Thin-Layer Chromatography

The following procedure is applicable as an aid in verifying the identities of many compendial drug substances as such and in their respective dosage forms.

Thin-layer chromatography is a technique in which a solute undergoes distribution between two phases, a stationary phase and a mobile phase in the form of a liquid. The stationary phase is in the form of a relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may be achieved on the basis of adsorption, partition or a combination of partition and adsorption, complexation, or ion exchange, depending on the particular type of the stationary phase, its preparation and its use with different solvent.

Identification can be affected by observation of spots of identical *Rf* value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots or bands usually serves for semi-quantitative estimation.

Apparatus

(a) Flat glass plates of appropriate dimensions which allow the application at specified points of the necessary quantities of the solution being examined and appropriate reference solutions and which allow accommodation of the specified migration path-length. The plates are prepared as described below; alternatively, commercially available pre-coated plates may be used.

(b) An aligning tray or a flat surface on which the plates can be aligned and rested when the coating substance is applied.

(c) The adsorbent or coating substance consisting of finely powdereddry materials, normally 5 µm to 40 µm in diameter, suitable for chromatography. It can be applied directly to the plate or can be bonded to the plate by means of Plaster of Paris (Hydrated Calcium Sulphate) or with any other suitable binders. The adsorbent may contain fluorescing material to help in visualizing spots under ultraviolet light.

(d) A spreader which, when moved over the glass plate, will apply a uniform layer of the coating material of desired thickness over the entire surface of the plate.

(e) A storage rack to support the coated plates during drying and transportation.

(f) A developing chamber that can accommodate one or more plates and can be properly closed and sealed. The chamber is fitted with a plate support rack that supports the plates, back to back, with the lid of the chamber in place.

(g) Graduated micro-pipettes capable of delivering microlitre quantities say 10 µl and less.

(h) A reagent sprayer that will emit a fine spray and will not itself be attacked by the reagent.

(i) An ultraviolet light, suitable for observation at short (254 nm) and long (365 nm) ultraviolet wavelengths.

Preparation of plates. Unless otherwise specified in the monograph, the plates are prepared in the following manner. Prepare a suspension of the coating substance in accordance with the instructions of the supplier and using the spreading device designed for the purpose, spread a uniform layer of the suspension, 0.25 to 0.30 mm thick, on a flat glass plate 20 x 20 cm or of appropriate dimensions. Allow the coated plates to dry in air, heat at 100º to 105º for at least 1 hour (except in the case of plates prepared with cellulose when heating for 10 minutes is normally sufficient), allow to cool, and protect it from moisture. Store the plates protected from moisture and use within 3 days of preparation. At the time of use, dry the plates again, if necessary, as prescribed in the monograph.

Method

Unless unsaturated conditions are prescribed, prepare the tank by lining one side of the walls with a sheet of filter paper; pour into the tank, saturating the filter paper in the process, sufficient volume of the mobile phase to form a layer of solvent 5 to 10 mm deep, close the tank and allow to stand for 1 hour at room temperature. If self prepared plates are used, remove a narrow strip of the coating substance, about 5 mm wide, from the vertical sides of the plate. Apply the solutions being examined in the form of circular spots about 2 to 6 mm in diameter, or in the form of bands (10 to 20 mm x 2 to 6 mm, unless otherwise specified) on a line parallel with, and 20 mm from, one horizontal end of the plate, and not nearer than 20 mm to the two vertical sides; the spots should be 15 mm apart. If necessary, the solutions may be applied in portions, drying between applications. Mark the sides of the plate 15 cm, or the distance specified in the monograph, from the starting line. Allow the sample solvent to evaporate and place the plate in the tank opposite to the filter paper, ensuring that it is as nearly vertical as possible and that the spots or bands are above the level of the mobile phase. The stationary phase should face the filter paper. Close the tank and allow to stand at room temperature, until the mobile phase has ascended to the marked line. Remove the plate and dry it.

For two-dimensional chromatography dry the plate after the first development and carry out the second development in a direction perpendicular to the first.

When the method prescribed in the monograph specifies ‘protected from light’ or ‘in subdued light’ it is intended that the entire procedure is carried out under these conditions.

