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APPLICATIONS OF HPTLC IN HERBAL DRUGS ANALYSIS

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Abstract:
The analysis and quality control of herbal medicines are going a step ahead towards an integrative and comprehensive direction, in order to equipment the complex nature of herbal medicines. High-performance thin layer chromatography (HPTLC) is one of the sophisticated instrumental techniques for quantitative and qualitative analysis of the herbs and herbal drugs. The reported HPTLC method was found to be rapid, simple, and accurate for substantiation of the herbal plant materials. Multidimensional chromatography is often a good choice in case of very multiplex samples, offering many advantageous features in the analysis of medicinal plants. First of all, multidimensional TLC is the only real multidimensional method in which, after the first separation in the first direction, all compounds can be passed to a second direction. A Quality Control for Authentication 107 fingerprint analysis methods to control the quality of herbal drugs have gradually come into being, such as GC, TLC, HPLC, etc. chromatographic fingerprint analysis of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality, and ensuring the consistency and stability of herbal drugs and their related products. For example, Ginseng and American ginseng, the HPTLC is difficult to distinguish them by only selecting single ginsenosides, but the HPTLC images with digital scanning profiles can easily be differentiated between them.

KEYWORDS: *Herbal drugs, High-Performance Thin Layer Chromatography (HPTLC), Analytical methods*

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INTRODUCTION:

In the present epoch, 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 nature and mankind. [3] As per WHO, there are three kinds of herbal medicines: processed plant material, raw 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]
- ✓ The active principle(s) is (are), in most cases unknown
- ✓ Extracts are usually mixtures of many constituents.
- ✓ Selective analytical methods or reference compounds may not be available commercially. [11-13]

Herbal drugs are obtain popularity nowadays as it has a reputation of being clean, organic, and safe for consumption due to their natural source and ingredients. Herbal drugs are used as supplements or medicinal uses. They consist of health systems such as Ayurveda, Siddha, Unani, Homeopathy and Naturopathy. Herbal medicines/drugs are plant-derived materials with therapeutic and different health benefits for humans, which are acquired or captured from the plants and their part. [11,14]

Any drug before being conduct into the market has to be tested and clinical trials have to be taken. Herbal drugs are more prone to adulteration. Many times, it is a polyherbal formulation meaning it is made up of different plants/ herbs. [10]

HPTLC is an efficient tool for herbal drug analysis. The herbal matrix is complex in nature and since HPTLC has expendable layers there is no damage to the instrument. Hence, HPTLC is used in herbal drug standardization. These medicines are prone to adulteration by infamous dealers and manufacturers who mix sub-standard material for profits. HPTLC has an application for the detection of adulteration of herbal drugs. For example, CAMAG's HPTLC systems (HPTLC & HPTLC Pro) in herbal drug analysis offers the tools and expertise required to withstand any scrutiny regarding botanical materials. [10,11,14]

All pharmacopoeias set standards for the strength, quality, purity, and consistency of these products–critical to the public health. USP–NF contains approximately 4550 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). [15] The Indian Pharmacopoeia 2007, which was made effective from last July, has already over 1600 monographs. [16] The British Pharmacopoeia 2012 contains approximately 3375 monographs for substances, preparations and articles used in the practice of medicine. [17]

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. [18] It is the process of developing and agreeing upon technical standards. Specific standards are worked out by observations, and experimentation which would lead to the process of prescribing a set of characteristics exhibited by the particular herbal medicine. [19]

HPTLC is a modern adaptation of TLC with better and advanced separation efficiency and detection limits. The table 1 compares HPTLC and TLC. [20-29]

Table no 1: Difference between HPTLC and TLC

PARAMETERS	HPTLC	TLC
Technique	Instrumental	Manual
Efficiency	High (Due to smaller particle size)	Less
Layer	Pre-coating	Lab Made/ Pre-coating
Sample Spotting	Auto sampler (Syringe)	Manual Spotting (Capillary/Pipette)
Solid Support	Silica Gel- Normal Phase C8 and C18- Reverse phase	Silica Gel, Alumina, Kiesulguhr
Plate Hight	12um	30um
Layer Thickness	100um	250um
Mean particle size	5-6 um	
Sample Volume	0.1-0.5 ul	1-5 ul
Shape of sample	Circular (2-4 nm Dia)	Rectangular (6 mm L × 1mm W)
Separation	3-5 cm	10-15 cm
Separation time	3-20 Min	20-200 Min
Sample tracks per plate	≤ 36 (72)	≤ 10
Detection Limits (Absorption)	100-500 pg	1-5 pg
PC connectivity, Method Storage	Yes	No
Detection limits (Fluorescence)	5-10 pg	50-100 pg
Validation, Quantitative Analysis, Spectrum Analysis	Yes	No
Analysis Time	Shortage Migration Distance and the analysis time is greatly reduced	Slower
Wavelength Range	190 or 800 nm, Monochromatic	254 or 366 nm, Visible
Scanning	Use of UV/ visible/ fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and scanner is an advanced type of Densitometer	Not Possible

