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REVIEW ON ANALYTICAL METHOD DEVELOPMENT, VALIDATION AND TROUBLESHOOTING OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

High performance thin layer chromatography (HPTLC) is a sophisticated instrumental technique based on the full capabilities of thin layer chromatography. This paper provides information regarding HPTLC-based analytical method development and evaluation of validation characteristics in accordance with related issues such as development of thin-layer chromatography, basic principle, protocol, resolution, validation process, recent developments, separation of elements and modifications on TLC leading to the HPTLC, optimization, process control, automation, and hyphenation. It says that HPTLC has stronger potentials over another method. As a result it meets standards comparable with alternative chromatographic techniques with specific aim to avoid confusion and methodological failure. The poor performance to the method development may be caused by systematic and scientific approach for the selection of separation mode, stationary phase and mobile phase not taken into the thought. The poor method validation may be caused by incomplete validation guidelines which are not fully understood[1].

KEYWORDS

HPTLC, Planer Chromatography, Method Development, Method Validation and Troubleshooting.

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INTRODUCTON

High Performance Thin Layer Chromatography (HPTLC) is a powerful advanced form of Thin Layer Chromatography (TLC) and consists of chromatographic layers of utmost separation efficiency and the application of sophisticated instrumentation for all steps in the procedure include accurate sample application, standardized reproducible chromatogram development and software controlled evaluation[1]. HPTLC is a

technique which encorporate a widely standardized methodology which is based on scientific facts as well as the use of validated methods for qualitative and quantitative analysis. HPTLC meets all the quality requirements for today's analytical labs, to increases the resolution and to which allow more accurate quantitative measurements. Applications of HPTLC, such as identification of the chemical constituents, impurities, active substances, process development and optimization, process monitoring, and cleaning validation have been demonstrated. HPTLC is a superior to other analytical methods in terms of the total cost and time required for analysis. It is an offline process in which the various stages are carried out independently. Important features of HPTLC include the ability to analyze crude samples containing multi-components, application of choice of mobile phase solvents. The newer instrumentation like scanners, densitometers, and new action saturation chambers, furthermore as gradient extraction techniques, high-resolution sorbents with selective particle size or chemically changed surface, the possibility of mixing with another instrumental methods, and also development of computer programs for method optimisation, all build HPTLC a vital analytical technique[2]. Various stages of HPTLC method development are fully automated by use of available commercial instruments, and the entire process can be controlled using software compliant with requirements of drug regulatory agencies[3].

BASIC DRUG SELECITION CRITERIA FOR NEW ANALYTICAL METHOD DEVELOPMENT

1. Analytical method for the quantisation of the drug in body fluids may not be available.
2. Analytical methods for a drug in combination with another drugs may not be available.
3. The existing analytical method may require expensive reagents and solvents. It may also involve extraction and separation procedures and these may not be reliable[4-6].

ANALYTICAL METHOD DEVELOPMENT

Analytical method development lastly results in official test methods. Consequently quality control the laboratories used these methods to check the efficacy, identity, purity, safety as well as performance of products of the drug. Regulatory authorities give utmost importance on the analytical methods in manufacturing. Shown in Fig No. 1.

Development in scientific and analytical method have been resulted from the advancements of analytical instruments. The enhancements of the analytical method development and analytical instruments have reduced the time and costing of analysis and determine precision and accuracy. Techniques pertaining to analysis are developed and validated for active pharmaceutical ingredients, excipients, related substances, drug products, degradation products and, residual solvents, etc. Resulting that become an important part of the required necessities for restrictive organization.

The number of novel drugs are being introduced and are constantly growing day by day. Therefore it is fully imperative to evolve novel ways and introduced them for dominant their quality. Modern pharmaceutical analysis needs the following requirements.

1. The analysis ought to take a minimum time and should be economical.
2. The accuracy of the analysis should accept the Pharmacopoeial guidelines.
3. The chosen method should be precise and selective[6].