Adjustment of chromatographic conditions

Minor adjustments to the parameters of the test may be made in order to satisfy the system suitability criteria. These may be:

Mobile phase. Minor solvent component of a mixture: ± 30 percent relative or ± 2 percent absolute, whichever is the larger; no other component altered by more than 10 percent absolute;

Concentration of salts. In the buffer component of the mobile phase; ± 10 percent;

Visualisation

After development, the plate should be examined under white light and under ultraviolet light having a maximum output at about 254 nm or at about 365 nm, as the case may be. Alternatively, it may be visualised as directed in the monograph; where a spraying technique is prescribed, it is essential that the reagent be evenly applied as a fine spray.

The termsecondary spot means any spot other than the principal spot. Similarly, a secondary band is any band other than the principal band.

Semi-quantitative estimation

Identification. The principal spot in the chromatogram obtained with the test solution is visually compared to the corresponding spot in the chromatogram obtained with the reference solution in respect of the colour, the size and the $R_f$of the spots.

Test for Related substances. The secondary spot(s) in the chromatogram obtained with the test solution is (are) visually compared to either the corresponding spot(s) in the chromatogram obtained with the reference solution containing the impurity (ies) or the spot in the chromatogram obtained with the reference solution prepared from a dilution of the test solution.

Quantitative measurement

The substances that have been separated after development of the plate and that respond to UV-Vis irradiation can be estimated directly on the plate with suitable instrumentation. Measurement is of the reflectance of the incident light from the spots by moving the plate or

the measuring device. Likewise, fluorescence may be measured using an appropriate optical system.

Apparatus. The apparatus for direct measurement consists of:

— a device for exact positioning and reproducible application of the amount of solutions onto the plate,

— a mechanical device for moving the plate or the measuring device along the *x*-axis or the *y*-axis,

— a recorder and a suitable integrator or a computer, and

— a photometer with a source of light, an optical device for generating monochromatic light and a photo cell of adequate sensitivity; for measurement of fluorescence, a suitable filter to prevent light used for excitation from reaching the detector while permitting emitted light or a specific portion thereof to pass.

Method. Prepare the test solution and reference solutions as prescribed in the individual monograph. Use the same solvent for all the solutions and apply the same volume of each and develop the plate. Prepare and apply not fewer than 3 reference solutions of the substance under examination, the concentrations of which span the expected value in the test solution (about 80 per cent, 100 per cent and 120 per cent). Treat with the prescribed reagent, if necessary, and record the reflectance, the transmittance or fluorescence in the chromatograms obtained with all the solutions. Use the measured results to calculate the amount of substance in the test solution.

The requirement for resolution and separation are prescribed in the individual monograph.

Procedure for Bacitracin, Neomycin and Polymyxin B

The following thin-layer chromatographic procedure is applicable as an aid in verifying the identities of bacitracin, neomycin, and polymyxin B active ingredients and in dosage

forms when present singly and in two- and three-component mixtures. Prepare atest solutionas follows, unless otherwise directed in the individual monograph.

Test Solution

For Drug Substances. Dissolve a portion of Bacitracin, Bacitracin Zinc, Neomycin Sulphate, or Polymyxin B Sulphate in *0.1 M hydrochloric acid* to obtain a solution containing about 500 Bacitracin Units per ml, 3.5 mg of neomycin (base) per ml, or 10,000 Polymyxin B Units per ml.

For Solutions. Where the Solution contains neomycin and polymyxin B, dilute a portion of it with *0.1 M hydrochloric acid* to obtain a solution containing the equivalent of about 3.5 mg of neomycin (base) per ml. Where the Solution contains polymyxin B but not neomycin, dilute a portion of it with *0.1 M hydrochloric acid* to obtain a solution containing about 10,000 Polymyxin B Units per ml.

For Creams, Lotions and Ointments. Where the Cream, Lotion, or Ointment contains Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 500 Bacitracin Units, to a 15-ml centrifuge tube. Where the Cream, Lotion, or Ointment contains neomycin, but not Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 3.5 mg of neomycin (base) per ml to a 15-ml centrifuge tube. Add 4 ml of chloroform to the centrifuge tube, and shake well to disperse the Cream, Lotion, or Ointment. Add 1 ml of *0.1 M hydrochloric acid*, vortex for 4 minutes, centrifuge, and use the clear supernatant.

Standard Bacitracin Solution. Dissolve a portion of *bacitracin zinc RS* in *0.1 M hydrochloric acid* to obtain a solution containing 500 Bacitracin Units per ml.