There are several advantages of using HPTLC for the analysis of compounds as compared to other techniques, like HPLC, titrimetry, spectrophotometry etc. [27] Some of the advantages of HPTLC are:

- ✓ Ability to analyse crude samples containing multi-components.
- ✓ Choice of solvents for the HPTLC development is wide as the mobile phases are fully evaporated before the detection step.
- ✓ The separation process is easy to follow especially with coloured 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.
- ✓ Two-dimensional separations are easy to perform. Stability during chromatography should be tested using two-dimensional development. [29]
- ✓ Specific and sensitive colour reagents can be used to detect separated spots (Dragendroff reagent/Kedde reagent). [30]
- ✓ Contact detection allows radio labelled compounds to be monitored and microbial activity in spots to be assessed.
- ✓ HPTLC can combine and consequently be used for different modes of evaluation, allowing identification of compounds having different light absorption characteristics or different colours.
- ✓ HPTLC method may help to minimizes exposure risk of toxic organic effluents and significantly reduces its disposal problems, consequently, reducing environment pollution. [22-32]

Applications of HPTLC Separation:

HPTLC is one of the most widely applied methods in phytochemical analysis. It is due to its numerous advantages, e.g., it is the only chromatographic method offering the option of presenting the results as an image. Other advantages include simplicity, low costs, parallel analysis of samples, rapidly obtained results, high sample capacity, and possibility of multiple detection. HPTLC provides identification as well as quantitative results. It also enables the identification of adulterants. [33,34]

Multidimensional chromatography is often a good choice in case of very complex samples, offering many advantageous qualities in the analysis of medicinal plants [35]. In multidimensional separation, a sample is first subjected to separation via one method, and then the separated compounds are further resolved by at least one additional independent method [36]. According to Giddings, two conditions should be fulfilled to classify a chromatographic method as a multidimensional one [37]. Firstly, the separation mechanisms of the applied steps must be orthogonal, and secondly, the resolution gained in the first dimension may not be lost in any of the subsequent steps.

Multidimensionality can be realized in gas chromatography; however, in case of liquid chromatography, it is not a simple task. Planar chromatography gives a possibility of performing multidimensional separations with the use of several techniques. 2D-TLC has several unique features and thus is quite often a choice in the analysis of complex natural mixtures [35]. First of all, multidimensional TLC is the only real multidimensional method in which, after the first separation in the first direction, all compounds can be passed to a second direction [38]. Sophisticated equipment is not required, as in case of LC×LC separations, what is more plate is used once only, there is no need to worry about the adsorbed constituents that may cause column contamination. There is no need to perform complicated clean-up procedures, and multiple detection can be used to analyse the wide spectrum of compounds, which is impossible to realize in the sequential mode of HPLC. Proper chromatographic systems must be chosen in order to focus on the desired constituent group. The sample preparation step does not have to be modified even if one wants to focus on different substance classes present in the extract. [35].

Multidimensional planar chromatography has also several noticeable disadvantages. The amount of the analyzed samples is considerably reduced in multidimensional planar chromatography (MDPC), when compared to one-dimensional technique, as only one sample per plate can be analysed. Multidimensional techniques are usually more time-consuming than one-dimensional methods, and if there is no significant improvement, when compared to one-dimensional mode, they simply do not pay off. Sometimes, change in chromatographic conditions may bring pleasing results without the need to apply multidimensional separation, as in the case of fatty oil's resolution, according to the European Pharmacopoeia [39]. In case of multidimensional methods, there is always a possibility of artefact formation, due to chemisorptions or decomposition during chromatography. There are also several limitations as far as the solvents used are considered. Very polar and non-volatile solvents should be avoided as they are difficult to be removed from the adsorbent, e.g., trimethylamine, dimethyl sulfoxide, acetic acid, as well as ion-pair reagents and non-volatile buffer components [40]. In case of quantitative analysis, HPLC still remains a better alternative to TLC. Special modes of development can be classified as following:

A) Repeated multiple development techniques in one direction

- Incremental Multiple Development (IMD)
- Gradient Multiple Development (GMD)
- Unidimensional Multiple Development (UMD)
- Bivariant Multiple Development (BMD) and its automated version – Automated Multiple Development (AMD)

B) Multidimensional techniques

- Comprehensive 2D planar chromatography realized on one adsorbent or on bilayer plates
- Targeted (selective) 2D planar chromatography – only chosen spots, separated after the first development, are subjected to further analysis
- Graft TLC – the analysed compounds are transferred from the first adsorbent to another and redeveloped in orthogonal system
- Combination of MD-PC methods
- Modulated 2D planar chromatography – first eluents of decreasing strength are applied in the perpendicular direction and the sample is developed several times with solvent mixture of different selectivity at constant eluent strength
- Coupling of two chromatographic techniques realized in on- or off-line modes, e.g., HPLC–TLC, TLC–GC, TLC–MS, etc.

