Simple. No special requirements
Sensitivity	High to ultra	high Moderate to ultrahigh
Fluorescence data	Possible, optional	Possible, built-in
Detectors	UV, Fluor, electrochem Light scatter , MS	UV - Vis, bioluminescence , MS
Chromatogram image documentation	No	Yes. At 254 & 366 nm & visible
Sample clean-up	Through Column reusable	Not so imp. Layer disposable
Chromatographic fingerprint	Yes, but limited	Yes. Comprehensive
Cost per analysis	Very high	Low
Eqpt. maintenance	Very high	Low
Analyst's skills required	High to very high	Low (TLC) to high (HPTLC)

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