Standard Neomycin Solution. Dissolve a portion of *neomycin sulphate RS* in *0.1 M hydrochloric acid* to obtain a solution containing the equivalent of 3.5 mg of neomycin (base) per ml.

Standard Polymyxin B Solution. Dissolve a portion of Polymyxin B *Sulphate* RS in *0.1 M hydrochloric acid* to obtain a solution containing 10,000 Polymyxin B Units per ml. Where the article under test also contains Bacitracin or Bacitracin Zinc, dissolve a portion of Polymyxin B *Sulphate* RS in *0.1 M hydrochloric acid* to obtain a solution containing 500*J* Polymyxin B Units per ml, *J* being the ratio of the labeled amount of Polymyxin B Units to the labeled amount of Bacitracin Units in each g of Cream, Lotion, or Ointment.

Developing Solvent Solution. A mixture of 4 volumes of *methanol*, 2 volumes of *isopropyl alcohol*, 2 volumes of *methylene chloride*, 2 volumes of *ammonium hydroxide*, and 1.5 volumes of *water*.

Procedure. Apply 10 µl of the test solution and each of the relevant standard solutions to a suitable thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel. Place the plate in a presaturated chromatographic chamber,and develop the chromatogram with the Developing Solvent System until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry at 105°

for 10 minutes. Spray the plate with a 0.2 percent solution of *ninhydrin* in *butyl alcohol*, and heat at 105° for 5 minutes. The R_f value of each principal spot in the chromatogram of the test solution corresponds to that of the principal spot in the chromatogram obtained from each relevant standard solution as appropriate for the labeled active ingredient or ingredients specified on the label. If the chromatogram of the test solutionyields excessive streaking, proceed as directed for Modified Procedure.

Modified Procedure. Transfer the test solution to a 15-ml centrifuge tube, add 10 ml of *saturated aqueous picric acid solution (1.2 per cent, w/v)*, vortex for 1 minute, centrifuge for 10 minutes, and discard the supernatant. Wash the residue with 1-ml portions of *water* until no yellow color is observed in the washing. Discard the washings and dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 1 ml of *acetone*, add 1 ml of a freshly prepared solution of *sulphuric acid* in *acetone* (1 in 100), shake, centrifuge for 5 minutes, and discard the supernatant. Rinse the residue with 1 ml of *acetone*, centrifuge briefly, and discard the washing. Repeat the washing until no yellow color is observed. Dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 0.5 ml of *0.1 M hydrochloric acid*. Repeat the procedureusing this Modified Test Solutioninstead of the test solution. The R_F value of each principal spot in the chromatogram of the Modified Test Solution corresponds to that of the principal spot in the chromatogram obtained from each relevant Standard Solution as appropriate for the active ingredient or ingredients specified on the label.

Adoption of HPTLC for Herbs & Herbal Products

High-performance thin-layer chromatography (HPTLC) is the advanced and standardized version of TLC (see 2.4.17. Thin-Layer Chromatography). HPTLC is actively applied in qualitative and quantitative analysis of a wide range of articles, including herbs and herbal products. It is known for its high separation power, performance, reproducibility, simplicity and is considered modern replacement to classical TLC. Its main use is to identify herbal and their extracts by their broad “chemical composition fingerprint using HPTLC” from electronic images of chromatograms, detect adulterants and secondly, using scanning densitometryto quantify markers.

In HPTLC, the stationary phase consists of a uniform, typically 200-µm layer of porous (pore size 60 Å), irregular particles of silica gel with a size between 2 and 10 µm and an average particle size of 5 µm, plus a polymeric binder and a fluorescence indicator (F254) coated onto

a support, which is typically a glass plate or aluminum foil. Other stationary phases, such as chemically bonded phases (C8, C18, CN, NH2, DIOL) or microcrystalline cellulose, are also available with and without a fluorescence indicator. Because of the greater separation efficiency of the fine particles in HPTLC, the chromatographic system is miniaturized, using smaller developing chambers and shorter developing distances of 6–8 cm, in comparison to classical TLC, where 12–15 cm is required for best separation. Therefore, less mobile phase and less time are required for chromatogram development. Another effect of miniaturization is the use of smaller sample volumes and the resulting possibility of analyzing more samples per plate than in TLC. In addition, improved layer quality due to smaller particle size positively affects the signal-to-noise ratio, and therefore improves the detectability of separated sample components.