STEPS FOR METHOD DEVELOPMENT[7]

Various steps are involved in the development of an analytical method are as follows:

- Sample Preparation
- Selection of Chromatographic layers
- Plates
- Pre washing
- Conditioning
- Sample Application
- Pre conditioning
- Mobile Phase
- Chromatographic Development

- Detection of spot
- Scanning and Documentation

Sample Preparation

It needs a high concentrated solution, as very less amount of sample require to be applied. For normal phase chromatography using silica gel pre-coated Plates solvents should be non polar of volatile type. For reversed phase chromatography usually polar solvents are used for mixing the sample.

Selection of Chromatographic Layers

Layer of HPTLC plate are available in the form of pre coats silica gel of very finer particle size is widely used as adsorbent.

Plates

The plates are similar to conventional alluminium TLC plates. Here silica gel is widely used as adsorbent. The use of particle size helps in the greater resolution and sensitivity.

Plates are to be produced from 4 to 5 mm silica gel with an inert binder to form a 200mm layer. Plates of 20x20cms are 5x7.5cms is used. Silica gel F254 having a pore size of 6 mm which is a coat material. The difference between TLC and HPTLC plates is particle size of coated material, which is 5 to 20 mm of TLC and 4 to 8 mm for HPTLC

Pre Washing

Plates are need to be washed to remove water vapors or volatile impurities. Plates are handled at the upmost edge to avoid contamination. The methanol is used as a prewashing solvent, a mixture of methanol and ethyl acetate or even mobile phase is used, per trough in a 20 × 10 cm twintrough chamber (TTC). The two 20 × 10 cm or four 10 × 10 cm plates can be developed back-to-back in each trough of the TTC. Remove the plate and dry it for 20 min in a clean drying oven at 120°C[3].

Conditioning

The pre washed plates are placed in oven at 120°c for 15 to 20mins. This process is known as conditioning.

SAMPLE APPLICATION

The sample spot applied must not be exceed 1mm in diameter. There are various techniques for the spotting of the sample; one of them is self-loading Capillary in which lesser volume of samples may be applied to the plate. Surface using pt- iridium tubing fused into the end of a length of glass tubing.

LINOMAT 5: SAMPLE APPLICATOR

Quantitative Analysis

This analysis most oftenly used. Usual application volume is 6 – 20ul. Usual band size is 6 mm. For aq. samples 8 mm. Distance from side, 12 – 15 mm, distance from bottom 8 mm.

In-situ clean - up

Used to separate components from samples. Samples applied 110 mm from bottom, developed in ether, plate dried, cut 120 mm from bottom, turned 180˚ and developed with appropriate mobile phase.

Micro - prep isolation

For isolation of components on a mg scale for identification by spectroscopy. Usual band length - 190mm. The volume applied 2-10ul. Several similar plates are need to be chromatography.

Superimpose

Required for overlapping of internal std. or spiking or derivatizing reagent or improving quantification; Single method for application and superimpose as per GLP[8].

PRE CONDITIONING (Chambers Saturation)

The methanol is used as a prewashing solvent, a mixture of methanol and ethyl acetate or even mobile phase is used, per trough in a 20 × 10 cm twintrough chamber (TTC). The two 20 × 10 cm or four 10 × 10 cm plates can be developed back-to-back in each trough of the TTC. Remove the plate and dry it for 20 min in a clean drying oven at 120°C. Equilibrate plate with laboratory atmosphere (temperature, relative humidity) in a suitable container providing protection from dust and fumes.

Mobile Phase

The solution of correct mobile phase is by trial and error basis in which chemical properties of solute and solvent solubility of analyte absorbent layer are to be considered.

Chromatographic Development

- In this technique in addition of stationary and mobile phases, a gas phase is present in

chamber. This gas phase is significantly influence the result of the separation.

- The linear method development is most familiar technique in HPTLC here the plate is placed vertically in solvent system in a suitable chamber. The solvent is usually run by capillary action and chromatogram can be developed from the both sides.
- Circular development, anti-circular device and multiple development are of some others methods which are used for chromatographic development.

AUTOMATIC DEVELOPING CHAMBER

In ADC2 chromatogram development is fully automatic and qualitative, independent of environmental effects. The activity and pre-conditioning of the layer, chamber saturation, developing distance and final drying can be pre-set and automatically monitored by the ADC2 Two modes of operation are possible: stand-alone with input of parameters via keypad or remote operation from win CATS with process monitoring, documentation of operating parameters and reporting[2].

