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A Stability Indicating RP-HPLC Method for the determination of Troxipide in Bulk and Commercial Formulation

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ABSTRACT

The present study was undertaken to develop a validated, rapid, simple and economic stability indicating reverse phase HPLC method for estimating Troxipide in bulk and commercial preparation. The Reversed phase chromatographic analysis was performed on a Purospher® STAR RP-18 (250 X 4.6 X 5) with Acetonitrile: 25mM Phosphate buffer, pH 5.0, (30:70) at a flow rate of 1ml/min and detection wavelength of 258 nm. The method was validated for accuracy, precision, reproducibility, specificity, robustness, limit of detection (LOD) and limit of quantification (LOQ), as per International Conference on Harmonization (ICH) Guidelines. Approximately 5-20% degradation of the drug substance has been considered as reasonable and acceptable for validation of chromatographic assays. Forced degradation studies were performed on a bulk sample of troxipide using 0.1N hydrochloric acid, 0.1N sodium hydroxide, 10 % hydrogen peroxide, heat (100 °C), HPLC grade water and sunlight. The drug was found susceptible to basic, oxidative, hydrolytic degradation and unaffected by acidic, photolytic and thermal degradation. The method was found to be specific for Troxipide in the presence of degradation products with an overall analytical run time of 5 min. A single sharp peak was obtained for Troxipide at Rt of 3.5 ± 0.1 min. The polynomial regression data for the calibration plots exhibited good linear relationship (r = 0.9998) over a concentration range of 100 – 700 µg/ml and the linear regression equation was y=9559x– 668164. Accuracy ranged from 99.98 to 100.81 and the %RSD was found to be less than 2 for both intra-day and inter-day precision.

Keywords: Troxipide, RP-HPLC, Stability Indicating Method, Validation, Forced degradation.

INTRODUCTION

Troxipide is a novel gastric mucosal protective agent with chemical formula 3,4,5- trimethoxy-N-3-piperidyl benzamide [1,2,5]. Antiulcer effect of troxipide is due to increase in gastric mucosal blood flow, mucus secretion, glycoprotein excretion in the gastric mucosa and inhibition of inflammatory responses and mucosal injury mediated by neutrophils. It increases gastric mucosal prostaglandin (PG) levels as evidenced in animal studies. This finding is of interest because endogenous PGE_2 plays a regulatory role in gastrointestinal function. Moreover PGE_2, PGI_2 and PGD promote gastric ulcer healing. Troxipide promotes ulcer repair by increasing collagen regeneration of the ulcer base and causes healing of peptic ulcer. Production of reactive oxygen radicals is believed to be one of pathogenic factors involved in neutrophil induced gastric mucosal injury. Troxipide can prevent the generation of oxygen free radicals, thereby protecting the gastric mucosa. Troxipide has no effect on gastric acid output and its action is independent of the gastric pH [3,4]. Troxipide is also found to have anti-inflammatory properties and inhibits chemotactic migration of human neutrophils to the inflammatory sites [6]. Literature survey reveals that method is available for analysis of Troxipide using HPLC but in human serum [7]. There is no reported stability indicating method using HPLC in bulk drug or formulation. As the formulation, TROXIP (100 mg), film coated tablets is

available in the market, there is a need for coming up with analytical method, which is simple, sensitive, rapid and accurate for estimation of Troxipide in pure form and in pharmaceutical preparation. Stability-Indicating Method (SIM) is a quantitative analytical procedure is used to detect a decrease in the amount of the active pharmaceutical ingredient (API) present due to degradation. SIM is defined as a validated analytical procedure that accurately and precisely measures active ingredients (drug substance or drug product) free from potential interferences like degradation products, process impurities, excipients, or other potential impurities. For developing SIM, a forced degradation study is normally carried out under more severe conditions than those used in accelerated studies. Approximately 5-20% degradation of the drug substance has been considered as reasonable and acceptable for validation of chromatographic assays [9,10,11].