Similar to other planar chromatography techniques, in HPTLC, the plate is an open system affected by environmental factors, which must be controlled carefully. In HPTLC, variables are controlled within narrow ranges using a rigorously standardized methodology and appropriate equipment, thus HPTLC enables increased reproducibility of the chromatographic result from plate to plate.

For qualitative and semi quantitative analysis i.e. HPTLC fingerprinting for identification, a scanner is not essential. Scanner is required for quantification of markers. Each and every herbal sample is amenable to fingerprinting even if none of its constituents (markers) may be known or available.

HPTLC involves the following:

Handling of Plates

HPTLC plates are delicate and must be handled with care to avoid damaging the layer. When moving the plate, it should be touched only in the upper part above the region of chromatography. Labeling the plate should be with a soft pencil in the upper corner. The expected developing distance can be marked on the edge with a short pencil mark that goes down to the glass support. Marking the developing distance across the entire plate should be avoided. Aligning the front with a short mark on the edge is easier.

Plates should be stored in a place that is free of fumes and dust with the layer facing down. Shrink wrap of the package should not be in contact with the layer to avoid contamination

with volatiles from the foil. Generally, HPTLC plates are ready for use without any pretreatment. Older or improperly stored plates may have accumulated impurities and therefore require precleaning. This is the case when after development, the solvent front (or additional secondary fronts) can be seen under UV 254 nm as an intense, broad dark band across the plate. Precleaning can be achieved by developing the plate in methanol to the upper edge. Subsequently, the plate is dried in a clean oven at 120° for 20 min. For cooling down to room temperature and for storage prior to further use, plates can be kept in an empty desiccator or wrapped in aluminum foil. Precleaning may slightly change the selectivity of a plate. Therefore, it is important to define in a method whether precleaned plates are to be used. Precleaning with the mobile phase is usually not a good option because it may be difficult to completely remove all components during the drying step.

Test solution

Sufficient amount of the sample is dissolved in a suitable solvent at typical concentrations of 0.1 – 1 mg/ml for individual substances. For herbs and herbal products, concentrations are 100 mg/ml in methanol or 70% ethanol.

This is the sonicated for 15 min, centrifuges for 10 minutes and supernatant is used as test solution.

Sample under examination may alsoalso be prepared by processes such as infusion, decoction, maceration, sonication, soxhelation, centrifugation, percolation and other concentration techniques.

Reference solutions

A) Sufficient amount of the standard is dissolved, preferably, in the same solvent used for the Test solution and at a comparable concentration. For herbs and herbal products, selection of a suitable marker compound for a particular herb or herbal preparation for quantification is also of critical importance. The reference material used in the Standard solution should be an authenticated Botanical Reference Substance (BRS) and Phytochemical Reference Substance (PRS). Unless otherwise stated in an individual monograph, PRS is dissolved at a concentration of 1 mg/mL in methanol.

B) **For essential oils** fingerprint analysis, the PRS and samples are dissolved in toluene at a concentration of 50 µL/mL.

C) **For fixed oils**, 25 µL of fatty oil PRS as well as samples are dissolved in 3 mL of dichloromethane (methylene chloride) fingerprint analysis for identification.

Preparation of standard and sample solution: Dissolve about 20 mg of RS and samples in 3 mL methylene chloride.

Stationary phase

Selection of the stationary phase depends upon the nature of the compounds to be separated, however, 200 µm layers of irregular particles of HPTLC Si 60 F254 (silica gel with average particle size of 5 µm and pore size 60 Å and a fluorescence indicator F 254) is suitable for the majority of herbs and herbal products.

Mobile phase

The mobile phase should be freshly prepared. Selection of the mobile phase depends upon the nature of the compounds to be separated. The mobile phase may be a mixture of two or more solvents. A drop of acid or alkali may sharpen the bands. For identification of herbs and herbal products, a suitable mobile phase is one that creates a sequence of separated bands (fingerprint) well spread over the entire R_f range. For quantitative determination, target compounds should be betweenR_f 0.25 to 0.65 and well resolved from all other constituents of the samples. Migration of compounds generally correlates with their polarity. In normal phase separations, non-polar compounds migrate further and polar compounds migrate less far and *vice versa* in reverse phase chromatography.

