HPTLC Methods to Assay Active Ingredients in Pharmaceutical Formulations: A Review of the Method Development and Validation Steps

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Abstract: **High-performance thin-layer chromatography (HPTLC) is still increasingly finding its way into 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). In this review, the literature is surveyed for developed and validated HPTLC methods to assay active ingredients in pharmaceutical formulations published in the period 2005–2011. Procedures and approaches for method development, validation, and quantitative assays are compared with the standard ways of conducting them. Applications of HPTLC in some other areas are also briefly highlighted.**

INTRODUCTION

Although Thin-Layer Chromatography (TLC) is a longstanding technique, it continues to be widely used in pharmaceutical analysis for the assay of active ingredient(s) in various parts of the world. This sustained relevance is attributed to advancements in instrumentation, automation, and the development of novel adsorbents and supports [1]. Furthermore, TLC is employed in numerous applications, including the analysis of herbal medicines, dietary supplements, biological and clinical samples, food and beverages, environmental pollutants, and chemicals [2].

Like all chromatographic techniques, TLC operates on a multistage distribution process involving a stationary phase (adsorbent), a mobile phase (solvent or solvent mixture), and the sample molecules. High-performance thin-layer Chromatography (HPTLC) is an advanced form of TLC that incorporates high-performance adsorbent layers—such as silica gel with more uniform and refined particles (approximately 5 µm in diameter compared to 12 µm in traditional TLC)—along with specialized instrumentation, such as optimized development chambers. Additionally, HPTLC follows standardized methodologies for development, optimization, documentation, and validation. The technique is applied in both qualitative and quantitative separations of compounds in mixtures, with the quantitative approach being more optimized and standardized, making it suitable for the assay of pharmaceutical compounds [3,4].

HPTLC offers several advantages over other analytical techniques, such as High-Performance Liquid Chromatography (HPLC), spectrometry, and titration. These advantages include:

- Ease of monitoring the separation process, particularly for colored compounds,
- Parallel separation of multiple samples on the same plate, leading to high-throughput and cost-effective analysis,

- Capability for two-dimensional separations,
- Use of specific and sensitive color reagents for spot detection,
- The ability to combine different modes of evaluation, facilitating the identification of compounds with varying light absorption characteristics or colors,
- Contact detection for monitoring radiolabeled compounds and assessing microbial activity in spots,
- Disposable TLC plates, eliminating the need for regeneration or clean-up, and
- The ability to store separated plates for extended periods before performing detection at a later stage [3,4].

A review of the literature highlights numerous publications on the application of (HP)TLC in various analytical fields. Several older review articles have discussed the significance, challenges, and opportunities associated with the HPTLC technique [3,5,6]. A more recent review by Kaale et al. (2011) [7] provides an overview of the extensive number of (HP)TLC methods developed, validated, and applied in pharmaceutical analysis for active ingredient assay and stability testing. The study [7] summarizes the role of TLC in drug quality testing and therapeutic drug monitoring in resource-limited settings in Africa, while also addressing the challenges related to its use in the region. Additionally, it provides an overview of HPTLC methods and their application in drug analysis.

This review will specifically focus on the quantitative application of HPTLC in the assay of active ingredient(s) in pharmaceutical formulations. The discussion will cover the various methodological steps involved in method development, validation, and quantitative analysis, concerning studies published between 2005 and 2011. Unlike [7], this review takes a different approach by examining quantitative HPTLC applications more comprehensively. A significant proportion of the reviewed publications originate from Asia, particularly India, as well as from Western European countries. A smaller fraction, approximately twenty studies, were conducted in Africa, where HPTLC has played a crucial role in drug quality assurance, especially in the absence of more advanced and expensive analytical equipment such as HPLC and Liquid Chromatography–Mass Spectrometry (LC-MS). Given the regional context, it might be expected that most HPTLC methods would focus on quality assurance of drugs for diseases prevalent in these areas, such as malaria, tuberculosis, and HIV/AIDS. However, the studies reviewed cover a broad spectrum of diseases.

Thin-layer chromatographic methods and their classification

Thin-layer chromatographic (TLC) techniques can be categorized in various ways depending on the perspective considered. As described in Reference [8], one classification is based on the movement of the mobile phase through the stationary phase. This includes capillary-flow layer chromatography, the conventional approach where capillary forces drive the flow, and Forced-Flow Planar Chromatography, which employs pressure to propel the mobile phase. Another classification considers the nature of the stationary phase, distinguishing between paper chromatography and thin-layer chromatography. Additionally, TLC can be classified based on separation mechanisms, including adsorption, partition, ion exchange, and size exclusion. A further distinction is made based on the polarity of the sorbent layer, categorizing it into normal-phase and reversed-phase chromatography. Moreover, classification can be based on the objective of separation, whether qualitative or quantitative, and another differentiation is made between online and offline separation techniques.

High-performance thin-layer Chromatography (HPTLC) can be further divided into classical, high-performance, ultra, and preparative TLC, with the first three being analytical techniques. These classifications differ from classical TLC in terms of particle size distribution and sorbent layer thickness. Specifically, the average particle sizes are 12 µm, 5 µm, and 25 µm for classical, high-performance, and preparative TLC, respectively, while ultra-thin-layer chromatography consists of a monolithic layer with 1–2 µm macropores [9,10]. Additionally, the layer thickness varies, with classical, high-performance, ultra-thin, and preparative TLC having thicknesses of 250 µm, 200 µm, 10 µm, and 0.5–2 mm, respectively. This review primarily focuses on commonly used analytical HPTLC techniques with classical capillary mobile flow.

General steps in an HPTLC analysis

Introduction

Nyiredy [6] emphasizes that method development in thin-layer (planar) chromatography is a crucial step

for both qualitative and quantitative analysis. As outlined in [6], this process involves selecting the development mode, stationary phase, vapor phase, and appropriate solvents, as well as optimizing the mobile phase. Additionally, it may include transferring the optimized mobile phase to a suitable forced-flow planar chromatography (FFPC) method and determining other operational parameters. However, the transfer to an FFPC method falls beyond the scope of this review and will not be addressed.