Techniques commonly applied in the analysis of phytochemical samples are discussed in the subsequent sections.

HPTLC Fingerprint Analysis:
Herbal medicines have a long therapeutic history and are still serving many of the health needs of a large population of the world. But the quality assurance and quality control still remain a challenge because of the high variability of chemical components involved. Herbal drugs, singularly and in combinations, contain a myriad of compounds in complex matrices in which no single active constituent is responsible for the overall efficacy. This creates a challenge in establishing quality control standards for raw materials and standardization of finished herbal drugs [41]. Generally, only a few markers of pharmacologically active constituents were employed to assess the quality and authenticity of complex herbal medicines. However, the therapeutic effects of herbal medicines are based on the complex interaction of numerous ingredients in combination, which are totally different from those of chemical drugs. Thus, many kinds of chemical 107 HPTLC Fingerprint Analysis:

A Quality Control for Authentication 107 fingerprint analysis methods to control the quality of herbal drugs have gradually come into being, such as Thin-Layer Chromatography (TLC), Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), etc. chromatographic fingerprint analysis of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality, and ensuring the consistency and stability of herbal drugs and their related products. The uninterrupted pattern of compounds can then be evaluated to determine not only the presence or absence of desired markers or active constituents but the complete set of ratios of all detectable analytes. The chemical fingerprints obtained by electrophoretic and chromatographic techniques, especially by flair chromatography's, are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the "chemical integrities" of herbal medicines and therefore be used for identification and authentication of the herbal products. [42] and the techniques used for fingerprint analysis of herbal medicines as following:

- Thin-Layer Chromatography (TLC)
- Gas Chromatography (GC)
- Column chromatography
- High-Performance Liquid Chromatography (HPLC)
- Ion-exchange chromatography

HPTLC in Herbal Drug Quantification:
Standardized manufacturing procedures and suitable analytical tools are required to establish the necessary framework for quality control in herbals. Among those tools, high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), and capillary electrophoresis are the most common used to establish reference fingerprints of herbs, against which raw materials can be evaluated and finished products, can be assayed. High-performance thin-layer chromatography, also known as planar chromatography, is a modern technique with high separation power and reproducibility superior to classical TLC. Main difference of HPTLC and TLC is particle and pore size of sorbents. Special features of HPTLC are as follows:

- Several analysts can work simultaneously
- Lower analysis time and less cost per analysis
- Low maintenance cost
- Simultaneous processing of sample and standard – better analytical precision and accuracy and less need for internal standard
- Simple sample preparation – can handle samples of divergent nature
- Visual detection possible – open system
- Non-UV-absorbing compounds detected by post chromatographic derivatization
- No prior treatment for solvents like filtration and degassing
- Low mobile-phase consumption per sample
- No interference from previous analysis – fresh stationary and mobile phases for each analysis, no contamination

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 [43] i.e. primary information about the physicochemical characteristics of sample and nature of the sample (structure, polarity, stability, volatility, and solubility). It involves considerable trial and error procedures. [44] General steps involved in HPTLC method developments are as follow: [24,45]

Basic steps:

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

HPTLC has been well reported that several samples can be run simultaneously by use of a smaller quantity of mobile phase than in HPLC. It has also been reported that mobile phases of pH 8 and above can be used for HPTLC. Another advantage of HPTLC is the repeated scanning (detection) of the chromatogram with the same or different conditions. Consequently, HPTLC has been investigated for simultaneous assay of several components in a multi component formulation. With this technique, authentication of various species of plant is possible, as well as the evaluation of stability and consistency of their preparations from different manufactures. Various workers have developed HPTLC method for phytoconstituents in crude drugs or herbal formulations such asbergen in, catechin and gallic acid in Bergenia cilliata and Bergenia lingulate.

Table no 2: Example of mobile phase used in HPTLC for herbal compounds [46,47,48]

Chemical Compounds (Herbal)	**Mobile Phase**
Alkaloids	Toluene: Ethyl Acetate: Diethyl Amine [70:20:10]
Flavonoids	Ethyl Acetate: Formic Acid: Glacial Acetic Acid: Water [100:11:11:26]
Saponin	Chloroform: Glacial Acetic Acid: Methanol: Water [64:32:12:8]
Cardiac Glycosides	Ethyl Acetate: Methanol: Water [100:13.5:10] OR [81:11:8]
Terpenes	Chloroform: Methanol: Water [65:25:4]
Lignans	Chloroform: Methanol: Water [70:30:4] Chloroform: Methanol [90:10] Toluene: Ethyl Acetate [70:30]
Essential Oil	Toluene: Ethyl Acetate [93:7]
Triterpenes	Ethyl Acetate: Toluene: Formic Acid [50:50:15] Toluene: Chloroform: Ethanol [40:40:10]
Polar Compounds Anthraglycosides, Arbutin, Alkaloids, Cardiac Glycosides, Bitter Principles, Flavonoids, Saponin	Ethyl Acetate: Methanol: Water [100:13.5:10]
Lipophilic Compounds Essential oils, Terpenes, Coumarin, Naphthoquinones, Velpotriate	Toluene: Ethyl Acetate [93:7]