Plate Layout and Sample Application

The standard format of the HPTLC plate is 20 × 10 cm (width × height). Other sizes (e.g., 10 × 10 cm) could be used as well, but one should keep in mind that for obtaining reproducible results, each size of plate requires a different chamber to maintain the same geometrical aspects as, for example, a twin-trough chamber would have for the standard plate format. The optimum developing distance in HPTLC is 62mm. Cutting plates to less than 10 cm in height provides no additional advantages.

Exact positioning is essential for proper identification of separated zones. Figure 1 depicts the application of samples with respect to plate layout.

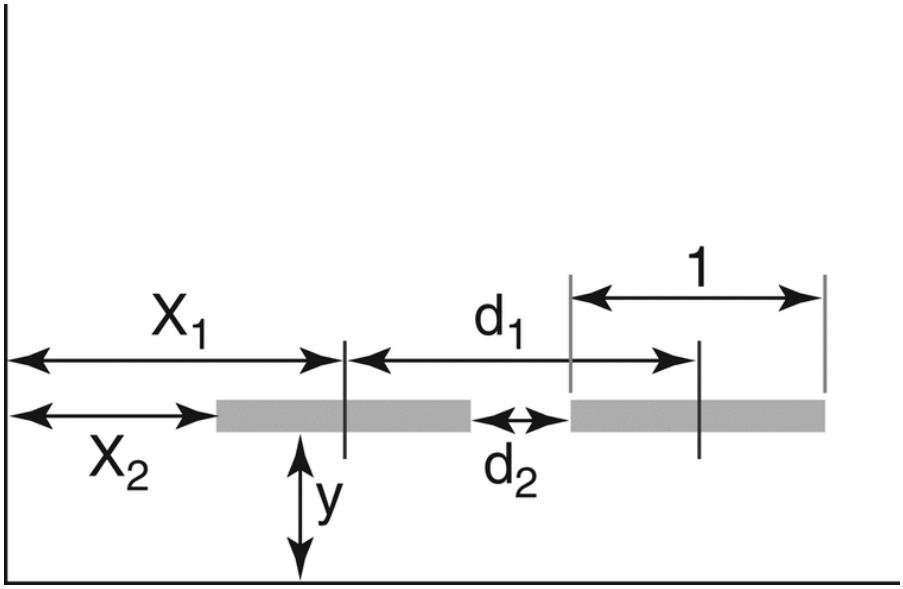

Figure 1. Plate layout

For proper identification (migration distance/R_f values), all samples must be applied on a (virtual) line parallel to the lower edge of the plate. The distance (y) must be large enough to avoid the sample being immersed in the mobile phase. During development, the velocity of the mobile phase decreases with increasing developing distance. Consequently, the result depends on the application position relative to the level of the developing solvent. For reproducible results, the distance from the lower edge (y) must be kept constant, and the amount of developing solvent placed into the chamber must also be fixed. To minimize solvent consumption, twin-trough chambers are used. The level of developing solvent in such chambers is typically 5 mm. A meniscus is formed on the plate at the immersion line. A sample applied at y = 8 mm will be well above this meniscus. The distance of the application position from the left and right edges of the plate (x) must be enough to avoid the so-called "edge effect" .

The position x1 is typically called "application position". To avoid the edge effect, the length of the applied band (l) has to be taken into account when defining x2 as the distance from the edge of the plate. A minimum of 15 mm is necessary for x2. The distance between two samples (d1) also considers the band length (l). For d2, a minimum of 2-3 mm is recommended so that samples do not interfere with each other if larger volumes are applied. The band length (l) must be fixed for each method because it affects the concentration of samples per band if a defined volume of sample solution is applied. The applied quantity (across a defined band length) influences the intensity of the separated zones and may affect

whether a particular zone of a fingerprint is visible. An argument for selecting shorter bands would be that more samples can be applied on the plate. However, because chromatogram evaluation is primarily performed visually, it must be noted that a band seems to look "sharper" (more like a band and less oblong) when it is >5 mm. A band length of 6-8 mm is a good compromise.

Sample application

Test and reference solutions can be applied by spraying the solutions without touching the plate (spray-on technique). During spray-on application, the sample's solvent is nebulized and evaporated, creating sharp zones regardless of the polarity of the solvent or the volume. Use of inert gas like nitrogen is recommended for nebulization since it also prevents the oxidation of the samples but air can also be used. Narrow homogeneous sample bands are applied using an automated applicator/sampler. This ensures high resolution.

