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### **A Review on Stability Indicating Analytical Method Development and Validation by HPTLC.**

**Shekhar Chaudhry<sup>1\*</sup>, Rajendra B.Patil<sup>1</sup>**

*1. Department of Quality Assurance Technique, Rajarshi Shahu College of Pharmacy & Research, Tathwade, Pune-33.*

#### ABSTRACT

Analysis of pharmaceutical and neutral compound and newer drugs commonly used in all the stages of drug discovery and development process. High performance thin layer chromatography (HPTLC) is one of the easiest instrumental technique based on the full capabilities of thin layer chromatography. It is still increasingly finding its way in pharmaceutical analysis with the advancements in the stationary phase and the introduction of the densitometers as detection equipment and the technique achieves high precision and trueness comparable to high performance liquid chromatography (HPLC).

**Keywords:** Force degradation, High performance thin layer chromatography (HPTLC), stability indicating method, validation (1).

\*Corresponding Author Email: [chaudhryshekhar12@gmail.com](mailto:chaudhryshekhar12@gmail.com)

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## INTRODUCTION

High Performance Thin-Layer Chromatography (or Planar Chromatography) is a modern separation technique, established worldwide and distinguished by flexibility, reliability and cost efficiency. Together with HPLC and GC it belongs to the micro-analytical methods, which play an important role in research and routine laboratories. HPTLC is the most simple separation technique today available to the analyst. It can be considered a time machine that can speed your work and allows one to do many things at a time usually not possible with other analytical techniques. In many cases instrumental Thin-Layer Chromatography offers a more suitable solution and often it is used as confirmatory or alternative technique.(2)

HPTLC is the most simple separation technique available today, because of following advantages:

1. Enormous flexibility
2. Parallel separation of many samples with minimal time requirement
3. Unsurpassed clarity and simultaneous visual evaluation of all samples and sample components
4. Simplified sample preparation due to single use of the stationary phase
5. Possibility of multiple evaluations of the plate with different parameters because all fractions of the sample are stored on the plate.
6. Required less mobile phase and sample amount.

TLC/ HPTLC are often found more troublesome than GLC/ HPLC as quantitative TLC is an off-line technique, hence automation is difficult and because of its open character, is highly influenced by environmental factors. It is therefore essential that each step may require specific approach and must be carefully validated to determine potential source of error (3).

### **General steps involved in HPTLC:**

#### **Sample preparation and derivatization**

The sample preparation procedure is to dissolve the sample with complete recovery of intact compounds of interest and minimum of matrix with a suitable concentration of analyte for direct application on the HPTLC plate. Therefore, the choice of a suitable solvent for a given analysis is very important. For normal phase chromatography using silica gel precoated plates, solvent for dissolving the sample should be non-polar and volatile as far as possible. Polar solvents are likely to induce circular chromatography at origin. For reverse phase chromatography usually polar solvents are used for dissolving the sample. Derivatization reactions are essentially required for detection when individual compound does not respond to UV or does not have fluorescence.

### Stationary and mobile phase

Generally Silica gel 60 F<sub>254</sub> having a pore size of 6 nm with fluorescent indicator is used as sorbent layer on different support material (glass, aluminium, plastic). Precoated plates in size of 20 x 20 cm having 100 - 250  $\mu\text{m}$  adsorbent thickness are generally used for quantitative analysis. The selection of appropriate mobile phase is based on the trial and error in which chemical properties of solute and solvent, solubility of analyte, adsorbent layer etc. are considered along with analyst's own experience.

### Activation of precoated plates

Plates exposed to high humidity for long time are activated by placing it in oven at 110°C- 120°C for 30 minutes prior to sample spotting. This step removes water that has been physically adsorbed on the surface of the sorbent.

### Sample application

It is the most critical step for obtaining good resolution for quantification by HPTLC. Usual application volume of 6–20  $\mu\text{l}$  and band size of 6 mm is recommended. Distance from side 12–15 mm and distance from bottom of 8 mm are usually practiced. Application of the sample and standard as a band gives better separation, equal R<sub>f</sub> values and less spot broadening. Band application is preferred than spots due to uniform distribution and larger volume can be applied. Most commonly used sample applicators are shown in figure given below.