DETECTION OF SPOTS

Immediately after the development is completed, the plated are removed from the chamber and dried with the help of dryer to remove the frees of mobile phase. Generally the detection can be known by iodine vapor in an iodine chamber.

SCANNING AND DOCUMENTATION

- The HPTLC equipments are attached with computer and data recording and storing devices. The development of HPTLC plates scanned at selected UV regions wavelength by the instruments and the detected spots are seen on computers in the form of peaks.
- The scanner which shows bond into peak and peak heights or area is related to concentration of the substance on the spot. The peak heights and the area under the spot are measured by the instrument and are recorded as percent on the printer and shows the proper result[2].

METHOD VALIDATION

According to ICH guidelines method validation define as, "Validation of an analytical procedure is the process by which it is established by laboratory studies, that the performance characteristics of the method gives the requirement for the intended analytical application."[9]

Parameters of Method Validation[9]

- Accuracy
- Precision
- Intraday Precision
- Interday precision
- Repeatability
- Specificity
- Limit of detection
- Limit of Quantitation
- Linearity
- Range
- Ruggedness
- Robustness

Principle of Method Validation

- Concentration range over which analyte can be estimated should be defined in the method. The relationship between response and concentration should be demonstrated to be continuous and linear with at least 5–6 points; extrapolation of the plot on either end is not desirable.
- A standard curve should be maintained.
- Accuracy and precision of the method over the range of the standard curve should be clearly established.
- Stability of the analyte during purification/extraction procedure needs to be established.
- Lowest concentration of analyte in the matrix for which a reliable and reproducible estimation can be obtained should be established (LOQ).
- Accuracy and precision of the method for estimation of sample of the known concentration should be established.

- A minimum of three concentrations, one near but more than LOQ, one near center, and one near upper portion of the curve, should be taken for validation.
- Recovery of the analyte should be evaluated and documented and should be constant.
- Available, they should be chromatographed[10].

ADVANTAGES OF ANALYTICAL METHOD VALIDATION

The advantages of the analytical method validation are given as below:

- The biggest advantage of the method validation is that it builds a degree of confidence, not only for the developer but also to the user.
- Although the method validation exercise may seems costly and time consuming, it results inexpensive, eliminates repetitions and leads to better time management in the end.
- Some changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to the obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process[4].

VALIDATION OF DEVELOPED METHOD

Validation should not seen differently from the development of a method. The entire process can visualized with the scheme in following Figure No.2. It starts from a clearly defined analytical goal, method choice, optimization, and development, which is called prevalidation method considerations before arriving at the elaboration of a validation protocol and is the starting point of the actual method validation[10].

It is possible that the validation method in different situations may require some changes in the standard validation protocol. Such changes may include restrictions with respect to relative humidity, waiting times, precision, etc. The validation protocol is a key instrument for structuring, regulating and documenting the validation processes, depending on the quality management system. The following elements must be included:

Selectivity

Ability of the developed analytical method is to detect analyte quantitatively in the presence of other components which are expected to be present in the sample matrix. Results are expressed as Resolution. If the expected impurities or related substances are available, they should be chromatographed along with the analyte to check the system suitability, retention factor, tailing factor, and resolution.

Linearity

Ability of the method within a given range to obtain test results in direct proportion to the concentration of analyte in the sample – calibration curve for the analyte.

Precision

Precision provides an indication of random error. Its results should be expressed as relative standard deviation (RSD) or coefficient of variation (COV). Precession is observed in terms of replication: precision under same conditions, same analyst, same apparatus, short interval of time and identical reagents using the same sample; measurement of peak area: RSD should not be greater than 1%, based on six times measurement of same spot; peak position: RSD should not be greater than 2% based on six times repositioning the instrument after each measurement.

Specificity

The specificity of the method was determined by analyzing standard analyte and test samples. The spot for analyte in the samples has to be confirmed by comparing the Rf and spectrum of the spot with that of a standard. The peak purity of analyte has to be assessed by comparing their respective spectra at the peak start (S), apex (M), and peak end (E) positions of that between the standard and sample spectra of analyte[10].