MATERIALS AND METHODS

Instrument, Reagents and Chemicals

A Waters HPLC containing Waters 515 HPLC pump, Waters 2998 Photo Diode Array Detector was used for the analysis. The method was carried out on a Purospher® STAR RP-18 (250 X 4.6 X 5) column as stationary phase and Acetonitrile: 25mM Phosphate buffer, pH 5.0, (30:70) as mobile phase at a flow rate of 1.0 ml/min. An automatic injector was used for the injection 10 µl of samples. Chemicals like Acetonitrile, Potassium dihydrogen orthophosphate, Triethylamine (TEA) of HPLC Grade, E.Merck and Hydrochloric Acid, Orthophosphoric Acid, Sodium hydroxide, Hydrogen Peroxide of LR grade, E.Merck were used. Gift sample of Troxipide was procured from Emcure Pharmaceuticals Limited, Pune, India. Tablets of 100 mg strength of Troxipide were procured from local pharmacy of brand (Troxip).

HPLC METHOD [14,15]

Preparation of mobile phase:

25mM phosphate buffer was prepared by dissolving 850.562 mg of potassium dihydrogen ortho-phosphate (KH_2PO_4) in 250ml water and adjusting the pH to 5.0 with triethylamine, added drop wise. Buffer solution was then finally filtered by passing through 0.45 µm membrane filter. Mobile phase was prepared by mixing HPLC grade acetonitrile and 25mM phosphate buffer in the ratio 30:70.

Preparation of stock solution of Troxipide:

An accurately weighed quantity of Troxipide (50 mg) was transferred to 50 ml volumetric flask, dissolved and diluted up to the mark with HPLC grade water (concentration: 1000 µg/ml).

Preparation of the Calibration Curve:

Subsequent dilutions of standard stock solution were prepared in HPLC grade water to give a concentration range of 100-700 µg/ml i.e. 100, 200, 300, 400, 500, 600 and 700 µg/ml. The HPLC was operated and equilibrated with the mobile phase containing acetonitrile and phosphate buffer (30:70). The prepared dilutions were filled in vials and placed in auto sampler carousel. The method was set in Empower software and samples were run. The peak area report thus obtained was recorded. A plot of concentration vs. peak area was prepared.

ANALYTICAL METHOD VALIDATION [8]

The method was validated according to ICH guidelines Q2 (R1).

Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

A standard curve was prepared over a concentration range of 100 – 700 µg/ml from a stock solution of Troxipide (1000 µg/ml). Dilutions were prepared in HPLC grade water. The data from peak area versus drug concentration plots were treated by linear least square regression analysis.

Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. The three components of precision, i.e., repeatability, intermediate precision and reproducibility, in accordance with ICH recommendations, were determined as follows:

Repeatability

Intra-day variation: Measurement of intra-day variation of Troxipide solutions of three different concentrations (200, 400 and 600 µg/ml) was carried out by injecting the samples on the same day at different time intervals at least four

times. The standard deviation, % relative standard deviation, and (±) confidence interval of the estimated concentrations based on standard curve were reported for each set of data.

Intermediate precision (Inter-day variation)
Measurement of inter-day variation of Troxipide solutions of three different concentrations (200, 400 and 600 µg/ml) for four times on three consecutive days determined the intermediate precision. The standard deviation, % relative standard deviation and (±) confidence interval of the estimated concentrations based on standard curve were reported for each set of data.

Accuracy
Accuracy is the measure of how close the experimental value is to the true value. Recovery studies by the standard addition method were performed with a view to justify the accuracy of the proposed method.

Previously analyzed samples of Troxipide (400 µg/ml) were spiked with 40, 80 and 120µg/ml Troxipide standard and the mixtures were analyzed by the proposed method. The experiment was performed for four times. Recovery (%), RSD (%) and (±) confidence interval were calculated for each concentration.