**Figure 1: CAMAG sample applicators in HPTLC**

### Preconditioning (Chamber saturation)

Chamber saturation has pronounced influence on the separation profile. When the plate is introduced into an unsaturated chamber, during the course of development, the solvent evaporates from the plate mainly at the solvent front. Therefore larger quantity of the solvent shall be required

for a given distance; hence resulting is increase in Rf values. If the tank is saturated (by lining with filter paper), solvent vapours soon get uniformly distributed throughout the chamber. As soon as the plate is placed in such a saturated chamber, it soon gets pre-loaded with solvent vapors, hence less solvent shall required to travel a particular distance, resulting in lower Rf values. Time required for saturation will depend on the nature and composition of mobile phase and layer thickness. For low polarity mobile phase there is no need of saturation, however, saturation is desirable in case of highly polar mobile phases.

### **Chromatographic development**

Ascending, descending, two-dimensional, horizontal, multiple over run (continuous), gradient, radial (circular), anti-radial, multi-dimensional, forced flow planar chromatography are the most common modes of chromatographic development. Twin trough chamber (Fig 2), Vario chamber (Fig 3), V-shaped chamber, Circular and Anti-circular U chambers are used in TLC development. For HPTLC plates, migration distance of 5 - 6 cm is sufficient. After development, plates are removed from the chamber and dried to remove traces of mobile phase.

### **Common problems encountered during chromatographic development are as follows:**

#### **Tailing:-**

This may occur due to the presence of traces of impurities or due to presence of more than one ionic species of substances being chromatographed. This can be reduced by buffering the mobile phase system with acidic (1-2 % acetic acid) or basic (ammonia) solution. It keeps the materials to be separated in non ionic forms.

#### **Diffusion:-**

This is seen as zones of chromatographic plates. This may arise due to non uniformity of mobile phase, longitudinal diffusion between mobile phase and stationary phase or due to non-equilibrium of stationary phase.



**Figure 2: Twin Trough chamber**



**Figure 3: Vario chamber**

### **Zone detection**

As soon as the development process is complete, the plate is removed from the chamber and dried to remove traces of mobile phase. The zones can be located by physical, chemical, biological-physiological methods. In HPTLC, detection is done by visual examination at 254 nm or 366 nm in UV cabinet.

### **Photo and Video documentation**

To assist the analysts and researchers in the traceability of analytical data, photo and video documentation are necessary.

### **Chromatogram evaluation by densitometry**

Densitometry is the in situ instrumental measurement of UV-Visible absorbance, fluorescence or fluorescence quenching directly on the layer without resorting to scrapping and elution. To carry out a HPTLC densitometric analysis, three or four standard and purified samples are applied on the same plate. After development, detection (if necessary), the chromatogram is scanned.

A calibration curve consisting of scan area of standard versus amount of analyte is constructed and amount of analyte in the sample represented by scan area is interpolated from the standard curve.

### **Factors influencing HPTLC separation and resolution of spots:**

#### **Type of stationary phase (sorbent)**

Silica gels of different grades, aluminium oxide of different grades, florisil, kieselguhr G are commercially available sorbents for HPTLC.

#### **Type of pre-coated plate**

For quantitative analysis, use of HPTLC pre-coated plate is absolutely essential. The Precoated plates with different support material (glass, aluminum, plastic) with different layers are available in different format and thickness by various manufacturers.

**Layer thickness**

Usually plates with sorbent thickness of 100 – 250  $\mu\text{m}$  are used for qualitative and quantitative analysis

**Mobile phase (solvent system)**

Mobile phases should be chosen taking into consideration chemical properties of analytes and sorbent layer. Use of mobile phase containing more than three or four components should normally be avoided as it is often difficult to get reproducible ratios of different components.

**Solvent purity**

Poor grade of solvent used in preparative mobile phases have been found to decrease resolution, spot definition and  $R_f$  reproducibility.