Accuracy

Accuracy of an analysis is determined by systematic error involved. It is defined as closeness of agreement between the actual value and mean analytical value obtained by applying the test method a number of times. The accuracy is

acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. This parameter is very important for formulated pharmaceutical dosage forms as it provides information about the recovery of the analyte from sample preparation and effect of matrix. It the recovery rate is found to be 100%, it implies that the proposed analytical method is free from constant and proportional systematic error. A blank matrix and known impurities must be available to test the accuracy of the method[4].

Ruggedness

This is one of the most important parameters for validation of HPTLC method. Experiments are usually recommended to evaluate ruggedness of a HPTLC method like sample preparation: composition, quantity of solvent, pH, shaking time, temperature and number of extractions; sample application: volume applied, spot shape and size, band and spot stability; separation: at least on three different plates; chromatographic conditions: chamber saturation, eluent composition, eluent volume, temperature, humidity and development distance; spot visualization: post chromatographic derivatization, spraying, dipping, reaction temperature and time; quantitative evaluation: drying of plates, detection and wavelength. Once the analytical method is developed, it should be performed independently by three analysts well conversant with practical aspects of the technique, analyzing the same sample under same experimental conditions to check reproducibility of the method.

Robustness

The robustness of associate in analytical procedure is outlined as a live of its capacity to remain unaffected by small but deliberate change in experimental parameters, providing an indication of the analytical method suitability and a reliability during the normal use. Some important parameters for testing the robustness of HPTLC methods include: The stability of an analyte in the solution which has analyzed and on the plate before and after development, the influence of temperature and humidity, The method of application, scanning, and evaluation, the spot shape and size, eluent composition, and pH, the batch/supplier of the HPTLC plate, the sample volume, the geometry of the chamber, and the drying conditions of the plate.

LIMIT OF DETECTION

Lowest amount of analyte that can be detected is not greater than 10% of the individual impurity limit. If this is not possible, then amount of analyte is applied has to be increased. Limit of detection (LOD) is determined on the basis of S/N ratio. Mean of noise peak areas and their absolute SD values are checked. LOD is the amount of applied sample producing a peak area which is equal to the sum of mean blank area and three times standard deviation. Stability Analyte should not decompose during development of the chromatogram and should be stable in solution and on the sorbent for at least 30 and 15 min, respectively. The intensity of the spot on the chromatogram should be constant for at least 60 min while optimization of the extraction/purification procedure and one must keep in mind the chemical properties and purity of the extraction solvent. Chemical reaction of the solvents and their impurities could turn out further spot/peak, thus leading to false assay values. Other vital factor is pH of the liquid mobile phase used for extraction/purification which can result in hydrolysis, oxidation and isomerization. The complete removal of organic solvent should be avoided[10].

LIMIT OF QUANTITATION

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantitated. It is a limit that took a look that specifies whether or not associate analyte is an top of or below a certain price. The limit of quantitation (LOQ) is outlined because the lowest concentration of associate analyte in a sample that may be quantitated with acceptable precision and accuracy beneath the expressed operational conditions of the method. For determination of LOD and LOQ, sample solutions ($n \geq 3$) are applied in decreasing quantities, in triplicate. The same volume of the pure solvent as a

blank is additionally applied. After development, a calibration graph is constructed by plotting the peak heights or areas against the applied quantities of the substances investigated. LOQ are calculated based on the signal-to-noise ratio. Results of LOQ should be reported as mass applied to plate instead of concentration[3].

TROUBLESHOOTING OF HPTLC

Troubleshooting is kind of problem solving of instrument which would applied to correct failed products or the processes. It is a logical and systematic search for the source of a problem so that it can be solved, so the product or process can be made useful or operational again. Troubleshooting is required to develop and maintain complicated systems wherever the symptoms of a problem can have several attainable causes. Troubleshooting is used in several fields like engineering, system administration, electronics, automotive repair, instrument repair and diagnostic medicine. Troubleshooting needs identification of the malfunction(s) or symptoms among a system.

TROUBLESHOOTING STRATEGY AND PROCESSES

Any troubleshooting strategy involves five steps;

1. Identification of the problem
2. Awareness of the cause(s) of the problem
3. Isolation of the precise reason for the matter
4. Rectifying the problem if able
5. Returing the unit to routine use or referring the matter to your maintenance manager.