Selection of the stationary phase

Nyiredy [6] highlights the availability of various stationary phases for high-performance thin-layer chromatography (HPTLC) in the separation of pharmaceutical active ingredients with differing chemical properties. These include silica, alumina, kieselguhr, cellulose, gypsum, and polyamides [4,6]. Despite this variety, approximately 90% of separations are reported to be conducted on silica, either in its bare or modified form [6,11]. In unmodified silica, the silanol groups of the silica gel layer interact with analytes in mixtures, facilitating their separation. Modified silica, on the other hand, can be categorized into nonpolar and intermediately polar layers, where the former includes silica modified with alkane (C8, C18), alkene, or phenyl groups, while the latter features functional groups such as amino, cyano, diol, or thiol, linked via short chains like propyl or substance-specific complexing ligands [6]. These modifications influence analyte interactions and, consequently, the separation process.

When an apolar mobile phase (e.g., hexane-based) interacts with unmodified silica, the process is referred to as normal-phase chromatography. In contrast, when polar mobile phases (e.g., water-based) interact with apolar-modified silica layers, the technique is termed reversed-phase chromatography. A third mode, hydrophilic interaction liquid chromatography (HILIC), applies to polar-modified silica layers with polar mobile phases (e.g., methanol-based) [6]. The choice of stationary phase should align with the chemical nature of the compounds to be separated [12]. For ionic compounds, adsorption chromatography is applicable, while ion-exchange chromatography—utilizing modified cellulose layers such as aminoethyl cellulose, diethylaminoethyl cellulose, or carboxymethyl cellulose—is suitable for separating inorganic ions, purines, and pyrimidines [12]. The primary distinction between adsorption and ion-exchange chromatography lies in the stationary phase: the former uses inorganic materials (e.g., bare silica), whereas the latter relies on organic materials. Thin-layer gel filtration is applicable for macromolecules like polymers and proteins, while partition chromatography is suitable for water-soluble compounds.

Nyiredy [6] also proposed a systematic approach for selecting the appropriate separation mode and stationary phase, as depicted in a flowchart (Fig. 1). This flowchart suggests methods based on the properties of the sample mixture. For mixtures with a broad polarity range, automated multiple development (AMD) is recommended (see Section 3.1.4 for a description of AMD), particularly for separating complex plant extracts, which often contain multiple compounds. The effectiveness of AMD in separating alcoholic extracts from medicinal plants—such as *Cinchona succirubra, Aesculus hippocastanum, Berberis vulgaris, Artemisia abrotanum, Carduus marianus, Thuja accidentalis, Baptisia tinctoria, Paulinia cupana, Lycopus europaeus,* and *Echinacea angustifolia*—has been demonstrated [13]. The flowchart further recommends reversed-phase thin-layer chromatography (TLC) for extremely polar compounds or homologous sample solutions, a strategy exemplified by the simultaneous analysis of levodopa, carbidopa, and entacapone in fixed-dose tablet formulations [14]. These structurally distinct drugs with varying polarities were successfully separated using reversed-phase HPTLC on silica gel 60 RP-18 F254, with a mobile phase comprising acetonitrile, *n*-butanol, water, and triethylamine (5:95:10:0.01, v/v/v/v).

Additionally, alumina (aluminum oxide) layers are recommended for samples containing dissociating compounds such as alkaloids, chlorinated hydrocarbons, and steroids. Depending on preparation methods, alumina can exist in acidic, basic, or neutral forms, affecting separation efficiency [10]. For aromatic or isomeric compounds, reversed-phase layers with buffer or ion-pair TLC are suggested. If a sample mixture does not exhibit any of these specific properties, silica gel remains the default stationary phase for separation. Bare silica has been extensively used for pharmaceutical analysis, as confirmed in multiple studies [1,4,7,11] and additional reviewed papers [15–61]. The continued preference for this stationary phase

highlights its effectiveness in addressing separation challenges.

Though bare silica dominates, modified silica layers—including reversed-phase stationary phases—have also been applied. Chiral separations have been performed using TLC, employing reversed-phase plates impregnated with complex-forming molecules such as *N, N*-di-*n*-propyl-*l*-alanine/copper(II) complex and copper(II) complexes of (2*S, 4R, 2*RS*)-4-hydroxy-1-(2'-hydroxy dodecyl)-proline. These techniques enabled the separation of chelate-complex forming compounds, including amino acids and heterocyclic compounds. Additionally, cellulose phenyl carbamate phases successfully resolved enantiomers of propranolol and bupranolol, while molecularly imprinted polymers served as chiral stationary phases for separating compounds such as *l*- and *d*-phenylalanine anilide, as well as adrenergic drugs like norepinephrine and epinephrine [62].

The recent introduction of ultra-thin layer chromatography (UTLC) silica gel plates has also been reported [9,63]. These plates feature a monolithic silica gel layer only 10 µm thick, with mesopores measuring 3–4 nm and macropores 1–2 µm in diameter—significantly smaller than the 0.6 µm pore diameter of conventional TLC and HPTLC silica layers. The advantages of UTLC include enhanced sensitivity, shorter migration distances (1–3 cm), reduced migration times (1–6 min), and lower mobile phase consumption (1–4 ml). While Hauck and Schulz [63] demonstrated the effectiveness of UTLC for pharmaceutical analysis, particularly in separating bromazepam, diazepam, and prazepam, further applications of this stationary phase have not been widely reported.

Regarding support materials for stationary phases, the reviewed literature identified glass [15,36,37] and aluminum [16–18,21–23,25,27,29,31,38–41,44,46,47,49–53,56,57,59] as the most commonly used substrates. These materials are preferred due to their resistance to heat and chemicals, surpassing alternative TLC support materials such as plastics [4]. Among them, aluminum is the most widely used due to its lower cost, ease of handling, and the ability to be cut into required sizes, making it a practical choice for many applications.

Mobile phase selection and optimization

Mobile phase optimization is a critical factor influencing the quality of separation in thin-layer chromatography (TLC) method development [64]. In many cases, the selection and optimization of mobile phase components are conducted through a trial-and-error approach, relying on the analyst's experience or insights from literature searches [2,4]. Additionally, manufacturers of high-performance thin-layer chromatography (HPTLC) plates, such as Macherey–Nagel (MN), provide data on mobile phases they have used for specific compound separations, offering another valuable resource for mobile phase selection [1].