Chromatogram development for

a) Herbals and Essential Oils:

The processes in a chromatographic chamber are highly complex and difficult to describe. Unlike column chromatography, which is assumed to take place in equilibrated chromatographic systems, planar chromatography always begins in a state of nonequilibrium and never reaches equilibrium. The sample has been applied onto the plate, which becomes the stationary phase only when it comes in contact with the mobile phase in the chromatographic chamber. The advancing mobile phase must be able to dissolve the sample for chromatography to begin. Driven by capillary action, the mobile phase velocity decreases with increasing migration distance because the resistance of the stationary phase against the flow is also increasing. The optimum migration distance for mobile phases on HPTLC plates is about 6 cm. Depending on the viscosity of the mobile phase, development takes place between 10 and 20 min. To further move the solvent front just 10 mm will extend the developing time by 5–15 min, while moving an additional 20 mm will increase the developing time by 15–40 min. The contribution of the extra developing distance to improved separation is usually not justifiable, because the diffusion that occurs at decreased velocity of the mobile phase often offsets any gain in distance of separated zones.

Chamber saturation is established before placement of the plate into the chamber. The mobile phase is placed in both troughs of the chamber, and the filter paper in the rear trough is wetted thoroughly. Chamber saturation occurs once the liquid in the tank is in equilibrium

with its own vapor, and this is a function of time. After 20 min, the chamber will generally achieve a reproducible degree of saturation. If the filter paper for saturation is to be re-used, it must be dried completely first. Different chambers may give different results, depending on the chamber's geometry, the presence or absence of liquid and filter paper in one of the troughs, and the waiting time before introducing the plate and so may be avoided.

The plate should be placed into the chamber so that it rests in a vertical position against the front wall while the layer faces the rear wall. The dry portion of the layer absorbs solvent vapor from the mobile phase in gas phase to achieve adsorptive saturation, which occurs when the surface is covered by a layer of solvent molecules. Generally, this process can lower R_f values and affect the selectivity of the separation. Depending on the degree of saturation, a part of the mobile phase may evaporate off the plate. Multicomponent mobile phases may be separated by the stationary phase because of different interactions of the constituents. This process is affected by the degree of preconditioning.

In an unsaturated chamber, the situation is different. The rear trough does not contain liquid or filter paper. The plate is introduced immediately after the solvent, which does not allow enough time to achieve gas–liquid saturation equilibrium. Preconditioning has little effect, while evaporation and possibly formation of secondary fronts dominate the chromatographic process. Both processes typically cause alterations in the chromatograms, thereby affecting the separation. Hence a 20 min saturation is desirable.

HPTLC standard conditions include the use of a 20x10 cm Twin trough chamber, saturated for 20 min with filter paper in the rear trough and solvent level of 5 mm in each trough. The standard developing distance is 70 mm from the lower edge of the plate.

b) HPTLC of Fixed Oils:

1st mobile phase – Ethyl ether

2nd mobile phase : Methylene chloride, glacial acetic acid and acetone 20:40:50

Stationary phase ; 20 x 10 cm, Silica gel C-18 precoated plates, 0.2 mm layer, average 4-8 um particle size .

Band Application volume: 1 ul

Development:

In the first mobile phase, develop plate upto 13 mm ie 5 mm above the point of application of 8 mm. Cool air dry for 2 min with hair dryer. Repeat the procedure a second time.

In the second mobile phase, develop the plate upto 80 mm and dry the plate for 5 min in cool air with hair drier. Repeat the procedure a second time. Heat the plate on a pre heated plate heater at 120 degrees centigrade for 1 min and examine in daylight.

Derivatization:

Spray reagent: 100 mg/mL of phosphomolybdic acid in 96% ethanol. Spray the plate.

Acceptance criteria: The Rf values of the principal spots of reference and sample and identical in Rf and intensity.

Drying

Drying the developed plate is best achieved while it is in the vertical position by exposure to a flow of cold air. Elevated temperature during drying could cause volatile sample components to diffuse or evaporate from the plate. During the evaporation of the mobile phase, sample components move from deeper areas of the layer to the surface. Uneven drying across the plate may result in different intensities for zones with equal amounts of substances.

