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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR DETERMINATION OF TROXIPIDE

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Keywords:

Troxipide (TRP), High performance thin layer chromatography (HPTLC), Method Optimization, Validation, Stability indicating, degradation

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ABSTRACT: A simple sensitive, accurate, precise and stability indicating high performance thin layer chromatographic method has been developed and validated for the estimation of troxipide in bulk drug and formulation. The method was developed using silica Gel 60 F_{254}TLC plates, with hexane: ethyl acetate: methanol: water: ammonia 1: 5: 5: 1.5:0.2 *(v/v)* as mobile phase. The densitometric scanning was performed at λ = 263 nm. The method was validated for linearity, accuracy, precision, specificity and robustness according to ICH guidelines. The calibration plots were linear in the range 200-700 ng/band with r^2= 0.998. The LOD and LOQ were found to be 20.39 and 61.81 ng/spot, respectively. Troxipide was subjected to acidic, alkaline hydrolysis, oxidation, photo degradation and thermal degradation. The drug undergoes degradation in acidic as well as alkaline hydrolysis and thermal degradation; however it was found to be relatively stable for photolytic and oxidative degradation conditions. As the drug peak and the degradation product peaks were well separated from each other; this method can be employed as stability indicating method for the determination of troxipide and its degradation products from API and formulation.

INTRODUCTION: Troxipide [3, 4, 5-trimethoxy-N-3-piperidylbenzamide] **(Fig. 1)**, is a gastroprotective agent with a molecular formula of $C_{15}H_{22}N_2O_4$ and molecular weight of 294.4. Troxipide is known to treat gastritis and gastric ulcer, through a multimodal action. Troxipide protects against mucosal fragility and disruption of gastric mucosal barrier by increasing the gastric mucosal content of glucosamine and mucopolysaccharides, stimulating the synthesis of cytoprotective prostaglandins and by stimulating the regeneration of collagen fibers [1, 2].

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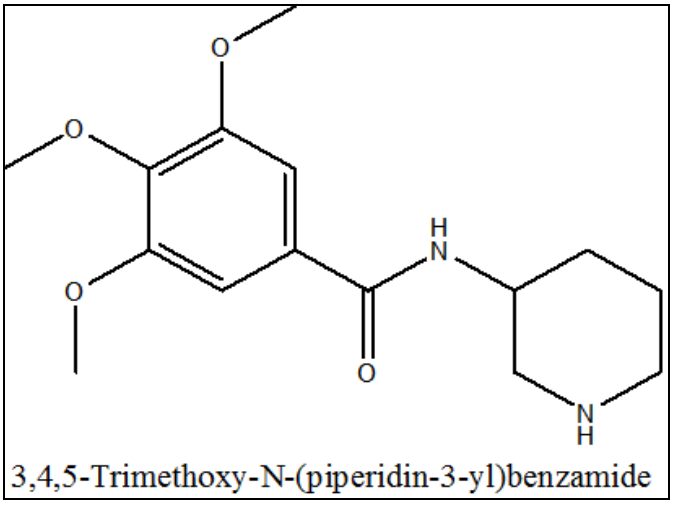

FIG. 1: STRUCTURE OF TROXIPIDE

Literature survey revealed that different analytical methods for the determination of troxipide have been reported, which include, spectrophotometric and high performance liquid chromatography

(HPLC) method [3] and a stability indicating LC method [4, 5]. However no HPTLC method has been reported for determination of troxipide and its degradation products. As compared to the other analytical techniques, HPTLC is the most simple and less time consuming technique. This technique has an advantage that many parameters can be performed at one time, which is not possible with other analytical techniques. Hence this article describes a simple, precise, accurate validated HPTLC method for quantitative estimation of TRP and its degradation products.

MATERIALS AND METHODS:

Materials & Reagents:

Troxipide pure analytical sample was obtained as a gift sample from Emcure Pharmaceutical Ltd., Jammu, India. Troxipide commercially available as tablet with the Brand name 'Troxip' containing Troxipide 100mg per tablet, Batch No. 16A13004, manufactured by Emcure Pharmaceutical Ltd., Jammu, India, and marketed by Zuventus Healthcare Ltd., Mumbai, India were purchased locally. All chemicals used were of Analytical reagent grade including acetone, methanol, hexane, ethyl acetate, ammonia and formic acid and were purchased from Research Lab Fine Chem Industries, Mumbai.