Building on Snyder's solvent classifications [65], Nyiredy [6,66] proposed a structured approach for selecting individual solvents for mobile phases. Snyder categorized over 80 solvents into eight groups for normal-phase liquid chromatography (NPLC) based on their proton acceptance, proton donation, and dipole interaction properties. From this classification, Nyiredy identified 27 commonly used solvents in planar chromatography, highlighting nine that can be applied individually for separating compound mixtures on silica TLC plates: diethyl ether, ethanol, tetrahydrofuran, acetic acid, dichloromethane, ethyl acetate, dioxane, toluene, and chloroform [66]. The ability of these solvents to induce compound migration is assessed, with an optimal retardation factor (Rf) distribution recommended between 0.2 and 0.8 to ensure high reproducibility in planar chromatography [2,67]. The Rf value, a qualitative parameter representing the ratio of the distance traveled by a compound to that of the solvent front, is adjusted by modifying solvent strength. This adjustment is achieved by adding hexane (solvent strength = 0) to reduce migration or a small amount of water (solvent strength = 10.2 in NPLC) to enhance it, thereby ensuring the spots migrate within the optimal Rf range. The best-performing solvents within this region are then selected for systematic mobile phase optimization [66].

For mobile phase optimization, solvents are mixed to fine-tune selectivity. Nyiredy outlined several systematic optimization strategies in planar chromatography, including the window diagram approach, the sequential simplex method, Geiss's structural approach, and the PRISMA model. The window diagram approach identifies the worst separation results for various experimental conditions and determines the conditions yielding the best performance among the worst-case scenarios, which then serve as the basis for optimization

[68,69]. The simplex method, a sequential approach, involves defining a geometrical figure (simplex) based on the number of variables examined and systematically adjusting experiments to refine the solvent composition toward an optimal outcome [70]. Geiss's structural approach treats selectivity and solvent strength as independent factors, using strong solvents such as methyl tert-butyl ether, acetonitrile, and methanol, diluted with a weaker solvent like 1,2-dichloroethane, to systematically determine the ideal solvent strength for separation [71]. The PRISMA model incorporates a three-dimensional geometric approach, addressing solvent strength and selectivity in a stepwise manner, ultimately leading to the selection of an appropriate development mode, such as overpressured planar liquid chromatography (OPLC) or rotational planar chromatography (RPC) [71].

Despite the availability of systematic optimization methods, a literature review indicates that mobile phase selection is frequently based on solvent availability and trial-and-error approaches [38]. For instance, Ekiert et al. [72] noted that the mobile phase used for analyzing azole antifungal agents (ketoconazole, fluconazole, bifonazole, and itraconazole) was derived from the Polish Pharmacopoeia VI, originally designed for ketoconazole purity analysis, without detailing any optimization procedures. Many studies reference trial-and-error as the primary approach to mobile phase optimization [15,17,18,21–24,27,29,30,36–39,41,43–51,53,54,59,61], while others provide no information on solvent composition origins or optimization methods [16,19,20,25,26,31–35,40,42,52,55–58,60].

In TLC applications, where pharmaceutical formulations often contain only a few active ingredients (e.g., sulfamethoxazole/trimethoprim in tablets [15], rabeprazole/itopride in capsules [35], or cefuroxime axetil/ordinazole in tablets [51]), separations can typically be achieved without employing systematic optimization methods. However, as sample complexity increases, systematic optimization of the mobile phase becomes increasingly crucial [7]. This is exemplified in [28], where numerical taxonomy was employed for selecting an appropriate solvent system in the TLC analysis of five androstane isomers, followed by the use of simplex or PRISMA approaches for mobile phase optimization. Numerical taxonomy applies mathematical techniques to classify solvent systems based on Rf values, grouping similar mobile phases by calculating taxonomic distances (DJ,k), determined using the equation:

dj,k=∑i=1n(xi,j−xi,k)2/ndj,k = \sqrt{\sum_{i=1}^{n} (xi,j - xi,k)^2 / n}dj,k=i=1∑n (xi,j−xi,k)2/n

Where xi, xi, xi,j and xi,kxi,kxi,k represent the Rf values of compound iii in mobile phases J and K, and it is the number of analyzed compounds. The mobile phases with the smallest taxonomic distance are grouped, and subsequent taxonomic calculations refine the selection.

This systematic approach to mobile phase optimization highlights its significance when dealing with complex sample compositions, ensuring improved separation efficiency in planar chromatography.

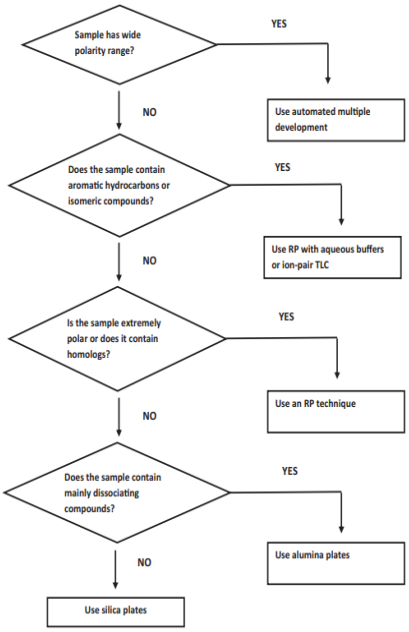


Fig 1. Flow chart for the selection of the stationary phase and separation mode

This process continues until all mobile phases are classified into a hierarchical, non-overlapping grouping system. The selection of the most effective mobile phase is based on the amount of information (I) and the objective function (Fobj), calculated using Equations (2) and (3), respectively. The amount of information is determined by:

I=−∑nknlog2nknI = - \sum \frac{n_k}{n} \log_2 \frac{n_k}{n}I=−∑nnklog2nnk

Where nkn_knk represents the number of separated compounds in a group, and nnn is the total number of

investigated compounds. The objective function, which quantifies the quality of chromatographic separation, is given by:

Fobj=aIp+bI+cIRc+dRRPFobj = aI_p + bI + cI_{Rc} + dRRPFobj=aIp+bI+cIRc+dRRP

Where a,b,c, a, b, c, a,b,c, and D are weighting coefficients (set as a=1a = 1a=1, b=10b = 10b=10, c=20c = 20c=20, and d=20d = 20d=20), IpI_pIp is the performance index, and RRP is the relative resolution index. The objective function (FobjFobjFobj) provides a numerical representation of the chromatographic separation quality, reaching its minimum value at the optimal system. The final mobile phase selection is based on both III and FobjFobjFobj. Readers can refer to [28,73] for a more detailed discussion on numerical taxonomy.