Effects of Humidity

Silica gel has an extremely high affinity for water and is used as an effective drying agent. An HPTLC plate is always at equilibrium with the water vapor in the laboratory atmosphere (relative humidity, RH). Chromatographic results are affected by variation in the amount of water adsorbed onto the silica gel surface. The higher the RH, the more water is adsorbed. This reduces the "activity" of the stationary phase and causes higher R_f values. Selectivity of the chromatographic system changes as the RH changes. It is nearly impossible to predict what effect a change in RH may have for a given separation. Some separations are more affected than others, and sometimes only parts of the chromatogram will change. In addition, there is no ideal activity of the plate that can solve all separation problems, and it is difficult to use defined activity changes of the stationary phase to influence separation in a desired way. The best option is to keep the RH constant at a moderate level (e.g., 33%) and then, try to adjust the selectivity of the mobile phase. One practical exemption is effective "drying off"

of the plate using a molecular sieve. Activating a plate by heat (120° for 20 min) is possible, because under these conditions water adsorbed onto the silica gel is effectively removed, but when the plate is cooled down and stored, or during sample application it re-equilibrates to ambient relative humidity. To achieve reproducible results independent of seasonal or regional changes in the RH in various laboratories, it is important to expose the plate to a defined RH just before development and with the samples already applied. This will keep the activity and the selectivity of the stationary phase constant. Defined RH can be created in closed containers such as a desiccator or specialized equipment and over saturated salt solutions. Between 20° and 25°, saturated solutions of magnesium chloride establish 33% RH; of potassium thiocyanate, 47% RH; and of sodium chloride, 75% RH.

Derivatization

Derivatization can be defined as a technique that primarily modifies analyte functionality in order to enable easy detection. Derivatization can be performed either by immersing the plates or by spraying the plates with suitable reagents carefully. For reproducible results, the amount of reagent of a given concentration applied needs to be defined either by volume (spraying) or by volume, and dwell time (immersion). Most derivatization reactions require a heating step performed either on a plate heater or in a drying oven. The plate should be dried in air before heating to avoid any disruption of the layer resulting from evaporation of residual solvent. Before the plate is heated, the heating device should reach the required temperature. For reproducible results, it is important that the entire derivatization process is strictly timed. This includes any waiting time (e.g., for cooling) before the detection step. Detection is then performed under white light or under UV at 366 nm.

NOTE—Precaution is exercised not to char the plate while kept in oven after derivatization.

Detectionby imaging of chromatogram

Generally, visualization of plates is either performed under white light, typically by using a light source above the plate (reflectance) in combination with light from below the plate (transmission), or under UV at 254 nm and 366 nm. Under UV 254 nm, the F254 indicator included in the stationary phase emits a green light (blue for F254s). Any substance that absorbs UV 254 nm will be visible as a dark “quenching” zone where the degree of quenching corresponds to the amount of that substance (and its extinction coefficient). Some substances can be excited to fluoresce by long wave UV light (UV 366 nm), particularly after

chemical derivatization. Such substances are seen as zones of specific colors on the dark background.Automated computer assisted HPTLC visualizers are available for photo documentation, reproducible visualization, and evaluation of HPTLC chromatograms. In addition to visualisation under white and UV light, derivatization by specific chemical reagents, if required, is specified in the individual monographs. Selection of such derivatization reagents depends upon the nature of compounds present in the sample.

HPTLC records electronic images of chromatograms that allow convenient visual comparison of results obtained for multiple samples against images of chromatograms generated with reference materials. The HPTLC fingerprint from the same chromatogram can be evaluated in multiple detection modes, e.g., white, UV 254 nm and UV 366 nm light without derivatization, and white and UV 366 nm light after derivatization, thus enables the production of significant amount of information about the sample without the need to repeat the chromatography. Digital images can be taken of the plate in different detection/illumination modes. Parameters to acquire such images should always be the same for a given analysis regardless of the operator and location. This ensures reproducibility and elimination of variations due to human factors. The use of image editing software to change certain aspects is not permissible.