Standard and Sample preparation:

Standard Solution: Standard stock solution containing 1000 µg/ml of Troxipide was prepared in methanol by weighing 10 mg of TRP dissolving it in sufficient quantity of methanol and finally volume was made up to 10 ml with methanol. Further it is diluted to get the concentration of 100 µg /ml.

Sample Solution:

Twenty TRP tablets were weighed and powdered and quantity of powder equivalent to 10 mg was weighed and transferred to 100 ml volumetric flask. The powder was dissolved in methanol by sonication for 5 min, the solution was filtered through a whatmann filter paper no. 41 and finally volume was made with the methanol. Further it is diluted to get the concentration of 100 µg /ml.

Chromatography: Chromatography was performed on 20 x10 cm aluminium backed silica gel 60 F_{254}TLC plates (Merck, Darmstadt, Germany). Before use, the plates were washed with methanol and activated at 110 ^{0}C for 5 min. hexane: ethyl acetate: methanol: water: Ammonia 1: 5: 5:1.5:0.2 *(v/v)* were used as the mobile phase. Samples (5 µl) were applied to the plates as 6 mm bands, keeping 17.7 mm distance between bands and 8 mm distance from plate side edge and 8 mm distance from bottom of plate. CAMAG (Muttenz Switzerland) Linomat V was used as sample applicator equipped with 100 µl syringe (Hamilton, Reno, Nevada, USA) with the spraying rate as150 nL/sec. The plates were developed in CAMAG 20 x 10 cm twin trough chamber lined with filter paper and saturated with mobile phase vapors for 10 min at room temperature. The solvent front position was set as 80 mm. After development of the plate in the mobile phase, it was dried in hot air oven at 50^0 C for 5 min.

Densitometric scanning of the developed plate was performed at wavelength λ= 263 nm using CAMAG TLC scanner 3 with win CATS Software and deuterium lamp as light source with the slit dimension 4.00 x 0.30 mm and scanning speed of 20 mm/s.

Optimization of Method:

HPTLC procedure was optimized to develop a method for identification and quantification of TRP. Based on the literature survey and the polarity of the TRP, initially individual solvents were tried as mobile phase, like hexane, ethyl acetate, and methanol. From the results of above trial, the mobile phase composition containing hexane: ethyl acetate (5:5) *v/v*; was tried. In this mobile phase, the bands were observed near to the spotting line. Therefore to increase the polarity of mobile phase, methanol was added to the above composition i.e. mobile phase containing hexane: ethyl acetate: methanol (5:4:1 *v/v*) was tried. In this also the TRP bands were not moving at all. Hence it is concluded that, polarity of the solvent in the mobile phase has to be increased. Hence hexane proportion was decreased and ethyl acetate proportion was increased i.e hexane: ethyl acetate: methanol (4:5:1) *v/v* was used as the mobile phase. But in this case TRP band was moving but very short distance. Further to increase the polarity of the mobile phase, water was incorporated as one of

the component of mobile phase i.e. hexane: ethyl acetate: methanol: water (4:5:1:0.5) *v/v*. In this TRP band was resolving but not to a considerable limit. Hence again the composition of the mobile phase was changed to hexane: ethyl acetate: methanol: water (2:5:3:1 *v/v*); here the band resolution was improved but still the peak shape was not proper. Hence, the composition was changed to hexane: ethyl acetate: methanol: water (1:5:5:1.5 *v/v*) were in this case the well resolved peaks were obtained but with tailing. Hence finally mobile phase containing hexane: ethyl acetate: methanol: water: ammonia (1:5:5:1.5:0.2) *(v/v)* was tried to reduce the tailing effect. This mobile phase showed well resolved peaks of drug with the reproducible results and the method was said to be optimized.

Method Validation:
The method was validated according to ICH guidelines for the parameters including accuracy, precision, linearity, specificity and robustness in analytical solution [6].

Linearity:
The equivalent volume of standard stalk solution (2.0-7.0 µl) was spotted on plate to cover the range of and 200-700 ng per band. Chromatographic plates were developed using optimized procedure and scanned as described above. The peak areas were recorded for troxipide. As the calibration can be accepted as linear, only if it is proven statistically, hence to prove the linearity of the data; non numerical test method (testing the residuals) was performed [7, 8, 9].

