# Degradation study of Nortriptyline HCl by HPTLC method

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## ABSTRACT

*Experimental analysis was performed on silica gel 60 F254 HPTLC plates (20cm x 10cm with 250 µm thickness; E Merck, Darmstadt, Germany, Batch-HX011551) using mobile phase consisting of toluene: ethyl acetate: methanol: glacial acetic acid in the ratio of 4:4:1.6:0.4 (v/v/v/v). Prior to chromatographic analysis the plates were washed in methanol, dried in a current of dry air and activated at 110$^0$C for 5 min. Samples were spotted in the form of bands of width 8mm with a Camagmicrolitre syringe. A constant application rate of 150nl/s was used and the space between two bands were 5mm. Monochromator band width was set at 20nm, each track was scanned six times and baseline correction was used. Linear ascending development was carried out in 20cm x 10cm twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for the mobile phase was 15 min at room temperature (26±2oC) and relative humidity (70±5%) and degradation study was carried out.*

## INTRODUCTION

An impure substance may be defined as any material that affects the purity of the material of interest, viz., an active pharmaceutical ingredient (API) or drug substance. Impurity control in pharmaceutical products is a primary goal of drug development. High performance thin layer chromatography (HPTLC) 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.

## EXPERIMENTAL AND RESULTS

### Development of Optimum Mobile Phase

The HPTLC method was optimized with an aim to develop a stability-indicating assay method. During mobile phase optimization various mobile phase compositions including toluene: methanol (8:2), toluene: methanol: glacial acetic acid (8:1.5:0.5), toluene: ethyl acetate: methanol (4:4:2) and different proportions of toluene: ethyl acetate: methanol: glacial acetic acid were tried. Both pure drug as well as forced degradation samples were spotted onto precoated TLC plates and tried with the afore mentioned mobile phase systems. Higher concentrations of toluene resulted in low Rf values of 0.32. Increasing the solvent strength of the mobile phase decreases retention and increases the Rf value. As a result, ethyl acetate was used in equal proportions as toluene along with methanol to have considerable. The spot obtained was diffused with an Rf value of 0.34. Glacial acetic acid was used in the mobile phase as a modifier to reduce the diffusion of the spots. Amongst

the combinations tried toluene: ethyl acetate: methanol: glacial acetic acid (4:4:1.6:0.4, v/v/v/v) was finalized as the mobile phase. The spots developed were dense and compact with an Rf value of 0.54± 0.02. Typical peak of nortriptyline is shown in Fig.2. The peak obtained was sharp and symmetrical in nature. The spectrum obtained after densitometric scanning showed peak at 279 nm

**Calibration Plot of Nortriptyline**

A stock solution of Nortriptyline (1mg/ml) was prepared in chloroform. 0.5 ml of stock solution was further diluted to 10 ml with chloroform to get standard solution of 0.05mg/ml. Appropriate volumes of standard solution were spotted to obtain Nortriptyline in the concentration range of 200-700ng (n=6). The plate was developed and scanned as described above. Well resolved compact band of drug was scanned at 279nm. The linearity plot was obtained by plotting average peak area at each concentration against corresponding band concentrations of Nortriptyline (ng/spot). Linear regression analysis was employed to calculate the regression equations and the correlation coefficients.

## METHOD VALIDATION

The proposed method was validated in compliance with ICH Guidelines. The method was validated for linearity and range, limit of detection (LOD), limit of quantitation (LOQ), precision, specificity, accuracy, repeatability and robustness.

**linearity**

The linearity of an analytical method is its ability, within a given range, to obtain test results which are either directly or through mathematical transformation, proportional to concentration of the analyte in the sample. The method was found to be linear (r2= 0.9965±0.00083) in the concentration range of 200-600 ng/spot (n=6), with respect to peak area. Peak area and concentration was subjected to linear regression analysis to determine calibration equation and correlation coefficients. No significant difference was observed in the slopes of standard curves (ANOVA, P< 0.0001 (P value, one sample t test). The three-dimensional overlay of densitograms of the calibration spots of nortriptyline at 279nmThe regression data is reported in Table 1

Table No.1: Peak area for different concentration nortriptyline

| Sr. no. | Concentration | Peak area |
|---|---|---|
| 1 | 200ng/band | 2750.69±0.32 |
| 2 | 300ng/band | 4126±3.04 |
| 3 | 400ng/band | 5501.33±0.21 |
| 4 | 500ng/band | 5787±0.02 |
| 5 | 600ng/band | 7285.3±1.93 |