**Size of developing chamber**

First, Stahl drew attention to importance of degree of saturation of atmosphere in the chamber and showed that it was necessary to adhere to certain conditions. He introduced the ratio Evaporation surface area: Chamber Volume as a characteristic. This ratio is 1:20 in usual rectangular chamber but 1:0.1 to 0.5 for narrow chamber.

**Saturation of chamber (pre-equilibrium)**

Chamber saturation has pronounced influence on the separation profile. When the plate is introduced into an unsaturated chamber, during the course of development, the solvent evaporates from the plate mainly at the solvent front. Therefore larger quantity of the solvent shall be required for a given distance; hence resulting in increase in  $R_f$  values.

**Sample volume to be spotted**

Substance zones which are too large from the beginning cause poor separation as during development spots do tend to become large and more diffused. This difficulty is more pronounced in case of substances with high  $R_f$  values. It is therefore recommended that solution should be applied in small increments with intermediate drying.

**Size of initial spot (band width)**

Greater the distance between different spots and smaller the initial band width of the sample, better the resolution.

**Solvent level in the chamber**

Solvent level should be lower than the position of the spots applied on the plate so that they would not be washed away while placing the plate into the chamber.

**Temperature**

$R_f$  value usually increases with rise in temperature.

### **Stability Indicating Method**

A stability indicating method (SIM) is an analytical procedure used to quantitates the decrease in the amount of the active pharmaceutical ingredient (API) in drug product due to degradation. According to an FDA guidance document, a stability-indicating method is a validated quantitative analytical procedure that can be used to detect how the stability of the drug substances and drug products changes with time. A stability indicating method accurately measures the changes in active ingredients concentration without interference from other degradation products, impurities and excipients. Stress testing is carried out to demonstrate specificity of the developed method to measure the changes in concentration of drug substance when little information is available about potential degradation product. The development of a suitable stability. Indicating method provides a background for the pre-formulation studies, stability studies and the development of proper storage requirements.

### **Importance of SIM:-**

- SIM helps to determine the intrinsic stability of drug molecule by establishing degradation pathway.
- SIM determines and identifies the likely degradation products.
- SIM helps to designed to assess the API stability requirements.
- SIM helps to monitoring the stability of a given drug in a finished product.
- SIM also use in cleaning validation.

### **Steps involved in development and validation of stability-indicating methods:-**

Development and validation of stability-indicating methods is neither provided in the regulatory guidelines nor in the pharmacopoeias. Therefore, the practical steps involved in the development of stability indicating assay methods (SIAMs) are discussed below.

Step I: Critical study of the drug structure to assess the likely decomposition route(s).

Step II: Collection of information on physicochemical properties.

Step III: Stress (forced decomposition) studies.

Step IV: Preliminary separation studies on stressed samples.

Step V: Final method development and optimization.

Step VI: Validation of SIAMs.

### **Validation of Analytical Method**

**Validation** is defined as "documented evidence which gives a high degree of confidence that a process, system, facility will consistently produce a product meeting its predetermined specifications and quality attributes."

#### **Method validation:**

Method validation is the process of proving that an analytical method is acceptable for its intended purpose. For pharmaceutical methods, guidelines from the United States Pharmacopoeia (USP), International Conference on Harmonization (ICH), World Health Organization (WHO) and the Food and Drug Administration (FDA) provide a framework for performing such validations.

#### **Parameters for method validation:**

The parameters for method validation as defined by ICH (International Conference on Harmonization) guidelines are summarized below:

##### **Accuracy**

The accuracy of an analytical procedure expresses the closeness of agreement between the values, which is accepted either as a conventional true value or an accepted reference value found. The results of the accuracy studies are expressed as percent recovery (The results must be followed within range of 98 % - 102 %)

##### **Precision**

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. It is normally expressed as % relative standard deviation.