Method sensitivity (LOD and LOQ):
From the calibration curve of TRP, LOD and LOQ of the method was calculated. All the data of calibration curve was processed as per the formula for limit of detection LOD and limit of quantitation LOQ given as LOD= 3.3x σ/S and LOQ=10x σ/S, where σ is standard deviation and S is slope of calibration plot.

Assay:
The concentration selected to perform assay was 300ng/band. The area of this band was measured six times and % assay was calculated. The area under curve for the sample solution was compared with the data of calibration curve and the % assay was calculated.

Accuracy (Recovery):
The accuracy of the method was determined by calculating recoveries of troxipide by the standard addition method. Recovery was performed at three levels i.e. 80, 100 and 120 %. Known amount of standards as 240, 300 and 360 ng per band was spiked to the sample (300 ng). The amount of troxipide was determined by measuring the peak areas.

Precision:
For the precision both repeatability and intermediate precision of the method for drug was checked. For repeatability the peak area of sample band was measured repeatedly (n=6) and the % relative standard deviation (% RSD) was calculated. For intermediate precision, the peak area of sample at three different concentration were measured for three times (n=3) on the same day for intraday precision and on three different days for inter day precision and the % relative standard deviation (% RSD) was calculated.

Robustness:
Robustness of the method was evaluated by deliberately changing the parameters which include volume of mobile phase, saturation time, composition of mobile phase, TLC plate form different lot and time from spotting to development and development to scanning. By changing the above parameters the peak area of the TRP was recorded and % assay was calculated.

Specificity:
The specificity of the method was determined by analyzing standard drug, test sample and its three impurities. The spot for standard and sample drug was confirmed by comparing the R_f and spectrum of the spot to that of a standard. The peak purity was determined by comparing r value of the spectrum at different regions of the spot, i.e, peak start (S) to peak apex (M) and peak apex (M) to peak end (E).

Stress degradation studies [10, 11]:
Acidic Hydrolysis: To the 10 ml of standard stalk solution of TRP standard (1000 µg/ml), 10 ml of

0.5 M HCl was added. The above mixture was refluxed for 4 hrs at 60^0C. After refluxing the solution was allowed to cool at room temperature and it was neutralized with 0.5 M NaOH and finally volume was made up to 50 ml with methanol.

Alkaline Hydrolysis: To the 10 ml of standard stalk solution of TRP standard, 10 ml of 0.5 M NaOH was added. The above mixture was refluxed for 4 hrs at 60^0C. After refluxing the solution was allowed to cool at room temperature and it was neutralized with 0.5 M HCl and finally volume was made up to 50 ml with methanol.

Oxidative degradation: To the 10 ml of standard stalk solution of TRP standard, 10 ml of 3 % H_2O_2 was added. The above mixture was refluxed for 4 hrs at 60^0C. After refluxing the solution was allowed to cool at room temperature and finally volume was made up to 50 ml with methanol.

Photolytic degradation: 10 ml standard stalk solution was exposed to sunlight for 24 hrs and volume was made up to 50 ml with methanol.

Thermal degradation: 10 ml of standard stalk solution was refluxed at 40^0C for 4 hrs. After refluxing the solution was allowed to cool at room temperature and finally volume was made up to 50 ml with methanol.

From all the above stress degradation sample solutions each of 10 µl solution was applied on the plate. Plate was developed in the optimized chromatographic conditions and scanned as explained in previous section.

RESULTS AND DISCUSSION:

Linearity: A good linear relationship between response (peak area) and concentration was obtained **(Fig. 2)**. Form the calibration curve of troxipide, linear regression equation was computed and a correlation coefficient was obtained **(Table 1)**. The correlation coefficient was found to be 0.998, which shows excellent linearity between the concentration and response. From non numerical test; the plot of residuals was obtained which doesn't show any trend in it that implies results are satisfactory. This proves the linearity of calibration.