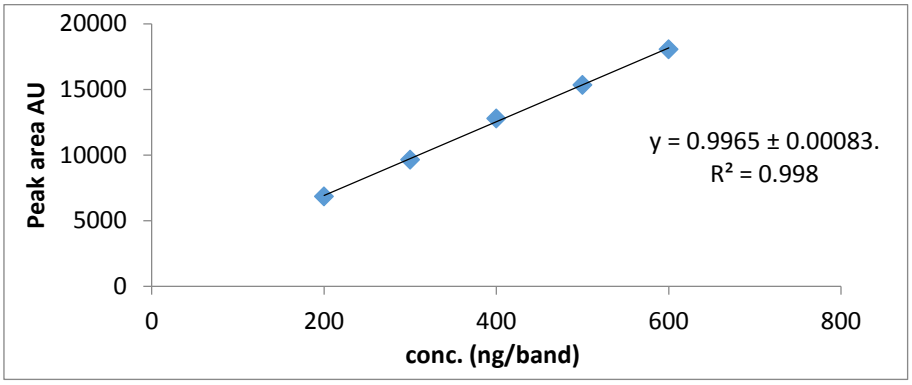


**Fig No.1: Standard calibration curve of nortriptaline.**

Table. No.2: Linear regression data for the calibration curve

| Sr no | Parameters | Value |
|---|---|---|
| 1. | Detection Wavelength (nm) | 279 |
| 2. | Beer's Law Limit (ng/band) | 200-600 |
| 3. | Correlation Coefficient (r2 ± SD) | 0.9965 ± 0.00083 |
| 4. | Intercept (c) ± SD | 0.9965 |
| 5. | Confidence limit of intercept | 1385.5-1804.1 |
| 6. | Slope (m) ± SD | 0.00083 |
| 7. | Confidence limit of slope | 7.901-8.771 |
| 8. | SD of residuals from line | 58.608 |

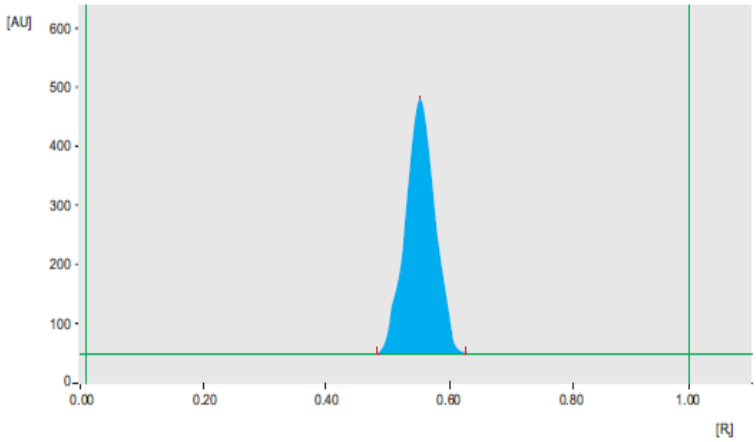


Fig No.2: Densitogram of nortriptyline, Wavelength: 279 nm, Mobile phase: toluene: ethyl acetate: methanol: glacial acetic acid (4:4:1.6:0.4, v/v/v/v)(Rf value is 0.54)

**Limit of detection and limit of quantitation**

Based on the standard deviation of the response and the slope, LOD and LOQ were estimated using the formulae:

LOD= 3.3 σ/S

Where σ = the standard deviation of the response

S = the slope of the calibration curve

LOQ = 10 σ/S
Where σ = the standard deviation of the response
S = the slope of the calibration curve
LOD and LOQ were determined from the standard deviations of the responses for six replicate determinations.
The LOD and LOQ were found to be 23.20 and 70.30 ng respectively.

**Precision**

Precision is a measure of the reproducibility of the analytical method under normal operating conditions. Precision is expressed as relative percent standard deviation (% RSD). The intra- and inter-day precisions were carried out at three different concentration levels, 300, 500 and 700 ng/spot (n=6) respectively. Repeatability of measurement of peak area and % content can be defined as the precision of the method when repeated under same operating conditions (same equipments, reagents, settings and laboratory) over a short interval of time. Repeatability was assessed by chromatography of six replicates of 100% of the test concentration (500ng/spot) and expressed in terms of coefficient of variation (CV).
The repeatability of measurement of peak area and % content was expressed as CV and found to be 0.011 and 0.016 for NOR. Results for intra- and inter-day variation studied at three levels 300, 500 and 700 ng/spot The % RSD was found to be<2% in all cases and thus indicate that the method is highly precise.