LOD and LOQ
Calculated according to the formulae given in the ICH guidelines.

LOD= 3.3 σ/S
LOQ = 10 σ / S

Where,
σ = the standard deviation of the response
S = the slope of the calibration curve

Robustness
The robustness of the method was determined to assess the effect of small but deliberate variation of the chromatographic conditions on the determination of Troxipide.

Effects of variation in pH of the mobile phase and λ_{max} were studied. Standard deviation, % recovery, (±) confidence interval as well as % relative error were reported. Three different concentrations were 200, 400 and 600 µg/ml were prepared and the measurement was done in four replicates.

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.

Three different concentrations of Troxipide (200, 400, 700 µg/ml) were prepared in HPLC water. Lactose, talc, starch, magnesium stearate and microcrystalline cellulose, hydroxyl propyl methyl cellulose were added as excipients and analysed by the proposed method. The mean area under curve and chromatogram of the mixtures were compared with that of pure Troxipide solution of the same strength. T-Test (Paired Two Sample for Means) was applied to compare the results.

Specificity of the developed method was also determined in presence of degradation products of pure drug: [12,13]

i. **Acid Degradation:** 10 ml of standard stock solution (1000 µg/ml) was mixed with 10 ml of 0.1N HCl solution and the resulting mixture was refluxed at 80ºC for 7 days. Sample was withdrawn after every 5 hrs, diluted with HPLC grade water to get final concentration of 400 µg/ml and then analyzed for degradation.

ii. **Basic Degradation:** 10 ml of standard stock solution (1000 µg/ml) was mixed with 10 ml of 0.1N NaOH solution and the resulting mixture was refluxed at 80ºC for 7 days. Sample was withdrawn after every 5 hrs, diluted with HPLC grade water to get final concentration of 400 µg/ml and then analyzed for degradation.

iii. **Oxidative Degradation:** 10 ml of standard stock solution (1000 µg/ml) was mixed with 10 ml of 10% H_2O_2 solution. Sample was withdrawn after 5 hrs, diluted with HPLC grade water to get final concentration of 400µg/ml and then analyzed for degradation. There was no need of doing reflux.

iv. **Hydrolytic Degradation:** 10 ml of standard stock solution (1000 µg/ml) was mixed with 10 ml of HPLC grade water and the resulting mixture was refluxed at 80ºC. Sample was withdrawn after every 5 hrs, diluted with HPLC grade water to get final concentration of 400 µg/ml and then analyzed for degradation.

v. **Photolytic Degradation:** 25 ml of standard stock solution (1000 µg/ml) was exposed to direct sunlight. Sample was withdrawn after every 5 hrs, diluted with HPLC grade water to get final concentration of 400 µg/ml and then analyzed.

vi. **Dry Heat Degradation:** 1 gm of pure drug was placed in a hot air oven at 100ºC on a petridish. 50 mg sample was withdrawn after every 5 hrs, dissolved in 50ml HPLC grade water to make standard stock solution (1000 µg/ml) which was further diluted with HPLC grade water to give final concentration of 400 µg/ml and then analyzed for degradation.

System Suitability Tests

System suitability parameters like tailing factor, number of theoretical plates, resolution etc were calculated and compared with standard value.

Analysis of Troxipide in marketed tablet

Twenty tablets (strength: 100 mg/tablet) were crushed and triturated well in a mortar. A powder sample, equivalent to 50 mg of Troxipide, was accurately weighed and transferred to a 50 ml volumetric flask, dissolved in 10ml water and shaken for 15-20 minutes. The volume was made up to the mark with water to prepare a stock solution of 1000µg/ml. The solution was then filtered through 0.22 µ nylon syringe filter.