TABLE 1: SUMMERY OF VALIDATION

Parameter		TRP
Linearity Range (ng)		200-700
Correlation Coefficient		0.998
Slope		5.454
Intercept		350.6
LOD (ng)		20.39
LOQ (ng)		61.81
Precision % RSD	Intra day	0.5377
	Inter day	0.399424
Specificity	r, s, m	0.999906
	r, m, e	0.995012
Robustness % RSD		< 2

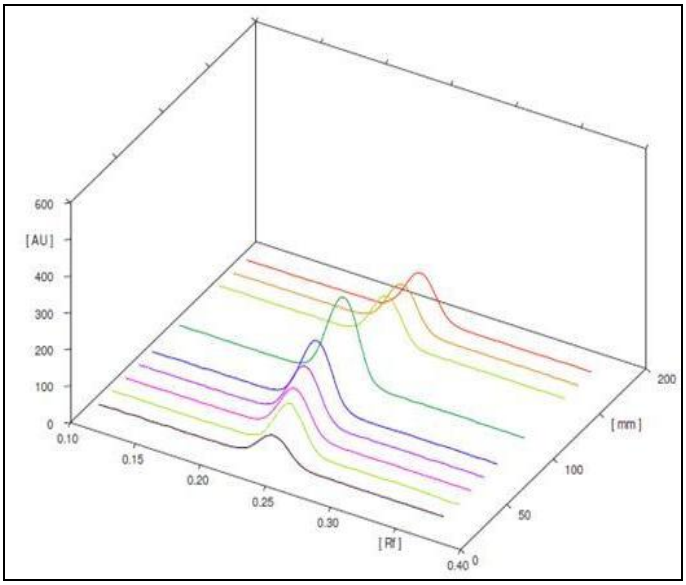

FIG. 2: CALIBRATION CURVE DENSITOGRAM

Method sensitivity (LOD and LOQ):
The LOD and LOQ were found to be 20.39 ng and 61.81 ng **(Table 1)**. The low values of LOD and LOQ reveals that the method is sensitive.

Assay:
The drug content obtained from standard and the sample was found to be comparable with no interference from excipients commonly present. The R_f value was found to be 0.35 and the assay value was found to be 100.1 % and % RSD value as 0.74. The result indicates that the method is suitable for routine analysis of TRP in pharmaceutical dosage form.

Accuracy:
The % recovery was found in the range of 99.25–101.001% and % RSD value was less than 2. The result shows that the method is accurate **(Table 2)**.

TABLE 2: RECOVERY AND PRECISION STUDY

Accuracy (n=6)			Precision (n=3)		
Recovery level	% recovery	%RSD	Concentration (ng/band)	Intra Day (% RSD)	Inter Day (% RSD)
80 %	101.001	0.704	300	0.7483	0.4727
100 %	99.254	0.547	400	0.5798	0.3363
120 %	100.05	0.038	500	0.2848	0.3891

Precision:

To check the repeatability, the response of sample solution was recorded six times. The intraday and inter day precision was performed as per the procedure given in previous section. For both repeatability and intermediate precision, the %RSD value was found to be always less than 2; which reveal that the method is precise **(Table 2)**.

Robustness:

By deliberately changing some of the parameter, robustness of the method was evaluated. The results after Statistical analysis show that the % RSD value was found to be always less than 2. Thus method was found to be robust (**Table 3**).

TABLE 3: RESULTS OF ROBUSTNESS STUDY

Parameter		% Assay,	% RSD
Volume of mobile phase (±1 mL)		99.83	0.7564
Time of saturation (±5 min)		100.38	0.5389
Composition of mobile phase Acetonitrile (±0.1 mL)		100.11	0.2252
Time from application to chromatography		99.95	0.2256
Time from chromatography to scanning		99.98	0.1323
Plate from different lot:	Lot HX004548	100.46	0.3239
	Lot HX004619	100.28	0.2285

Specificity:

The value of peak purity for TRP was found to be in the range of 0.999906 to 0.995012 **(Table 1)** which shows that the method is specific for the determination of drug **(Fig. 3)**.