Following the selection, the best mobile phase identified in [28] was further optimized using two different approaches: the simplex method and the PRISMA model. This comparison aimed to evaluate the optimal compositions obtained through each approach, ultimately revealing that both methods led to similar optimal mobile phase compositions.

Table 1. Solvent specification table for the mobile phase

Solvent	UV-cutoff (nm)	Boiling Point (°C)	Density (g/mL) at 25°C	Refractive Index at 25°C	Melting Point (°C)	Polarity Index	Elutropic Value on Silica (D°)	Viscosity at 20°C (cP)	Flash Point (°C)	Molecular Weight
1-Butanol	215	117.7	0.8098	1.3972	-88.6	3.9	-	2.98	35	74.12
2-Propanol	205	82.3	0.7855	1.3772	-90.0	3.9	0.63	2.40	-12	60.10
Acetone	330	56.1	0.7857	1.3568	-94.3	5.1	0.53	0.36	20	58.08
Acetonitrile	190	81.6	0.7780	1.3415	-50.0	5.8	0.52	0.36	2	41.05
Chloroform	245	61.7	1.4840	1.4445	-63.3	4.1	0.26	0.58	None	119.38
Cyclohexane	202	80.7	0.7740	1.4247	-6.5	0.2	0.03	0.90	-20	84.16
Dimethyl Sulfoxide	262	189.0	1.1014	1.4783	18.5	7.2	-	2.24	87.8	78.13
Ethyl Acetate	255	77.1	0.8940	1.3695	-83.9	4.4	0.38	0.45	-4	88.11
Ethyl Ether	218	34.6	0.7134	1.3500	-116.3	2.8	0.43	0.24	-45	74.12
Glycerol	205	290.0	1.2613	1.4746	18.2	-	-	193	N/A	92.09
Heptane	197	98.4	0.6838	1.3855	-90.6	0.2	0.01	0.40	-4	100.20
Hexanes	195	69.0	0.6630	1.3759	-95.3	0.1	0.01	0.31	-23	86.18
Isooctane	205	99.2	0.6919	1.3895	-109.5	0.1	0.01	0.50	28	114.23
Methanol	205	64.7	0.7915	1.3288	-97.8	5.1	0.73	0.55	12	32.04
Methylene Chloride	233	39.5	1.3180	1.4215	-96.7	3.1	0.32	0.30	N/A	84.93
N-Methylpyrrolidinone	275	202.2	1.03	1.469	-24.4	-	-	1.67	95	99.13
N, N-Dimethylformamide	268	153.0	0.9440	1.4280	-61.0	6.4	-	0.92	58	73.09
Pentane	190	36.1	0.6264	1.3555	-129.7	0.0	0.00	0.22	-49	72.15
Petroleum Ether	-	35-60	0.6400	1.3610	-	0.1	-	-	-18	-
Tetrahydrofuran	210	66.1	0.8892	1.4060	-108.3	4.0	0.35	0.55	-14	72.11
Toluene	285	110.6	0.8660	1.4940	-95.0	2.4	0.22	0.59	-4	92.14
Water	-	100.0	0.9982	1.3330	0.0	10.2	-	1.00	N/A	18.02

Sample application

Sample Application Devices

Various manufacturers offer devices for applying sample solutions onto chromatographic layers. Notable examples include the Nanomat 4, Linomat 5, and the Automatic TLC Sampler 4 (ATS 4) (all from CAMAG, Muttenz, Switzerland). The Nanomat 4 is a manually operated device designed for spot sample application, utilizing a disposable capillary pipette held by a universal capillary holder. This device can deliver sample volumes ranging from 0.5 to 5 µL per spot on the chromatographic layer [74].

The Linomat 5, on the other hand, is a semi-automated device that applies samples in bands, making it suitable for qualitative, quantitative, and preparative separations. It accommodates 100 and 500 µL sample syringes and can dispense between 0.5 and 500 µL per band. This device primarily employs the spray-on technique with nitrogen gas during application, ensuring rapid solvent evaporation and the formation of narrow bands that concentrate the sample efficiently [75].

For fully automated sample application, the Automatic TLC Sampler 4 (ATS 4) offers both spotwise and bandwise applications using the spray-on technique. It allows multiple applications of different solutions at the same location, facilitating pre-chromatographic derivatization. This device is particularly beneficial for high-throughput analysis involving multiple samples across multiple TLC plates [76].

Proper sample application is critical for achieving high resolution and accurate quantification in High-Performance Thin-Layer Chromatography (HPTLC). Bandwise application offers greater flexibility regarding solvent choice (even highly polar solvents such as methanol and water) and allows for precise control over application volumes. Spotwise application, in contrast, may lead to broadening effects that reduce resolution. The precision of bandwise application ensures compact application zones and maximizes resolution, making it the preferred method for quantitative analysis [4].

Studies indicate that the Linomat 5 is the most commonly used application device, as reported in multiple publications [15,18,19,22–24,26,27,29,32,34–40,42–45,47–49,51–53,55,56,60]. The Linomat 4 has also been widely used [16,17,20,21,25,30,31,33,41,50,54,57–59], along with the Desaga AS 30 TLC applicator (Wiesloch, Germany) [41,61]. In contrast, the Nanomat 4 and Automatic TLC Sampler 4 were not reported in any of the reviewed studies. The use of a micropipette for sample application was mentioned in [28].

Chromatographic Development Techniques

Comprehensive descriptions of HPTLC development techniques can be found in [4,8,77]. These techniques include ascending, descending, two-dimensional, multiple, stepwise development, radial, antiradial, and forced-flow planar chromatography.