For HPLC, the area under chromatogram was read and amount of drug was estimated by comparison with working standard using formulae

$$\% \text{ of Labeled Claim} = \frac{C_u \times \text{Avg. Wt.}}{\text{Wt. Taken} \times \text{Label Claim}} \times 100$$

$$C_u = \frac{A_u}{A_s} \times C_s$$

C_u = Wt. of Unknown Sample
C_s = Wt. of Standard
A_u = Area under Curve of Unknown Sample
A_s = Area under Curve of Standard

RESULTS

Method Development

Acetonitrile: 25mM Phosphate buffer, pH 5.0, (30:70 %v/v) was selected as the optimum mobile phase. Under these conditions the retention time and tailing factor were 3.5 ± 0.1 min and 1.435062e+000 respectively. A typical chromatogram is represented in Fig. II and Optimum Experimental Conditions for SIM by RP- HPLC is shown in Table I.

Table I: Optimum Experimental Conditions for SIM by RP- HPLC

Mobile phase	Acetonitrile : Phosphate buffer (30:70) final pH adjusted to 5.0
Column	Purospher® STAR RP-18 (250 X 4.6 X 5)
Wavelength	258 nm
Flow rate	1.0 ml/min
Temperature	25 °C
Injection volume	10 µl
t_R optimized	3.5 ± 0.1

Method Validation

Linearity and Range

Peak area versus drug concentration was plotted to construct a standard curve for Troxipide. The polynomial regression for the calibration plots showed good linear relationship with coefficient of correlation, r = 0.9998; slope = 9559 and intercept = 668164 (n = 3) over the concentration range studied. The range was set at 100 – 700 µg/ml.

The linear regression data for the calibration plot is indicative of a good linear relationship between peak area and concentration over a wide range. The correlation coefficient was indicative of high significance. A calibration curve is represented in Fig. I and The Statistical Data of Calibration Curve are listed in Table II.

Fig. I: Calibration Curve of Troxipide on HPLC

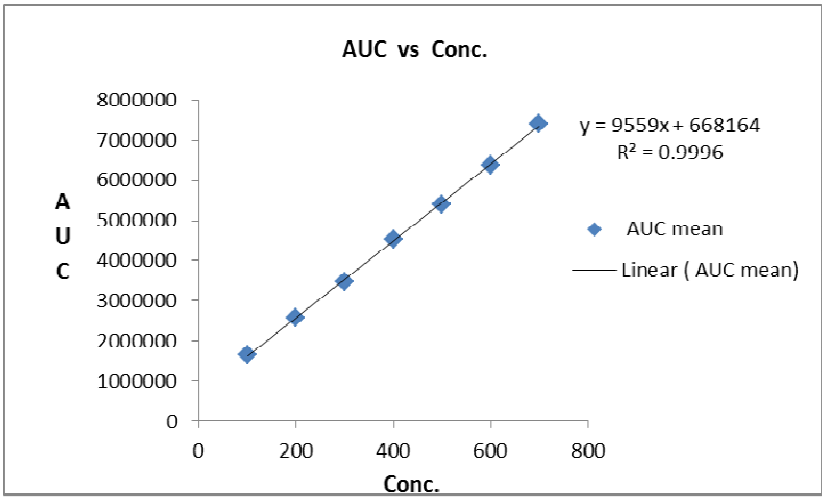


Table II: Statistical Data of Calibration Curve on HPLC

Regression equation	AUC = 9559 conc.– 668164
Regression coefficient (R^2)	0.9996
Correlation coefficient (R)	0.9998
Range	100 – 700 µg/ml
Slope	9559
Intercept	668164

Precision

Precision was measured in accordance with ICH recommendations. Repeatability of sample injection was determined as intra-day variation while intermediate precision was determined by measuring inter-day variation for four times of Troxipide of three different concentrations on three consecutive days. The results of the determination of repeatability and intermediate precision are listed in Table III and IV. The low RSD values indicate the repeatability and intermediate precision of the method.