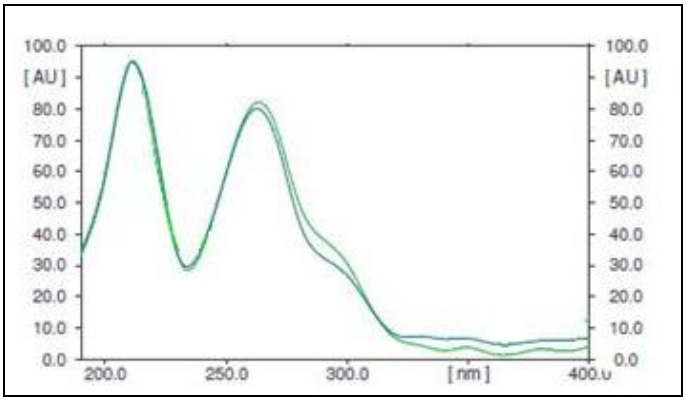

FIG. 3: OVERLAIN UV SPECTRA OF TRP FOR PEAK PURITY

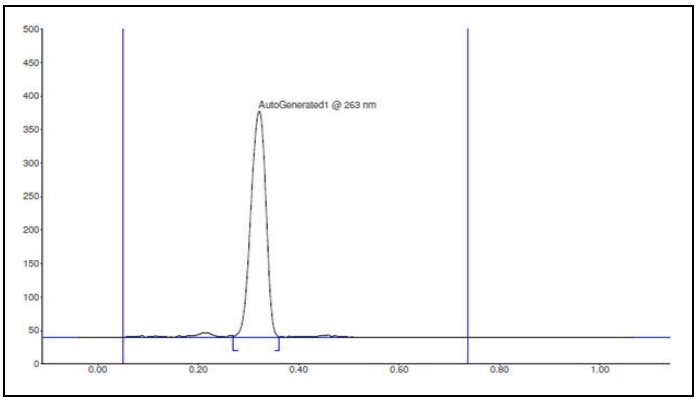

FIG. 4: DENSITOGRAM OF TRP (R_F: 0.35)

Stress degradation studies:

From the stress degradation studies it was found that TRP is susceptible to degradation in acidic and alkaline hydrolysis which gives TRP peak at R_f 0.35 and degradation product peak at R_f 0.48 and 0.58. But TRP was found to be relatively stable to Photolytic and oxidative degradation as it doesn't give any extra peak but there is decrease in area under curve. In case of thermal degradation it shows two distinct peaks at R_f 0.54 and 0.68 apart from the TRP peak at R_f 0.35. Here the peak purity values for all the degradation peaks was found to be greater than 0.99 ($r > 0.99$), which proves that the method is specific **(Table 4)**.