Table No.3: Statistical evaluation of precision (repeatability) of developed method at 500 ng/spot

| **Conc.(ng/spot) area** | **Peak area** | **% Content ± SD** | **± SD** | **CV** |
|---|---|---|---|---|
| 500 | 5803.09±68.44 | 101.88±1.65 | 0.011 | 0.016 |

Table No.4: Summary of Intra-day and Inter-day method precision

| **Amount (ng/spot)** | **Intra-day precision by peak area** | | | **Inter-day precision by peak area** | | |
|---|---|---|---|---|---|---|
| | **Mean ± SD** | **% Content ± SD** | **%RSD** | **Mean ± SD** | **% Content ± SD** | **%RSD** |
| 300 | 4126±3.04 | 101.37±0.10 | 0.073 | 4134.06 ± 7.96 | 101.53±0.16 | 0.192 |
| 500 | 5787±0.02 | 101.34 ±0.001 | 0.003 | 5786.73 ±0.27 | 101.33 ±0.01 | 0.004 |
| 700 | 8499.3±1.84 | 98.47 ± 0.02 | 0.026 | 8486±0.70 | 98.48 ±0.015 | 0.009 |

**selectivity and specificity**

Selectivity of an analytical method is its capability to determine precisely and specifically the analyte in the presence of components that may be expected to be present in the sample matrix like matrix components, impurities or degradation products. UV spectrum of standard drug and sample at selected wavelength was compared. Specificity ensures that the signal measured comes from the analyte of interest and that there is no interference from excipients, impurities or degradation products. Specificity was determined by analyzing standard drug and test sample. The spot for nortriptyline in the sample was confirmed by comparing the Rf value and spectrum of the spot with that of the standard. The peak purity of nortriptyline was judged by comparing the spectrum at three different regions of the spot i.e. peak start, peak apex and peak end positions of the spot.
The method was selective and specific for the determination of nortriptyline. Good correlation (r=0.998) was obtained between the standard and sample spectra of nortriptyline. The overlay spectrum of standard and sample is shown in Fig. A single spot with an Rf value of 0.54 was observed in nortriptyline standard and samples. In degradation studies the spots of degraded products were well resolved from the drug spot.

**Accuracy (recovery studies)**

Accuracy of an analytical method is the closeness of test results to true value. It was determined by the application of analytical procedure to recovery studies, where known amount of standard is spiked in preanalyzed sample solutions. The amount of drug recovered in accuracy study was in the range, which indicated that the method is accurate. Accuracy was studied at different levels i.e. 0%, 80%, 100% and 120% by addition of pure drug to previously analyzed test samples. The mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate and percent recovery was calculated. The proposed method for extraction and estimation of nortriptyline from pharmaceutical dosage form after spiking with 80%, 100% and 101.96, 101.45, 101.97
120% showed good recoveries in the range of 101.45-101.97% as listed in Table.

Table No.5: Recovery studies of Nortriptyline

| Recovery Level | Initial amount (ng) | Nortriptyline added (ng) | Total amount (ng) | Nortriptyline found (ng) ± SD | % Recovery ± SD |
|---|---|---|---|---|---|
| 0 | 300 | 0 | 300 | 305.12 ± 0.75 | 101.70 ± 0.25 |
| 80 | 300 | 100 | 400 | 407.85 ± 11.52 | 101.96 ± 2.88 |
| 100 | 300 | 150 | 450 | 456.53 ± 11.59 | 101.45 ± 2.58 |
| 120 | 300 | 200 | 500 | 509.85 ± 16.46 | 101.97 ± 3.29 |

**Robustness**

The robustness of an analytical method may be defined as the measure of its ability to remain unchanged by small, but purposeful variations in the method parameters and provides an indication of its ependability during normal usage. The robustness of the method was determined by variations in mobile phase composition (±0.2ml), volume of mobile phase (±2ml), chamber saturation period (±5min), development distance (±0.5cm), and time from development to scanning (0, 10, 20 and 30 min). One factor at a time was changed at a concentration level of 400ng/band (n=6) of nortriptyline to study the effect on peak areas of the drug.
Robustness was studied by determining the effect of small variations in the mobile phase composition, mobile phase volume, chamber saturation time, migration distance and time from chromatography to scanning. The % RSD of peak areas was calculated for each variable and was found to be less than 2%. The low values of % RSD as listed in Table indicate that the method is robust.