Authentication of the Species Prone to Confusion:

For example, Ginseng (root of Panax ginseng) and American ginseng (root of Panax quinquefolium) are two close species containing very similar chemical ingredients. The functions of the two species are different according to TCHM in clinical use. It is difficult to distinguish them by only selecting single ginsenosides, but the HPTLC images with digital scanning profiles as a whole can easily be differentiated between them [49] (Fig. 1).

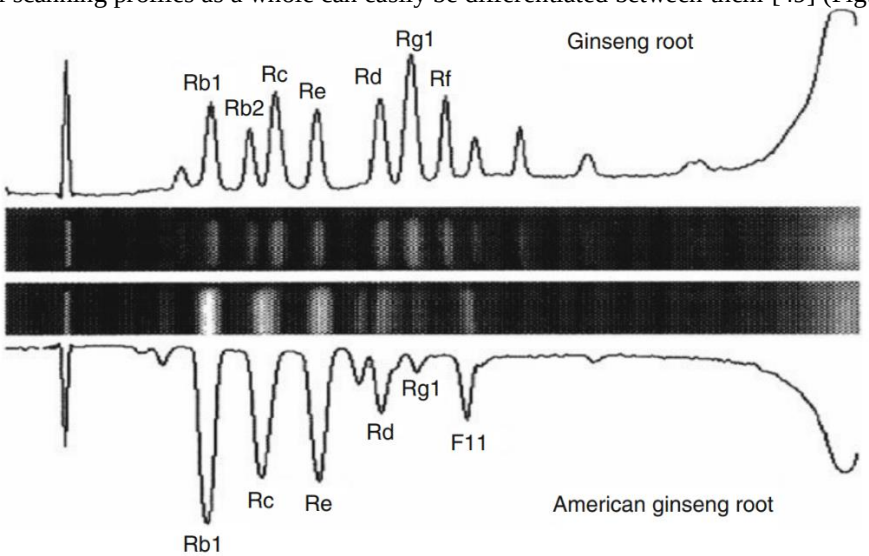

Fig no 1: HPTLC fingerprint of Ginseng and American ginseng

Table no 3: List of spices and potential adulterants as well as the suggested method

Product/Herbs	Adulterant	Recommended methods
R. graveolens	E. dracuveuloids	HPTLC profiling
C. angustifolia	C. tora	HPTLC profiling
C. reflexa	C. chinesis	Microscopic
P. nigrum	C. papaya	Microscopic
G. glabra	A. precatorius	TLC
Z. jujube	Z. mauritiana	Microscopic
Capsicums	Sudan red and related dyes, mono or disaccharides	HPTLC profiling
Oregano	Noncompliant herbs, i.e., Savory, thyme, marjoram	Microscopic
Saffron	Added artificial color	TLC
Nutmeg	Coffee husks	Microscopic
Cinnamon	Coffee husks	Microscopic
Cloves	Essential oils may have been removed	TLC
Chilli powder	Saw dust and color may be added	HPTLC
Coffee	Chicory	HPTLC
Cinnamon	Cassia bark which resembles cinnamon in taste and odor	HPTLC

CONCLUSION:

Application of HPTLC for Pharmaceutical analysis, Herbal drug quantification, phytochemical analysis, analytical analysis, fingerprint analysis, and HPTLC future to combinatorial approach, HPTLC scanning Diode Laser made HPTLC a power analytical tool in the field of analysis. It is noteworthy that utilization of instrumental HPTLC toward the analysis of drug formulations, natural products, Bulk drugs, clinical samples food stuffs, environmental, and other relevant samples will increase in the future. HPTLC is one of the sophisticated instrumental techniques for qualitative and quantitative analysis of the herbs and herbal drugs. In this article emphasizes on HPTLC based analytical method development and its applications.

The reported HPTLC method was found to be rapid, simple, and accurate for authentication of the herbal plant materials. The described method is suitable for routine use and authenticates the herbal plant materials. The processing of samples and standards together at the same time (in-system calibration) leads to improved reproducibility and accuracy. Fingerprint developed by HPTLC can be used for routine authentication of various herbal plant materials purchased from market. Biochemical markers also can be developed by this method to authenticate the herbal plant materials.

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