1. Ascending Development: This is the most widely used method, where a spotted plate is placed in a chamber containing the mobile phase, which moves upward through the chromatographic layer via capillary action [77]. During this movement, solutes in the sample migrate at different rates, leading to separation.

2. Descending Development: In this technique, the mobile phase enters the chromatographic layer from the upper edge of the plate, enabling downward movement.

3. Two-Dimensional Development: This method enhances separation by developing the plate twice—first in one direction and then after a 90° rotation, using a different solvent system. This approach is particularly useful for separating structurally similar compounds such as amino acids, which tend to have similar Rf values [77].

4. Multiple and Stepwise Development: Multiple-run development involves repeated development with the same solvent, while stepwise development uses different solvent systems with decreasing polarity and increasing development distances. These methods improve resolution by concentrating separated compounds in each successive run. They are particularly beneficial for detecting analytes in complex mixtures, such as plant extracts [77,78]. The automated version of this technique is known as Automated Multiple Development (AMD). However, no published studies were found describing AMD in pharmaceutical analysis.

5. Radial and Antiradial Development: In radial chromatography, the sample is applied to the center of a circular TLC plate, with the mobile phase introduced at the center via a wick. As the mobile phase spreads outward, it forms concentric rings where separation occurs [8,77]. Conversely, in antiradial development, the mobile phase enters the plate from the edges, moving inward toward the center. This method is particularly useful for separating compounds with high Rf values [8].

6. Forced-Flow Planar Chromatography (FFPC): FFPC techniques use external forces to enhance mobile phase movement. These include:

- Rotational Planar Chromatography (RPC) – Uses centrifugal forces

- Overpressured Layer Chromatography (OPLC) – Uses pumps
- Electro-planar chromatography (EPC) – Uses electric fields [79,80]

These approaches optimize mobile phase flow, reduce diffusion, and allow for faster separations. For instance, an OPLC method was developed for the analysis of five hormones (allylestrenol, estradiol, ethynodiol diacetate, levonorgestrel, and norethisterone) in a cleaning validation study. The method utilized a silica gel stationary phase, a mobile phase of diethyl ether and n-hexane (6:4, v/v), and a pressure of 5 MPa, completing the separation within 15 minutes using only 7 mL of mobile phase—significantly less than the 20 mL typically required for standard HPTLC [9,81].

Despite the availability of multiple chromatographic techniques, ascending development was found to be the preferred choice across all reviewed studies.

Chromatographic Chambers

HPTLC chambers are broadly classified into normal (N) type and sandwich (S) type. The distinction between these is based on the distance between the chamber wall and the plate—if greater than 3 mm, it is a normal chamber, while distances less than 3 mm define a sandwich chamber. Chambers can also be classified based on whether they are saturated or unsaturated with mobile phase vapor [4,7,8,66,73].

For quantitative analysis and method development, chamber selection depends on the number of compounds in the sample. Studies suggest that if a sample contains fewer than seven analytes, a saturated N chamber is preferable. However, if a sample contains more than seven compounds or features structurally related compounds with broad polarity ranges, an S chamber is recommended, as it enables efficient mobile phase transfer to forced-flow systems [7,66].

Among the various chamber types, the twin-trough chamber was the most frequently used in the reviewed studies [15–18,21–39,44,45,47–51,53,54,56–59,61]. This chamber is divided into two compartments by a glass ridge at the bottom, allowing the mobile phase to saturate the chamber before development begins. This design ensures low solvent consumption (10–15 mL) and reproducible pre-equilibration of the stationary phase, solvent system, and vapor phase [3]. The flat-bottom normal (N) chamber, which operates similarly to the twin-trough chamber, was reported in [47].