Table No.6: Results of Robustness studies

| Parameter | Mean Rf | %RSD of peak area |
|---|---|---|
| Mobile phase composition (±0.2ml) | 0.54 ± 0.007 | 1.15 |
| Mobile phase volume (±2ml) | 0.57 ± 0.009 | 1.19 |
| Chamber saturation time (±5 min) | 0.58 ± 0.009 | 1.42 |
| Migration distance (±0.5 cm) | 0.55 ± 0.003 | 1.18 |
| Time from chromatography to scanning (±10 min) | 0.56 ± 0.002 | 0.59 |

**Forced Degradation Studies**

A stock solution containing 10 mg/ml nortriptyline was prepared. This solution was used for stress degradation to indicate specificity of the proposed analytical method and its stability indicating property. In all the degradation studies the average peak area of three replicates was obtained.

**Acid and Base Induced Degradation Studies**

Degradation studies were carried out by refluxing 5ml of drug solution with 5ml each of 1N HCl and 1N NaOH. The mixtures were refluxed for 3h respectively at $80^0$C. The solutions (0.5ml) were taken and neutralized and subsequently diluted to 10 ml with Neutralizing solvent (HCl and NaOH). The resulting solutions were applied to TLC plate in such a way that the final concentration attained was 5000 ng/spot for both acid and base degradation products and the chromatograms were run as described in previous section.

The rate of degradation in acid was slower as compared with alkali. The chromatogram of the acid degraded sample showed peak at Rf value of 0.42, 0.49, 0.53, 0.55, 0.62, 0.82 was observed in acid degradation peak are shown in Fig. 3

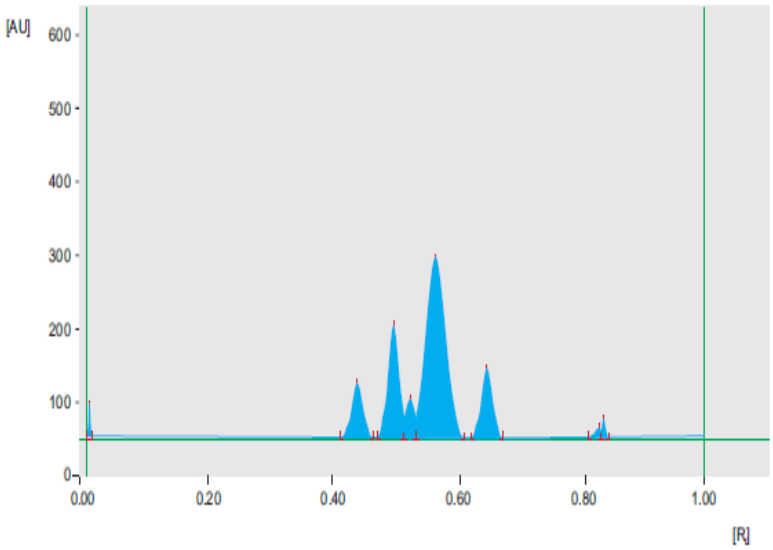


Fig No.3 Densitogram of nortriptaline bulk drug after acidic degradation of 1N HCl

For **base degradation**study different peak value observed at various Rf values of 0.40, 0.45, 0.49, 0.58, 0.62 showen in fig. The results indicate that nortriptyline undergoes degradation under acidic and basic conditions.

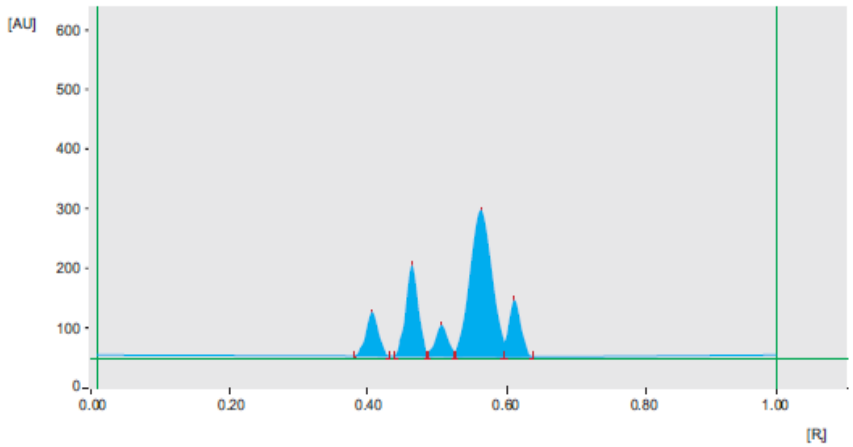


Fig No.4: Densitogram of bulk drug after a base degradation of Nortriptyline

**Hydrogen peroxide induced degradation product**

The sample showed an additional peak with 6 % (w/v) at Rf value of 0.39, 0.48, 0.57, 0.73.