Precision may be considered at three levels:

##### Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. It is also termed as intra-assay precision. (Relative Standard Deviation of repeatability studies must be <2 %)

##### Intermediate precision

Intermediate precision expresses the precision within laboratories variations, different days, different analysts, different equipments, reagents etc. (Relative Standard Deviation of Intermediate precision studies must be <2 %)

##### Reproducibility

Reproducibility expresses the precision between different laboratories, (collaborative studies, usually applied for standardization of methodology).

##### **Specificity**

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically these might include impurities, degradants, matrix etc.

### **Limit of Detection**

The detection limit of an individual analytical procedure is the lowest amount of analyte in sample, which can be detected but not necessarily quantitated as an exact value. Limit of detection (LOD) is expressed as a concentration at a specified signal to noise ratio. In chromatography detection limit is the injected amount that results in a peak with a height at least twice or three times as high as baseline noise level.

$$S/N = 2/1 \text{ or } 3/1$$

Where, **S**= Signal

**N**=Noise

It may be calculated based on standard deviation (SD) of the response and slope of the curve(S).

$$LOD = 3.3(SD)/S$$

Where, **SD**= Standard deviation

**S**= Slope

### **Limit of Quantitation**

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in sample, which can be quantitatively determined with suitable precision and accuracy. Limit of quantitation (LOQ) is expressed as a concentration at a specified signal to noise ratio. In chromatography quantitation limit is the injected amount that results in a peak with a height ten times as high as baseline noise level.

$$S/N = 10/1$$

Where, **S**= Signal

**N**=Noise

It may be calculated based on standard deviation (SD) of the response and slope of the curve(S).

$$LOQ = 10 (SD)/ S$$

Where, **SD**= Standard deviation

**S**= Slope

### **Linearity**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample. (The correlation coefficient for linearity studies must be,  $r > 0.999$ ).

### **Range**

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

### **Robustness**

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

### **Ruggedness**

The robustness/ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

### **Force Degradation Studies**

Forced degradation studies provide data to support identification of possible degradants; degradation pathways and intrinsic stability of the drug molecule and validation of stability indicating analytical procedures<sup>14,15</sup>. A draft guidance document suggests that results of one-time forced degradation studies should be included in Phase 3 INDs (Investigational New Drugs)<sup>16</sup>. NDA (New Drug Application) registration requires data of forced degradation studies as forced degradation products, degradation reaction kinetics, structure, mass balance, drug peak purity, etc. This forced degradation study provides information about degradation pathways of API, alone and in drug product, any possible polymorphic or enantiomeric substances and difference between drug related degradation and excipient interferences.

### **Role of forced degradation:-**

Trial and error needed to find the proper combination of stress agent concentration and time to effect degradation, preferably in the 20-30 % range. Achieving 100 % degradation would be too strenuous and could possibly cause secondary degradation, giving degradation product(s), which are not likely to be formed under normal storage conditions. Under-stressing may fail to generate important degradation products. The degradation studies should be terminated after the maximum recommended time/stress conditions, even if sufficient degradation has not been achieved. It is unnecessary and even unwise to try to degrade the drug at all cost as it would only increase the

complexity of the method development with little or no benefit in the quality of the data generated by the method. The initially developed method should achieve a suitably retained peak, with  $k'$  of about 4 to 10. This range allows a suitable time space in the chromatogram for degradation products to elute before or after the active (major) peak.

#### **Acid/ Base hydrolysis:-**

Functional groups likely to introduce acid/ base hydrolysis are amides (lactams), esters (lactones), carbamates, imides, imines, alcohols (epimerization for chiral centers) and aryl amines. The degraded test samples are often neutralized using acid/base/buffer to avoid further decomposition.

#### **Thermal and thermal/humidity stress testing :-**

The goal of thermal and thermal/humidity studies is to force the degradation of drug substance over time to determine the primary thermal and/or humidity degradation products.

#### **Oxidation:-**

Oxygen which is required in most oxidation reactions and is abundant in the environment to which pharmaceuticals are exposed during either processing or long term storage. Drug substance functional groups that are susceptible to oxidation reactions include heteroatom (nitrogen: n-oxides and sulphur: sulfoxids and sulfons), alkenes, 2° and 3° amines, benzylic sites, and aldehydes and ketones. Acetonitrile is preferred reaction solvent over methanol because alcohols slow the reaction by competing with drug for initiator radicals.