Evaluation of fingerprint image chromatograms:

In the evaluation step, the analytical data obtained from samples are compared against specifications in the monograph. HPTLC for identification of herbs and herbal products typically produces fingerprints, i.e., sequences of zones that have specific positions, colors, and intensity. These fingerprints are compared against fingerprints obtained for the authenticated Botanical Reference Substance (BRS) and Phytochemical Reference Substance (PRS). Ideally, comparison is based on images rather than descriptions. Reference and sample images are compared on one computer display. Because of the natural variability of herbal materials, there will always be a range associated with the acceptance criteria for a fingerprint to pass the identity test. Fingerprints of related herbal species or adulterants should have distinguishing features not present in samples that pass the evaluation.

Quantification

The quantitative evaluation is performed by measuring the predefined bands obtained in the sample under examination and reference standards (PRS) using a scanner. External standard

methods are mostly used but internal standard methods can be used. Limit tests require a single concentration standard level. The scanner needs to be set to the desired wavelength(s), either in absorption mode or in fluorescence mode (with a suitable cut-off filter in place to remove light required for excitation). Scanning at desired wavelength(s) reflects the bands in the form of peaks on area/ height or on both (area and height) basis and is representative of the concentration of substances in the bands and their extinction coefficients. The software stores this data and draws the calibration curve.The beam for scanning samples is 65 to 80 % of the band length.

System Suitability Test

A system suitability test is required to qualify each developed plate before data obtained is considered reliable. On each plate, the analyst applies a track with 2 - 4 reference substances in defined concentrations covering the R_f range of 0.2 -0.8. Two of these have similar but just separable R_f values under the method's chromatographic conditions. The substances are designated to check the system suitability for position, resolution, and colors of the bands after the plate is developed. Only when the system suitability requirements have been satisfied can the results obtained with the samples on the same plate be evaluated further to determine compliance. The system suitability test is of great importance especially for the identification of herbs, which have an intrinsic natural variability in chemical composition.

Documentation

Documentation of HPTLC is to be done with the help of a suitable automated system coupled to appropriate software. While doing so, samples and reference materials need to be suitably identified (numbered) and a suitable labelling system needs to be developed and adopted for proper storage/archiving of the documents/images/profiles/densitometric data for easy retrieval in a controlled quality assurance system and for auditing.

Apparatus

The equipment used for HPTLC technique consists of the following:

- Plates: Unless otherwise specified in the individual monograph, use plates coated with a uniform 200-µm layer of porous (60-Å pore size) silica gel having irregular particles of 2–10 µm and an average particle size of 5 µm, a polymeric binder, and a fluorescence indicator (F254) of 20 × 10 cm. [NOTE—Chromatographic methods

using high-performance thin-layer chromatographic glass plates are preferred over aluminum-backed sheets because of greater mechanical stability.]

- A device suitable for the application of specified volumes of samples as bands with specified length at the specified positions
- A suitable chromatographic chamber (for example, a twin trough chamber) allowing for control of saturation and developing distance
- A device suitable for controlling the activity of the stationary phase via relative humidity
- A device suitable for reproducible drying of the developed plate
- A device suitable for treatment of the plate with derivatization reagent, if required
- A device suitable for heating as part of the derivatization procedure, if required
- A system suitable for documentation of chromatograms under UV 254 nm, UV 366 nm, and white light
- A suitable HPTLC software to integrate the instruments into one HPTLC system, store and process the analytical information
- A scanner suitable for scanning plates for quantitative analysis

Each of these devices as well as the system as a whole should pass installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) in order to assure that the instruments are working according to their specifications.

HPTLC Standard Conditions (applicable unless specified otherwise in the individual monograph):

Plate: 20x10 cm or 10 x 10 cm glass or aluminum foil plate with HPTLC Silica gel 60 F 254 and polymeric binder

Plate layout: Bands of 6-8 mm length, 8 mm from lower edge of plate, minimum 2 mm apart, center of first band about 20 mm from the left edge of plate and similarly on right edge.

Plate conditioning: at 33% RH immediately before development

Chamber: 20x10 cm twin trough chamber, solvent level of 5 mm in both troughs, 20 min saturation with filter paper in the rear trough, developing distance 70 mm from lower edge of plate

Drying: 5 min in a stream of cold air, immediately after removal from chamber. Plate held vertically.

Image Documentation: under UV 254 nm, UV 365 nm, and white light prior to derivatization, under UV366 and white light after derivatization.

Densitometric scanning for quantification: A scanner for plates, with UV and Visible range, suitable for fluorescence measurements, monochromator, variable scanning slit sizes, different calibration modes for quantitative analysis.