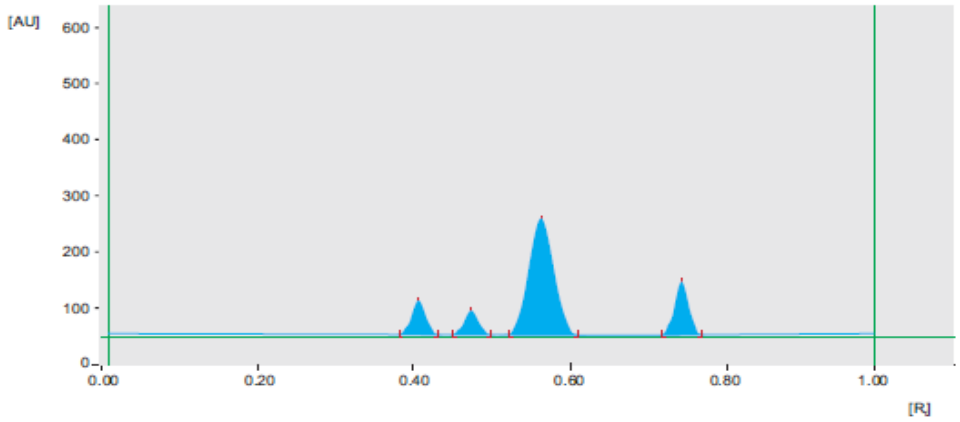


**Fig No.5: Densitogram of nortriptaline after oxidative degradation (6%$H_2O_2$)**

**Dry heat degradation**

The standard drug was stored in oven at 50oC for 72h to study dry heat degradation

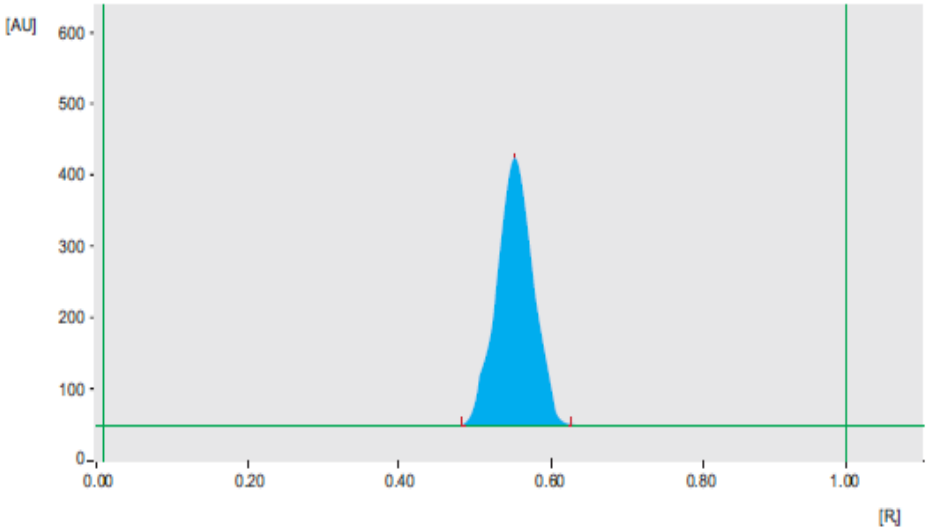


Fig No.6:Densitogram of Nortriptyline after thermal degradation

**Photo degradation**

The photochemical stability of the drug was studied by exposing the stock solution to direct sunlight for 24 hr. After suitable dilution, concentration of 5000ng/spot was applied and the chromatograms were run as described in previous section. Oxidative degradation (Hydrogen peroxide induced) Studies were performed in 30% (v/v) hydrogen peroxide at room temperature for 48h respectively.

applied to TLC plate to achieve a final concentration of 5000ng/spot and the chromatograms were run and peak obtained RF value at 0.54.