#### **Photo stability:-**

Any light source that is designed to produce an output similar to the D65/ID65 emission standard such as an artificial daylight fluorescent lamp combining visible and ultraviolet (UV) outputs, xenon, or metal halide lamp. D65 is the internationally recognized standard for outdoor daylight as defined in ISO 10977 (1993). ID65 is the equivalent indoor indirect daylight standard. Functional groups likely to introduce drug photo reactivity are carbonyl, nitro aromatic, N-oxide, aryl halo derivative, aryl acetic acid, aromatic nitro group.

#### **Overview of regulatory guidance:-**

According to the available guidance, forced degradation studies are carried out for the following reasons:

- Development and validation of stability-indicating methodology.
- Determination of degradation pathways of drug substances and drug products.
- Discernment of degradation products in formulations that are related to drug substances versus those that are related to non-drug substances (e.g., excipients).

- Structure elucidation of degradation products.
- Determination of the intrinsic stability of a drug substance molecule.

Stress type	Conditions	Time
Acid hydrolysis	1 mg/ml in 0.1 N (up to 1.0 N) HCl; RT or higher	1–7 days
Base hydrolysis	1 mg/ml in 0.1 N (up to 1.0 N) NaOH; RT or higher	1–7 days
Oxidative/solution	0.3% (up to 3%) H <sub>2</sub> O <sub>2</sub> ; RT; protected from light	Few hours to 7 days
Thermal/Humidity	70 degC / 75 % RH	Up to 2 weeks
Photo-degradation	Fluorescent and UV light	ICH

### Recommended stress conditions for drug substance

#### Acid and Alkali Hydrolysis:-

The hydrolytic degradation of a new drug in acidic and alkaline condition can be studied by refluxing the drug in 0.1 N HCl or 0.1 N NaOH. If reasonable degradation is seen, testing can be stopped at this point. However in case no degradation is seen under these conditions, the drug should be refluxed in acid/alkali of higher strength & for longer duration of time. Alternatively, if total degradation is seen after subjecting the drugs to initial condition, acid/alkali strength can be decreased along with decrease in reaction temperature.(Fig 4)

#### Oxidation:-

To test for oxidation, it is suggested to use hydrogen peroxide in the concentration range of 3 to 50 %. In some drugs extensive degradation is seen when exposed to 3% of hydrogen peroxide for very short time period at room temperature. In other cases exposure to high concentration of hydrogen peroxide, even under extreme condition does not cause any significant degradation. The behavior is on expected lines, as some drugs are fast oxidisable, while others are not. The latter are not expected to show any change even in the presence of high amount of oxidizing agent.

#### Photolytic Degradation:-

**UV light:** The photolytic studies should be carried out by exposure to light using either a combination of cool white & UV fluorescent lamp. Exposure energy should be minimum of 1.2 million lux hrs fluorescent light and if decomposition is not seen the intensity should be increased by 5 times. In case still no decomposition takes place, the drug can be declared photo stable.

**Sunlight:-**The photolytic studies should cover the exposure of drug solution to sunlight. The exposure period ranges from few hours to several months. The photolytic studies are carried out at room temperature.

**Hydrolysis:-**

Stress testing under neutral condition can be started by refluxing the drug in water for 12 hours. Refluxing time should be increased or decreased as per the degradation obtained in 12 hours.

**Dry Heat:-**

Heating the drug powder at higher temperature in oven can be followed to carry out stress testing for dry heat degradation.

**Wet Heat:-**

Wet heat degradation can be studied by keeping the drug solution at 50°C, 75% relative humidity for 3 months in humidity chamber.

**CONCLUSION:**

The review reveals that the HPTLC method developed and validated comply with the general procedure pertaining to the quantitative mode of this technique HPTLC is generally used with an unmodified silica layer as 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 analyst's own experience .However , in case a complex mixture of drugs needs to be separated, we advocate for a systemic approach.

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