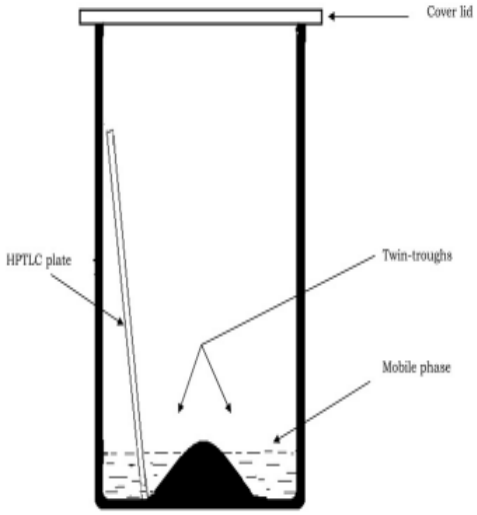


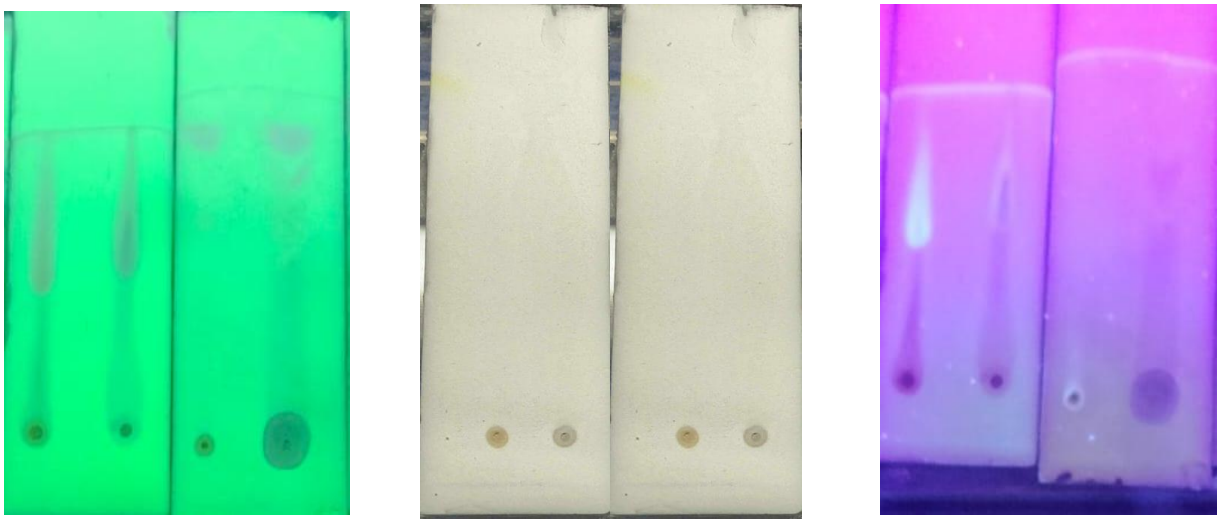
Fig 1. Twin trough chamber

Detection

Detection of separated compounds on sorbent layers in HPTLC analysis is enhanced by the incorporation of fluorescence indicators with excitation wavelengths of 254 and 366 nm. When exposed to these wavelengths, the plates fluoresce in green or blue, respectively [77]. Detection occurs due to fluorescence quenching by UV-absorbing compounds, allowing for clear visualization of separated components. Electronic instruments such as densitometers are employed to measure the quenching intensity and convert it into chromatographic peaks. The peak heights or areas can then be utilized for quantitative analysis [4].

Densitometers function by scanning chromatographic tracks using monochromatic light through a rectangular slit of selectable dimensions. Light from a deuterium (UV) or tungsten (visible) lamp is directed at the sample at a right angle, and a photomultiplier positioned at a 30° angle measures the reflected light [76,77]. The absorbance mode quantifies the difference between incident and reflected light, while the transmission mode measures light passing through the sample spot. Reflectance/absorbance mode is more commonly used in densitometry due to its precision, as it is less affected by variations in layer thickness and uniformity compared to transmission mode [77]. Among reviewed studies, reflectance measurements were the predominant detection method [15–27,29–45,47–61].

Additional detection techniques include video densitometry [82,83], mass spectrometry, Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy, nuclear magnetic resonance (NMR), and radioactivity-based methods [84–86]. For compounds lacking UV-vis absorption properties, such as ethambutol, chemical derivatization is necessary [77]. However, since many pharmaceutical active ingredients exhibit UV absorption, they can typically be detected without derivatization.



A B C

Fig 2. Detection of. the chromatographic sample with UV light.

A. Sample detection under 254 nm light, B. Sample detection under Day light/Normal light, C. Sample detection under 554 nm light

Quantitation

Quantitative determination of separated compounds on HPTLC plates can be performed using two approaches: (1) extracting and analyzing scraped compounds, or (2) measuring compounds directly on the stationary layer [8]. The first method involves dissolving scraped compounds in a solvent and analyzing the resulting solution using techniques such as UV spectrophotometry, HPLC, GC, or Liquid Scintillation Counting. In contrast, the second method employs optical densitometric scanners linked to computers for in situ measurements. All reviewed publications on formulation analysis applied the second approach [15–61], as it is simpler and avoids the need for additional extraction equipment.

For online extraction and mass spectrometry analysis, semi-automated systems such as the ChromeX-trakt (ChromAn, Holzhausen, Germany) [86] and TLC-MS interface (CAMAG, Muttenz, Switzerland) [87,88] are available. Accurate and precise sample application is critical for successful quantitation in HPTLC [4], with standard and sample solutions chromatographed on the same plate to minimize variability [2,4]. After development and drying, densitometers are used to measure the areas of separated bands [15–27,29–45,47–61].

Calibration graphs are constructed by plotting peak areas or heights against the amounts of standards rather than their concentrations. These graphs typically yield linear or quadratic relationships, which are then used to quantify unknown compounds. Although a linear calibration is preferred, quadratic relationships have also been reported [19,36]. Bands applied using the spray-on technique result in homogeneous sample distribution, enabling partial band measurement [76]. Densitometer slit dimensions are typically set at 70–90% of the band size to ensure accuracy. For instance, a 6 mm band requires a slit size of approximately 5.0 mm × 0.45 mm. Scanners offer customizable slit settings ranging from 0.5 to 20 mm in length and

0.025 to 1.2 mm in width, depending on band size [76]. Most studies adhered to the recommended slit dimension settings [15,17,21–25,27,29,32–37,40,43,45,48,49,51,53,56,57,59], though some deviated by using 100% of the band size [48–50,60].

HPTLC Method Validation for Pharmaceutical Analysis

Like all analytical techniques for pharmaceutical analysis, HPTLC methods require validation to ensure their suitability for regulatory compliance.

Classical Validation Approach

Several guidelines outline validation procedures for analytical methods, including Quality Assurance in Analytical Chemistry [89], ICH guidelines [90], FDA Guidance for Industry [91], the US Pharmacopeia [92], and AOAC guidelines for single-laboratory validation [93]. Among these, ICH guidelines were the most frequently referenced in publications [15,17–19,21–24,26,27,29–32,34,36–44,46,48,50–57,60], while some papers did not cite any references.

Method performance parameters such as specificity, linearity, precision, trueness, range, limits of detection (LOD), limits of quantitation (LOQ), and robustness are defined, with recommendations for their evaluation [68,90]. However, explicit acceptance criteria for each parameter are not always specified. In pharmaceutical analysis, chromatographic methods commonly adopt an intermediate precision acceptance criterion of ≤5% relative standard deviation, as observed in certain studies [16,46]. A method is considered validated if all parameters meet predefined criteria. The extent of validation varies among studies [15–61], and it is unclear whether this variation is always based on the intended use of the method. While all publications validated linearity, trueness, and precision, other ICH-suggested parameters such as specificity and range were not always evaluated. Notably, the range is often unnecessary in formulation assays.