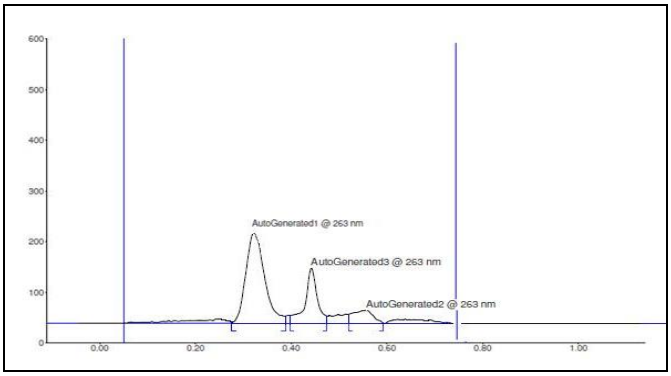

FIG. 5: DENSITOGRAM OF TRP AFTER ACIDIC HYDROLYSIS

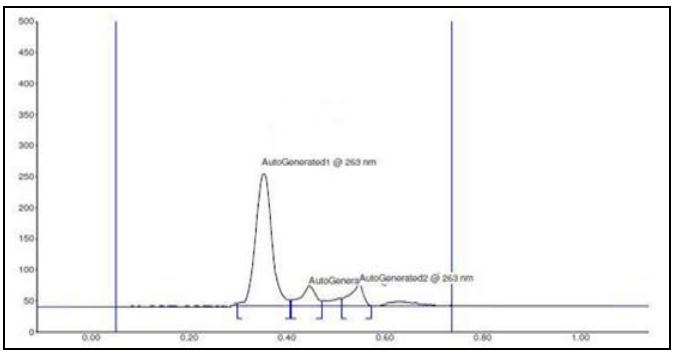

FIG. 6: DENSITOGRAM OF TRP AFTER ALKALINE HYDROLYSIS

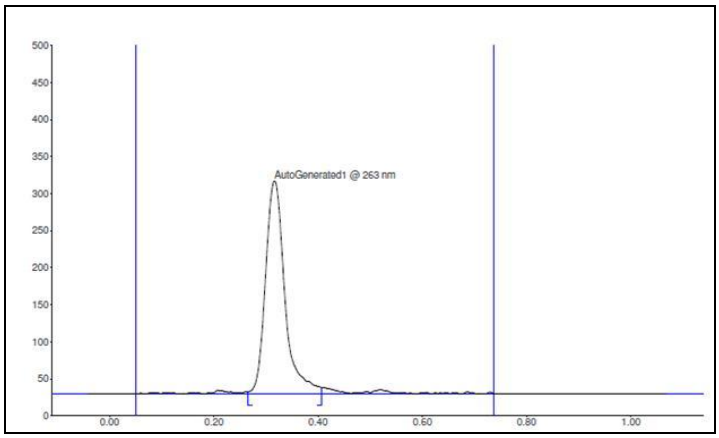

FIG. 7: DENSITOGRAM OF TRP AFTER OXIDATIVE DEGRADATION

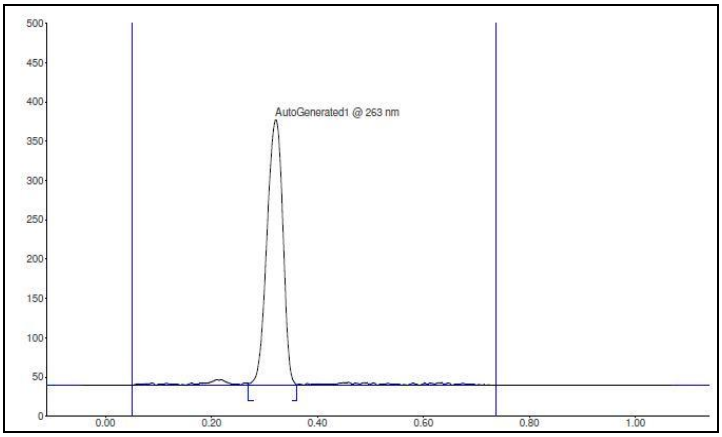

FIG. 8: DENSITOGRAM OF TRP AFTER PHOTOLYTIC DEGRADATION

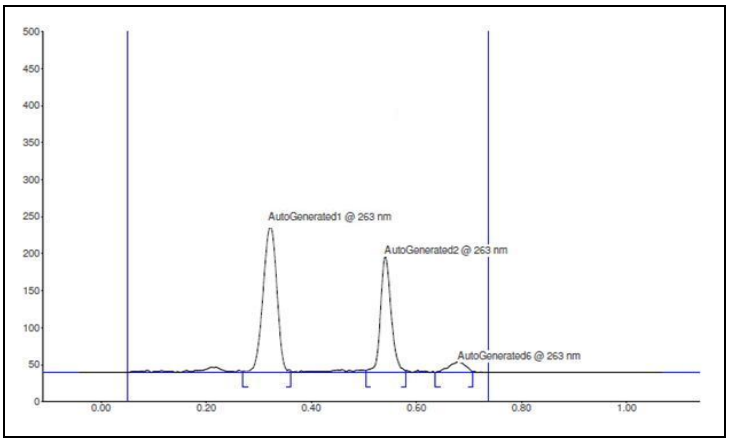

FIG. 9: DENSITOGRAM OF TRP AFTER THERMAL DEGRADATION

TABLE 4: SUMMARY OF STRESS DEGRADATION OF TRP

Sr. No.	Stress Degradation Parameter	R_f Value			% of Drug degraded
		TRP	Degradation product 1	Degradation product 2	
1.	Acidic Hydrolysis	0.35	0.48	0.58	13.36877
2.	Alkaline Hydrolysis	0.35	0.48	0.58	8.943522
3.	Oxidative Degradation	0.35	NA	NA	7.581395
4.	Photolytic Degradation	0.35	NA	NA	0.611296
5.	Thermal Degradation	0.35	0.54	0.68	43.93821

CONCLUSION: Proposed method describes stability indicating HPTLC method for the estimation of TRP from API as well as its formulation. The method was validated and found

to be simple, sensitive, accurate, precise and robust. Also the results of stress degradation study indicate the suitability of the method for acid, base, oxidative, photo and thermal degradation study. As the method separates the drug from its degradation products, it can be used for analysis of stability samples.

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