TROUBLE SHOOTING PROBLEMS

It is important to remember that once the problem is defined and possible corrective action is identified, only one change at a time should be made; after each change, the whole system should be checked again to determine whether the problem still exists or whether the change corrected the problem[11]. Fix the HPTLC problems before they show trouble. Ideally an all problems will be prevented by the good maintenance plan. One way to reduce problems is to anticipate them so they can be fixed at your expectations. Some problems, however, cannot be reduced, but good work practices will minimize the result of such problems. HPTLC is a Planar chromatography it is an ideal tool for analysis of medicinal plant and offers several advantages. In its traditional form, thin layer chromatography has a long record in almost all pharmacopoeias for its use in identification of botanical raw materials. However, HPTLC is not limited to identification. It can also be used for control of batch to batch consistency in stability testing of medicinal plants and for purposes of control overall the entire manufacturing process[12,13]. It helps to ascertain quality control of batch to batch consistency. This can be done by well photo documentation under light at 254 nm, 365 nm, and visible mode. Compare that scanned 3D chromatogram for further study (15). Short remedies for trouble shooting in HPTLC are discussed as follows:

GENERAL PROBLEM DIAGNOSTIC & TROUBLESHOOTIN GUIDE

Topic: Mobile phase problems

Possible Cause

Lower the sensitivity and drift, noise or spikes in the Chromatogram.

Solution

Contaminants in the eluent are especially troublesome in gradient elution. So therefore use of distilled water.

Topic: Baseline Symptoms (High baseline drift)

Possible Cause

Detector lamp/optics temperature not stable

Solution

Allow the detector to warm up. Depending on the optical design, this may take 30 minutes to a few hours. See the detector‘s operating in details.

Possible Cause

Mobile phase not homogeneous.

Solution

Gently swirl the eluent to mix eluents already in their reservoir[11].

Topic: Sample application symptoms

Possible Cause

Poor Band quality [Linomat 5 applicator].

Solution

To check the Nitrogen gas flow, check distance between needle tip/ TLC layer and Damaged or clogged needle, Check gas pressure and adjust to 2-5 bars, Remove syringe and fill it with solvent. Force out by hand

Topic: peaks symptoms [Ghost peak]

Possible Cause

Only some peaks broad: Late eluting peak from previous injection

Solution

Extend run time. Increase elution strength of gradient (higher organic content). At the end of the run, flush syringe with strong eluent.

Possible Cause

Contamination of eluents

Solution

Water quality: Run different amount of water over the sampling and measure enrichment time as a function of the volume. Replace water with distilled water. Contaminations may also occur due to improper liquid handling (e.g., amino acid mobile phase prepared without gloves).

Possible Cause

Interferences in sample

Solution

Use sample clean-up techniques.

Possible Cause

Microbial growth in a mobile-phase.

Solution

Use fresh mobile phase with microbicide. Cell may require electrochemical treatment and/or cleaning with freshly prepared mobile phase. Older cells may require replacement.

Peak shape problem

Possible Cause

Insufficient injection, detector insensitivity

Solution

Check the injection settings, Check sample concentration, Check the position of pate, Check detector settings.

Topic: Sample band symptoms

Possible cause

Poor band quality

Solution

Check Regulating the nitrogen gas flow, Check gas supply for spraying, Check distances capillary/nozzle and capillary/object. The applied solution may be too concentrated. [Use low application speed for polar solvents such as methanol and water.]

Topic: Leakage

Possible cause

syringe problem

Solution

If air bubbles are formed, when the syringe is filled the syringe piston has a leak, Another reason for leaks could be caused by the O-ring gasket in the connection between syringe and capillary, Check and replacing the O-ring (gasket) as well as syringe piston[14].