Linearity of the Calibration Line

Analysts typically favor utilizing straight-line equations to facilitate the straightforward estimation of ingredient quantities in samples. Consequently, numerous reviewed methods were optimized and validated within concentration ranges where a linear correlation between the active ingredient content in solutions or spot bands and the peak heights or areas was established [15,20–27,29–31,33,35,37,38,40,42–60]. Nevertheless, quadratic relationships have also been reported to yield satisfactory results. For example, Habte et al. [36] developed and validated a method for the simultaneous separation and quantification of lamivudine and zidovudine in pharmaceutical formulations. The calibration data conformed more accurately to a quadratic model, with r^2 values of 0.9997 and 0.9996 for lamivudine and zidovudine, respectively, compared to the linear model, where the data exhibited lower r^2 values of 0.9776 and 0.9616. The mean assay results obtained using the quadratic model were 99.74 ± 0.84% and 99.76 ± 0.88% for lamivudine and zidovudine, respectively. Similarly, Kaale et al. [19] participated in an interlaboratory study employing HPTLC to analyze composite samples containing lamivudine-zidovudine, metronidazole, nevirapine, and quinine. Linearity assessments for each compound were conducted across concentration levels spanning 50–150% of the target concentration, with the calibration data aligning well with quadratic models, all yielding r^2 values exceeding 0.998. The assay results obtained using these quadratic models ranged from 97.0% to 102.7%, with a relative standard deviation (RSD) not exceeding 3.21%. However, in the studies cited above [19,36], assay results derived from a straight-line model were not reported, precluding a direct comparison with the quadratic equation results. In both cases, an r^2 value of at least 0.99 was considered indicative of linearity, though this criterion is insufficient as values approaching 1.0 can also be observed for nonlinear relationships [85,94–97].

Precision (Repeatability and Intermediate Precision)

Precision in an analytical procedure refers to the degree of agreement (or scatter) among a series of measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions [90]. Regulatory guidelines define repeatability, intermediate precision, and reproducibility as key precision parameters to evaluate [90,91]. Since reproducibility assesses interlaboratory precision, it is excluded from this discussion as the considered case studies were validated within development laboratories. Given that analytical methods within a single laboratory are utilized on different days and by different analysts, sometimes using varied equipment, it is crucial to assess both repeatability and intermediate precision. Reference [22] only evaluated repeatability, which is insufficient for fully determining precision. Studies

on both repeatability and intermediate precision have been conducted [15,21–26,30,31,35,37,38,40,41,43–48,50–54], yielding %RSD values of ≤3% for repeatability and ≤5% for time-different intermediate precision—comparable to the thresholds recommended for HPLC methods, which should not exceed 5% [20,97]. Intermediate precision should be estimated through measurements taken on multiple days, ideally involving different analysts and equipment [90]. In most reviewed publications, time-different intermediate precision was assessed via multi-day measurements. Some sources advocate for simultaneous evaluation of repeatability and intermediate precision through experimental designs with replicate measurements on different days [68,90,98]. Analysis of variance (ANOVA) is used to estimate variance components, namely variance due to repeatability (s^2r) and variance due to between-day effects (s^2between), with time-different intermediate precision ($s^2I(t)$) calculated as $s^2I(t) = s^2r + s^2$between. Reference [98] demonstrated the underestimation of time-different intermediate precision when an experimental design is not utilized. Without ANOVA, %RSD was estimated at 5.2%, whereas incorporating ANOVA yielded a higher %RSD of 6.0% for the same dataset. Except for publications [15,37], that employed ANOVA to assess precision types, other studies did not specify how reported values were obtained. In repeatability assessments, studies adhered to the recommended $n \geq 3$ replicates across at least three concentration levels ($k \geq 3$) covering 80–120% of the target sample concentration [69,90] [15–18,21–27,30–33,36–41,43–45,47–53,56–59,61]. For intermediate precision, compliance with $n \geq 2$, $k \geq 3$, and multi-day measurements was observed [15,17,18,21–27,30–33,36–40,43–45,47–53,56–59,61]. However, some studies failed to specify the number of days included in intermediate precision assessments or did not thoroughly describe their methodologies. Furthermore, concentration levels used in precision assessments were not consistently reported. In some cases, only specific method components, such as chromatographic plate application repeatability or measurement repeatability, were evaluated, underestimating overall method repeatability, which should encompass all procedural steps from sample preparation to final measurements [1].

Trueness

Trueness in an analytical method represents the degree of agreement between the mean value obtained from a series of measurements and an accepted conventional true or reference value [99,100]. It is quantified through recovery or bias assessments [15,17,22,26,37,38,59,61]. While the International Council for Harmonisation (ICH) refers to this parameter as accuracy, ISO considers accuracy as a combination of precision (random error) and bias (systematic error). All reviewed studies conducted recovery tests by spiking prepared tablet matrices with reference standards [15–17,61] or using pre-analyzed samples via standard addition [18,20–27,29–31,33–36,38–45,47–49,51–54,56–60].

According to ICH guidelines, recovery studies for assay methods should be performed at a minimum of three concentration levels with at least three replicates per level. Most studies determined recoveries at 80%, 100%, and 120% of the expected sample concentration [15–18,21–27,29–34,36–43,45–54,56,57,59–61]. However, some deviations exist; for instance, in [35], a method for simultaneously quantifying rabeprazole and itopride hydrochloride in fixed-dose capsules only included two concentration levels (50% and 100%). Similarly, [43] reported a recovery study for cefetamet in tablets at just one concentration level. Nearly all reviewed studies complied with the requirement of $n \geq 3$ replicates per concentration level. Previous research has indicated that conventional point-interval hypothesis testing is not the optimal approach for bias detection. Instead, interval-hypothesis testing has been proposed, which requires predefined acceptance intervals within which bias at each concentration level must fall [101]. However, none of the reviewed case studies applied this approach [1].

Range

As outlined in the ICH guidelines [90], the range of an analytical method refers to the interval between the lowest and highest concentrations of an analyte in a sample where the method exhibits an appropriate level of precision, accuracy (trueness), and linearity. According to the ICH recommendations, concentration levels covering 80–120% of the target concentration should be evaluated to determine the method's range in the assay of formulations. Among the reviewed studies, only one [61] explicitly reported the method’s range, whereas all other studies mentioned linear ranges, which correspond to the lower and upper concentrations of the calibration curve. Typically, these linear ranges extend significantly beyond the 80–120% target concentration window. For example, in a

simultaneous determination of atorvastatin and ezetimibe in tablet formulations, the reported linear range covered 17–200% (0.4–2.4 ng/spot) of the target concentration (1.2 ng/spot) [50].