Table III: Repeatability (Intraday Precision) on HPLC (n=4)

Amount (µg/ml)	Found Mean Conc. (µg/ml)	SD	% RSD	CI (±)
200	200.03	1.83	0.92	1.79
400	400.38	5.31	1.33	5.21
600	599.19	3.79	0.63	3.72

Table IV: Intermediate Precision (Interday Precision) on HPLC (n=12)

Amount (µg/ml)	Days	Found Mean Conc. (µg/ml)	SD	% RSD	CI (±)
200	1	202.95	2.38	1.17	2.33
	2	200.61	3.59	1.79	3.52
	3	200.73	2.59	1.30	2.55
400	1	400.45	3.84	0.96	3.76
	2	401.70	2.44	0.61	2.39
	3	402.39	7.27	1.81	7.12
600	1	601.35	4.29	0.71	4.20
	2	598.59	6.10	1.01	5.98
	3	598.26	5.73	0.96	5.62

Accuracy

The recovery of the method, determined by spiking a previously analyzed test solution with additional drug standard solution, was found to be in the range of 99.98 to 100.81%. The values of recovery (%), REE (%) and **(±)** CI listed in Table V indicate the method is accurate.

Table V: Accuracy on HPLC (n=4)

Amount of formulation (µg/ml)	Amount of Bulk Drug (µg/ml)	Total amount (µg/ml)	Found Mean Conc. (µg/ml)	SD	% R	CI (±)	% REE (±)
400	40	440	443.56	2.27	100.81	2.23	0.81
400	80	480	479.94	5.69	99.98	5.57	0.01
400	120	520	521.38	3.32	100.26	3.25	0.26

Detection and Quantification Limits

The limit of detection was found to be 15.33 µg/ml where the drug could be detected. The limit of quantification was 46.46 µg/ml.

Robustness

There was no significant change in the retention time of Troxipide when there is variation in pH of the mobile phase and λ_{max}. The % RSD ranged from 0.717 to 1.170 % for pH 5.0 + 0.1, 0.978-2.098 for pH 5.0 - 0.1, 0.432-0.924 for λ_{max} 258+1 and 0.271-1.416 for λ_{max} 258-1 . The low values of the RSD indicated the robustness of the method. The results of robustness on HPLC by changing pH and λ_{max} are shown in table VI and VII respectively.

Table VI: Robustness on HPLC by Changing the pH (n=4)

pH	Amt. (µg/ml)	Found Mean Conc. (µg/ml)	SD	% RSD	CI (±)	% REE (±)
5.0 + 0.1	200	201.02	1.44	0.71	1.41	0.51
	400	399.51	4.46	1.11	4.37	0.12
	600	593.89	6.95	1.17	6.81	1.02
5.0 - 0.1	200	202.34	4.25	2.09	4.16	1.17
	400	403.19	3.95	0.97	3.87	0.79
	600	599.33	6.15	1.02	6.02	0.11

Table VII: Robustness on HPLC by Changing the λ_{max} (n=4)

pH	Amt. (µg/ml)	Found Mean Conc. (µg/ml)	SD	% RSD	CI (±)	% REE (±)
258 + 1	200	202.08	1.86	0.92	1.82	1.04
	400	398.86	2.61	0.65	2.56	0.28
	600	602.37	2.60	0.43	2.55	0.39
258 - 1	200	199.29	2.82	1.41	2.76	0.35
	400	396.23	1.93	0.48	1.89	0.94
	600	600.70	1.63	0.27	1.59	0.11

Specificity

The specificity of the method was determined by mixing sample solutions of Troxipide with excipients like lactose, talc, starch, magnesium stearate and microcrystalline cellulose, hydroxyl propyl methyl cellulose and analysed by the proposed method. The mean area under curve and chromatogram of the mixtures were compared with that of pure Troxipide solution of the same strength. T-Test (Paired Two Sample for Means) was applied to compare the results. The results of Specificity using Excipients are shown in Table VIII.