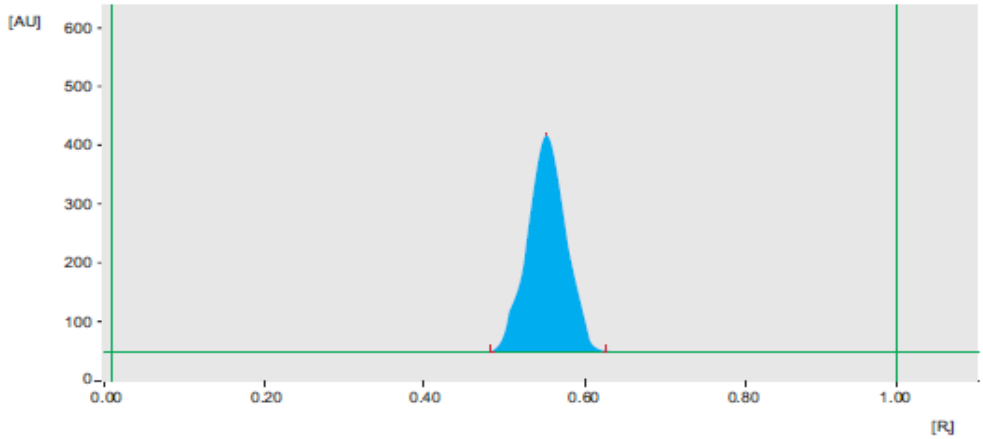


Fig No.7:Densitogram of Nortriptaline after photo degradation

Table No.7: Summary of forced degradation

| Sr no | Parameter | % Degradation | Peak area |
|---|---|---|---|
| 1. | Normal | 100 | 5786.73 |
| 2. | 1N HCl | 56 | 3240.16 |
| 3. | 1N NaOH | 34 | 1967.24 |
| 4. | 6% H2O2 | 52 | 3008.72 |
| 5. | Thermal | 99.91 | 5781 |
| 6. | Photolytic | 98.37 | 5692 |

## DISCUSSION AND CONCLUSION

Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was by peak areas with linear regression. Scanned peak areas were recorded for each sample at each concentration level. The average peak areas and variations in peak area obtained were expressed as percent relative standard deviation (% RSD). Linear regression data for the calibration curve was found to be Detection Wavelength (nm) 279, Beer's Law Limit (ng/band) 200-600, Correlation Coefficient (r2 ± SD) 0.9965± 0.00083, Intercept (c) ± SD 0.9965, Confidence limit of intercept 1385.5-1804.1, Slope (m) ± SD 0.00083, Confidence limit of slope 7.901-8.771, SD of residuals from line 58.608 respectively. Recovery studies of Nortriptyline was found to be for 80,100, 120 level to 101.70 ± 0.25, 101.96 ± 2.88, 101.45 ± 2.58, 101.97 ± 3.29 Degradation studies were carried out by refluxing 5ml of drug solution with 5ml each of 1N HCl and 1N NaOH. The mixtures were refluxed for 3h respectively at 800C. The solutions (0.5 ml) were taken and neutralized and subsequently diluted to 10 ml with Neutralizing solvent (HCl and NaOH). The resulting solutions were applied to TLC plate in such a way that the final concentration attained was 5000 ng/spot for both acid and base degradation products and the chromatograms were run as described in previous section. The rate of degradation in acid was slower as compared with alkali. The chromatogram of the acid degraded sample showed peak at Rf value of 0.42, 0.49, 0.53, 0.55, 0.62, 0.82 was observed in acid degradation. For base degradation study different peak value

observed at various Rf values of 0.40, 0.45, 0.49, 0.58, 0.62. The results indicate that nortriptyline undergoes degradation under acidic and basic conditions, the sample showed an additional peak with 6 % (w/v) at Rf value of 0.39, 0.48, 0.57, 0.73. The photochemical stability of the drug was studied by exposing the stock solution to direct sunlight for 24 hr. After suitable dilution, concentration of 5000ng/spot was applied and the chromatograms were run as described in previous section. Oxidative degradation (Hydrogen peroxide induced) Studies were performed in 30% (v/v) hydrogen peroxide at room temperature for 48h respectively applied to TLC plate to achieve a final concentration of 5000ng/spot and the chromatograms were run and peak obtained RF value at 0.54. The degradation of HPTLC was found to in 1N HCl 56 %, 1N NaOH 34 %, 6% H2O2 52 %, Thermal 0.09, Photolytic 1.63, Statistical analysis of the data establishes that the developed HPTLC method is specific, accurate, precise and stability-indicating. The validated method is suitable for analysis of nortriptyline in both bulk without any interference from the excipients. The method can be employed to determine the purity of the drug by detecting any related impurities present. The method is efficient in separating the degradation components from the main analyze nortriptyline and hence can be considered as stability-indicating study.

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