In [61], an HPTLC method was developed to quantify glucosamine in herbal dietary supplements, with validation performed using the accuracy profile approach. An accuracy profile is a graphical tool that visually represents the method's capability to meet analytical objectives and assess the associated risks in routine applications [102]. During validation, bias and standard deviation at each concentration level are determined, followed by the calculation of 95% tolerance limits for total error (bias + intermediate precision). These tolerance limits, along with predefined acceptance limits (e.g., ±5% for active ingredients in pharmaceutical products), construct the accuracy profile [98,100,102,103]. Figure 5a illustrates an example of an accuracy profile, where the solid lines at ±5% denote acceptance limits, dashed lines indicate 95% tolerance limits for total error, and the middle line represents relative error. The arrows highlight the upper and lower concentrations studied, confirming the method's range as the concentrations that fall within acceptance limits, thereby complying with the ICH definition.

Detection Limit (DL) and Quantitation Limit (QL)

According to the ICH guidelines, determining DL and QL is generally not required for assay methods [90]. Since most pharmaceutical formulations contain high levels of active ingredients, information on DL and QL is often unnecessary. However, in many cases, these limits were still assessed [22–25,27,30,33,35,38,40–46,48,51–53]. The signal-to-noise ratio is one commonly used approach for determining DL and QL [24,27,35,44,45,48,53], wherein signals from low-concentration sample solutions are compared with blank solution signals [90]. Ratios of 3:1 and 10:1 are generally accepted for DL and QL, respectively.

Another common approach is based on calibration curves [22,23,30,33,38,40,42,46,51,52]. According to [90], the formulas for DL and QL are as follows:

DL=3.3σS, QL=10σSDL = \frac{3.3\sigma}{S}, \quad QL = \frac{10\sigma}{S}DL=S3.3σ,QL=S10σ

Where σ\sigmaσ is the standard deviation of the response and SSS is the slope of the calibration curve. The slope is derived from the sample calibration curve, while σ\sigmaσ can be estimated from the standard deviation of blank solution signals or the y-intercept standard deviation of a regression line constructed with sample concentrations near DL and QL. Additionally, the residual standard deviation of these regression lines may serve as an estimate of σ\sigmaσ. A third method involves diluting a standard solution to the lowest detectable level [41]. However, some studies did not specify the procedure used to determine DL and QL [25,43].

When validating an analytical method using the accuracy profile, the DL and QL are identified as the points where the 95% expectation tolerance limits intersect with the acceptance limits [100]. If no intersection occurs, then the lowest and highest studied concentrations define the quantitation limits (Figure 5b). Within this range, measurements can be performed with known accuracy [100].

Additional Applications of HPTLC

HPTLC is widely applied in analytical sciences beyond pharmaceutical formulations, including stability testing. For example, Ali et al. [38] developed a stability-indicating method for analyzing impurities and degradation products in a fixed-dose antituberculosis tablet formulation containing isoniazid and rifampicin. The method used an aluminum-backed silica gel 60 F254 plate and a mobile phase composed of n-hexane, 2-propanol, acetone, ammonia, and formic acid (30:38:28:3:1, v/v/v/v/v), with detection at 254 nm. Similarly, Chitlange et al. [26] reported an HPTLC method for stability testing of dexibuprofen in pharmaceutical dosage forms, where the active ingredient was effectively separated from degradation products. The chromatographic conditions included a silica gel 60 F254 stationary phase and a mobile phase of n-hexane, ethyl acetate, and glacial acetic acid (7.5:2.5:0.2, v/v/v), with detection at 225 nm.

Ferenczi-Fodor et al. [104] provided a comprehensive review of the versatility, speed, and flexibility of HPTLC for pharmaceutical purity testing. Additionally, HPTLC methods have been explored for assessing content uniformity in tablets. For instance, [4] reported its application in uniformity testing, while Machale et al. [105] validated an HPTLC method for the content uniformity of diazepam tablets (2 mg). Using an aluminum-backed silica gel 60 F254 (20 cm × 10 cm) plate, 10 tablets were analyzed simultaneously in a short runtime. Results met USP specifications (85–

115% assay for individual tablets) with an RSD below 6%.

HPTLC has also been employed in environmental analysis, such as detecting pesticide residues in soil and water. Morlock [106] described a method for the simultaneous analysis of 283 pesticide residues in groundwater, raw drinking water, and mineral water, which has been standardized in Germany. This method is based on automated multiple development. Similarly, HPTLC has been used for pesticide residue detection in fruits, including chlorpyrifos, imidacloprid, and acephate in brinjal fruits [107].

In food analysis, HPTLC serves as a quantitative tool. Mennickent et al. [108] demonstrated its reliability for analyzing food additives like sucralose in milk-based confectioneries, adhering to European regulations (10–3000 mg/kg, depending on the food type). The repeatability (%RSD) of HPTLC methods in food analysis ranges between 1.5% and 6.4%, while correlation coefficients for calibration curves typically exceed 0.998, indicating high precision [108].

HPTLC is also valuable in drug monitoring, metabolism studies, and doping control. Mennickent et al. [108] developed a method to quantify fluoxetine in human serum, facilitating medication adherence monitoring. A 10 cm × 20 cm HPTLC plate was used to analyze 33 spots (6 standards + 27 patient samples), with protein precipitation performed using methanol. Similarly, Jain et al. [109] developed an HPTLC method for determining minocycline levels in human plasma. The method utilized silica gel 60 F254 plates and a mobile phase consisting of methanol, acetonitrile, isopropanol, and water (5:4:0.5:0.5, v/v/v/v), with detection at 345 nm. The method achieved a trueness of 95.8 ± 4.5%, with repeatability and intermediate precision (CV) of ≤3.49% and ≤4.14%, respectively. The quantitation limit was reported at 15.4 ng/spot.

CONCLUSION

This review reveals that most of the HPTLC methods developed and validated comply with the general procedures about the quantitative mode of this technique. HPTLC is generally used with an unmodified silica layer as a stationary phase on precoated plates and slit-scanning densitometry with UV–vis light as the detection technique. The most preferred way of mobile phase selection and “optimization” was found to be the trial-and-error approach and the analysts’ own experiences. However, in case a complex mixture of drugs needs to be separated, we advocate for a systematic approach.

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