The Standard stock solution (1000 µg/ml) was exposed with stress conditions like 0.1N HCl solution, 0.1N NaOH solution, H_2O, light, heat (100 °C) for 7 days and 10% H_2O_2 for 5 hours and the resulting mixture was refluxed at 80ºC for 7 days. Sample was withdrawn after every 5 hrs, diluted with HPLC grade water to get final concentration of 400 µg/ml and then analyzed for degradation. There was no Significant photolytic and thermal degradation of Troxipide as there is no significant change in peak area and retention time of Troxipide was observed. However, drug was found susceptible to basic, oxidative, hydrolytic degradation and there was a substantial change in the peak area of Troxipide but not in the retention time and peaks of degradation products were obtained. Chromatograms obtained from Troxipide after treatment with 0.1 N NaOH, H_2O and 10% H_2O_2 are shown in Fig. III, Fig. IV and

Fig. V respectively. The results from stress testing, including separation of the degradation product and quantification of Troxipide after exposure to stress conditions, show that the method is stability indicating and shown in Table IX.

Table VIII: Specificity using Excipients on HPLC (n=4)

Amt. (µg/ ml)		Found Mean Conc. (µg/ml)	SD	% RSD	CI (±)	% REE (±)
200	U.S	201.52	4.04	2.00	3.96	0.76
	S*	201.06	3.99	1.98	3.91	0.53
400	U.S	402.67	3.99	0.99	3.91	0.66
	S*	400.98	6.91	1.72	6.77	0.24
600	U.S	597.91	4.95	0.82	4.85	0.34
	S*	595.09	7.82	1.31	7.67	0.81

U.S- Unspiked; S- Spiked*

Amount (µg/ml)		Observed t-value
200	Unspiked	0.1630
	Spiked	
400	Unspiked	0.4246
	Spiked	
600	Unspiked	0.6093
	Spiked	
For degree of freedom= 3 t-critical value is 3.182		

Table IX: Forced Degradation Studies using HPLC

Stress Condition	Duration of Deg. Study	t_R of Pure drug	t_R of Degraded Products	% Deg.	R_S
Standard Drug (400 µg/ml)	—	3.5 (±) 0.1	—	—	—
Acidic (0.1N HCL)	7 days	3.5 (±) 0.1	2.7 (±) 0.1 3.5 (±) 0.1	~3%	— 1.5
Basic (0.1N NaOH)	7 days	3.5 (±) 0.1	1.7 (±) 0.0 2.4(±) 0.0 2.7 (±) 0.1 3.2 (±) 0.1 3.5 (±) 0.1	~50%	— 2.7 2.1 1.6 1.9
Oxidative* (3% H_2O_2)	5 hours	3.5 (±) 0.1	2.7 (±) 0.1 3.5 (±) 0.1	~65%	— 5.6
Hydrolytic	7 days	3.5 (±) 0.1	2.3 (±) 0.1 2.7 (±) 0.1 3.2 (±) 0.1 3.5 (±) 0.1	~25%	— 1.7 1.6 1.8
Photolytic	7 days	3.5 (±) 0.1	—	No Deg.	—
Thermal (100 °C)	7 days	3.5 (±) 0.1	3.2(±) 0.1 3.5 (±) 0.1	~2%	— 1.5

*[*degradation occurs at room temp. without reflux]*

Deg.- Degradation

Fig. II: HPLC Chromatogram of Troxipide (400µg)