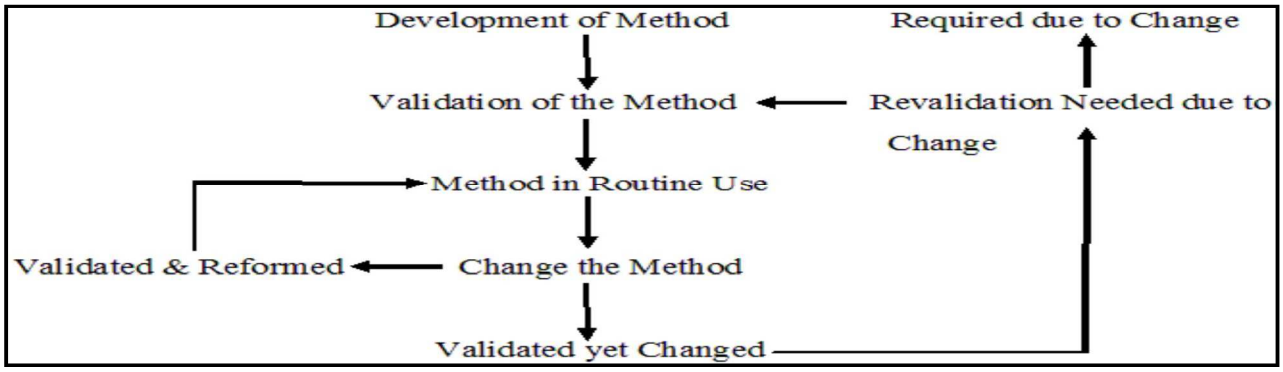

Figure No.1: Method development criteria

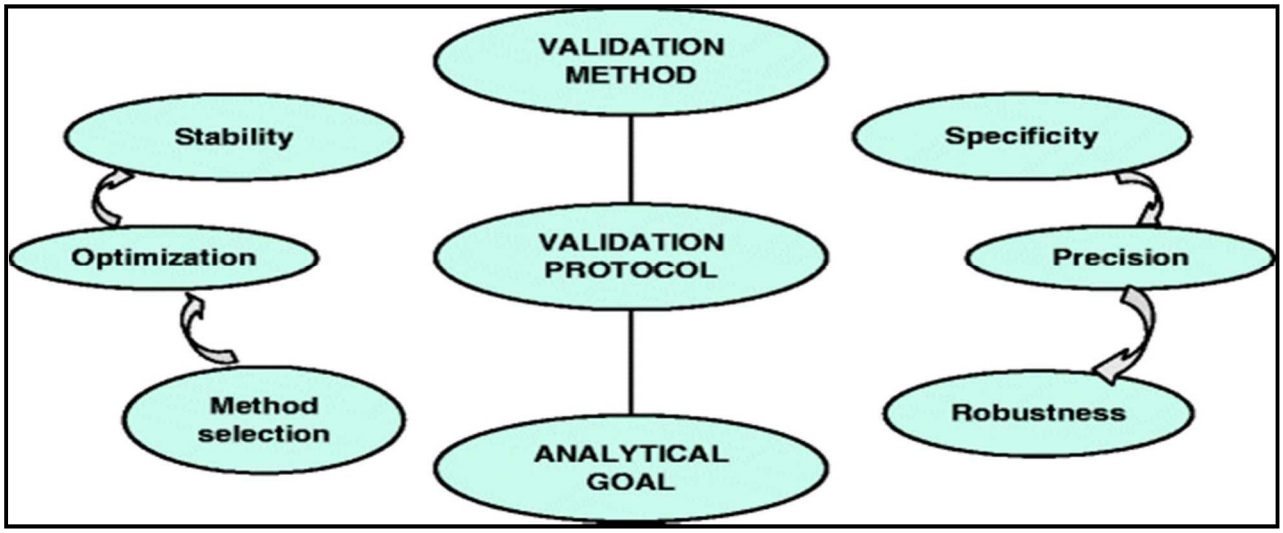

Figure No.2: Validation process involved in HPTLC

CONCLUSION

High Performance Thin Layer Chromatography has wide variety of applications in many fields such analysis and separations of pharmaceuticals, biochemistry, analyzing the air and water pollutants, monitoring the pesticides levels in the environment. Federal and state regulatory agencies use HPTLC to check for adherence to label claim, neutraceuticals, forensic department, and clinical diagnostics. HPTLC is suited to separating higher molecular weight compounds in order to provide qualitative and quantitative information. HPTLC made by several critical components. These guidelines will assist to maintain the HPLC system for routine problems. It leads to reduce the cost and enhance the performance of the system. This article provides the method development of HPTLC and common troubleshooting procedures for all types of manufacturers.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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