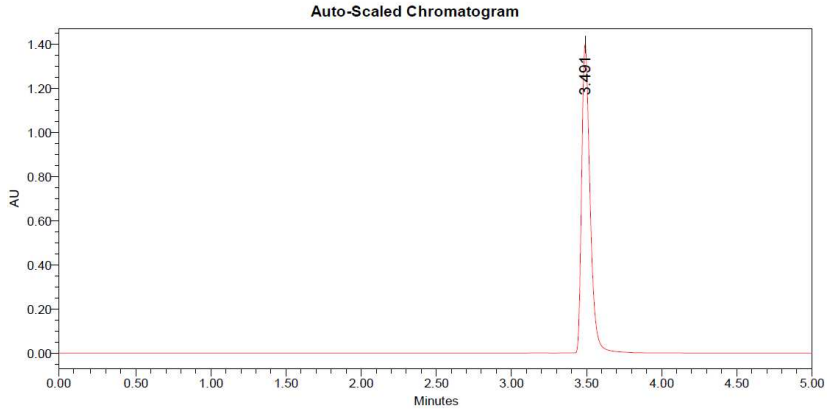


Fig. III: HPLC Chromatogram of Pure Drug with 0.1N NaOH (400µg)

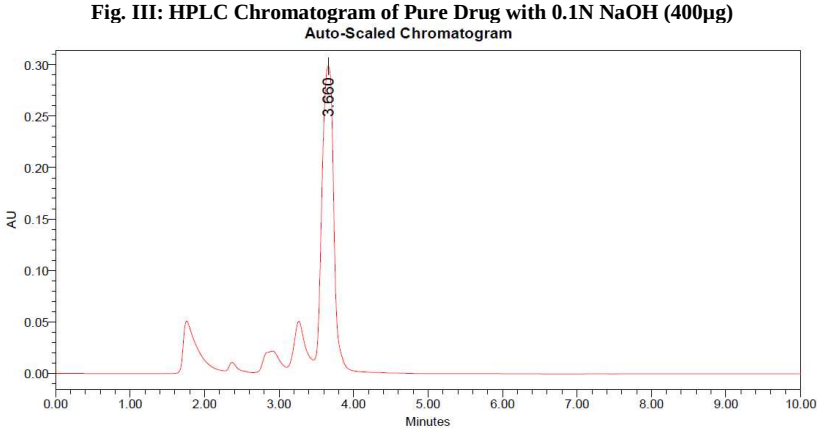


Fig. IV: HPLC Chromatogram of Pure Drug with H_2O (400µg/ml)

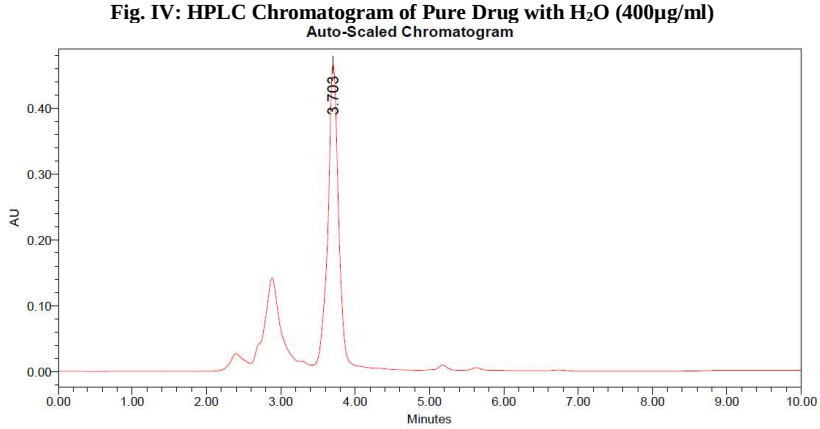
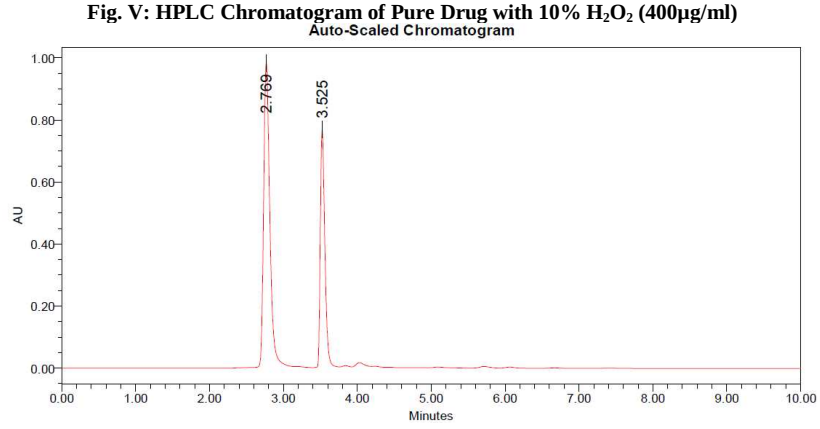


Fig. V: HPLC Chromatogram of Pure Drug with 10% H_2O_2 (400µg/ml)



System Suitability Tests

The results of the system suitability tests assure the adequacy of the proposed HPLC method for routine analysis of Troxipide. The Tailing factors, Plate count, Retention time for Troxipide peak were found to be 1.435062e+000, 1.935812e+004, 3.491 respectively, calculated by Empower 2 software. The resolution (Rs) for the principle peak and its oxidative degradation, basic, hydrolytic and acidic product were found to be 5.6, 2.7, 1.7 and 1.5 respectively.

Analysis of Troxipide from Marketed Tablet

A peak was observed at the retention time 3.550 of Troxipide when a suitably diluted solution of the tablet formulation was chromatographed. The Troxipide content was found to be 99.06% and the RSD was 0.44%. The low RSD indicated the suitability of this method for routine analysis of Troxipide in pharmaceutical dosage forms.

Table X: Formulation Assay using HPLC

Formu-lation	Amt (µg/ ml)	Found Mean Conc. (µg/ml)	SD	% R	% RSD	CI (±)	% REE (±)
TROXIP	400	396.23	1.76	99.06	0.44	1.72	0.94

Formulation	Amount Labelled	Amount Found
TROXIP	100 mg	99.63 mg

DISCUSSION

The final decision on mobile phase composition and flow rate was made on the basis of peak shape, peak area, tailing factor, baseline drift and time required for analysis. The solvent system selected [Acetonitrile: 25mM Phosphate buffer, pH 5.0, (30:70 %v/v)] gave good resolution of degraded product and drug peak. No internal standard was used because no extraction or separation step was involved. Methanol-water did not furnish a sharp, well defined peak and there is also high back pressure. Other mobile phases tried resulted either in much lower sensitivity, delayed retention time or poor peak shapes, and so were not considered. The proposed HPLC method of analysis was also found to be precise and accurate, as depicted by the statistical data of analysis. High values of correlation coefficients and small values of intercepts validated the linearity of the calibration plots and obedience to Beer's laws. The method was also found to be robust as there was no significant change in the peak area, peak shape and retention time of Troxipide. This method is also highly sensitive and could effectively separate the drug from its degraded product.

Forced degradation studies were performed on a bulk sample of troxipide using 0.1N hydrochloric acid, 0.1N sodium hydroxide, 10 % hydrogen peroxide, heat (100 °C), HPLC grade water and sunlight. The drug was found susceptible to basic, oxidative, hydrolytic degradation and unaffected by acidic, photolytic and thermal degradation. The method was also found to be highly sensitive and could effectively separate the drug from its degraded product. As the reported method could effectively separate the drug from its degraded product, it can be employed as a stability indicating one.

CONCLUSION

The HPLC method developed is accurate, precise, reproducible, specific, and stability indicating. The method is linear over a wide range, economical and utilizes a mobile phase which can be easily prepared. All these factors make this method suitable for quantification of Troxipide in bulk drug and in pharmaceutical dosage form. It can therefore be concluded that use of the method can save much time and money and it can be used even in small laboratories with very high accuracy and precision. The method can also be used for the routine analysis of Troxipide in bulk preparation of the drug and in pharmaceutical dosage form without interference.

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