International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.9, pp 189-199, 2016

Stability indicating thin-layer chromatographic determination of framycetin sulphate as bulk drug: Application to forced degradation study

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Abstract : The objective of present work was to develop a validated stability indicating high performance thin layer chromatographic method (HPTLC) for estimation of framycetin sulphate. The stationary phase used was precoated silica gel aluminium plates 60 F-254 with 250 µm thickness. The mobile phase used for separation was acetonitrile: methanol: water (7.5: 0.5: 2: v/v/v) gave a resolved peak at (R_f value of 0.46 ± 0.02). Framycetin sulphate was subjected to hydrolytic, oxidative, dry heat and photo treatment degradation. The drug was found to degrade in hydrolytic, oxidative, dry heat conditions and there was no degradation in photolytic conditions. All the peaks of degraded products were separated from the standard drug with significantly different R_f values of its degradants. The drug showed two degradant peaks in acidic medium at R_f value of0.02± 0.02, 0.38± 0.02. In basic medium two degradant peaks at R_f value of 0.23± 0.02, 0.36±0.02. Oxidative hydrolysis showed three degradants which were resolved at R_f value of 0.25± 0.02, 0.60± 0.02, 0.79± 0.02. Dry heat degradation was observed with two resolved peaks at R_f value of 0.09± 0.02, 0.60± 0.02. There was no degradation observed in photochemical degradation. The developed method can effectively separate the drug from its degradation products under accelerated degradation studies; it can be routinely employed as stability indicating method for framycetin sulphate.
Key words: Framycetin sulphate, Thin layer chromatography, Stability indicating method.

Introduction

Framycetin sulphate (fig.1) is chemically known as (2S,3S,4R,5R,6R)-5-amino-2-(aminomethyl)-6-{[(2R,3S,4R,5S)-5-{[(1R,2R,3S,5R,6S)-3,5-diamino-2-{[(2R,3R,4R,5S,6R)-3-amino-6 (aminomethyl)-4,5-dihydroxyoxan-2-yl]oxy}-6-hydroxycyclohexyl]oxy}-4-hydroxy-2-(hydroxymethyl)oxolan-3-yl]oxy}oxane-3,4-dio. Its chemical formula is $C_{23}H_{46}N_6O_{13}$ with a molecular weight 614.6437 g mol^{-1}. It is a white amorphous solid powder, soluble in water and insoluble in common organic solvent. Framycetin sulphate is used as an antibiotic antibacterial drug. Framycetin binds to specific 30S-subunit proteins and 16S rRNA, four nucleotides of 16S rRNA and a single amino acid of protein S12[1-3].

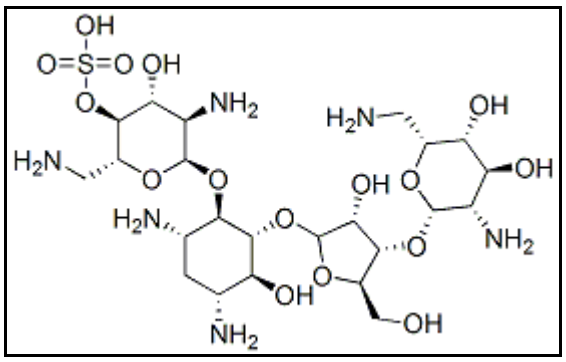

Fig. 1: Chemical structure of framycetin sulphate

Literature survey revealed various HPLC methods for determination of framycetin sulphate in bulk and formulation, analysis of neomycin sulphate and framycetin sulfate by high-performance liquid chromatography using evaporative light scattering detection, derivatization of primary amines by 2-naphthalenesulfonyl chloride for high-performance liquid chromatographic assay of neomycin sulphate, determination of neomycin sulfate and impurities using high-performance anion-exchange chromatography with integrated pulsed amperometric detection, development of a liquid chromatographic method for ear drops containing neomycin sulphate, polymycin B sulphate and dexamethasone sodium phosphate. However to the best of our knowledge, stability indicating HPTLC method has not yet been reported for framycetin sulphate[4-7.]

The International Conference on Harmonization (ICH) guidelines entitled stability testing of new drug substances and product requires the stress testing of the drug substance should be carried out to elucidate the inherent stability characteristics of the active substance. An ideal stability indicating method is one that quantifies the drug per se and also resolves its degradation product. HPTLC has become a part of routine analytical techniques in many product development and analytical laboratories due to its advantages. The major advantage of HPTLC is that several samples can be simultaneously using a small quantity of mobile phase unlike HPLC thus lowering the analysis time and cost per analysis with high sample throughput. The method of detection does not place any restriction on the choice of the mobile phase and unlike HPLC mobile phases having pH 8 and above can be employed [8-17].

The aim of the present work was to develop an accurate, specific, reproducible stability indicating TLC method for estimation of framycetin sulphate as bulk drug in presence of its degradation products.

Materials and Methods

Chemicals and reagents

Gift sample of pure drug framycetin sulphate was procured from Encube ethical Pvt Ltd., Ponda, Goa, India. All solvents and reagents used for the analysis were of (A R grade) and were purchased from Merck, Mumbai, India.

HPTLC Instrumentation

A Camag HPTLC system equipped with Linomat V applicator (Switzerland), TLC Scanner III and integrated software Win-Cats (V 3.15, Camag) was used for the analysis. The standard and the sample solutions were spotted in the form of bands of width 6 mm with a Camag 100 µl sample (Hamilton, Bonaduz, Switzerland) syringe, on silica gel pre-coated aluminum plate 60F-254 plates (20×10 cm, 250 µm thickness, E. Merck, Darmstadt, Germany) supplied by Anchrom technologist, Mumbai. The plates were prewashed with methanol and activated at 110°C for 5 min prior to chromatography. The slit dimension was kept at 5 mm × 0.45 mm with data resolution of 100 µ/m step and the scanning speed was 20 mm/s. The monochromatic band width was set at 258 nm, each track was scanned three times and baseline correction was used. Linear ascending development was carried out in a (20 cm × 10 cm) twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was (30 min) at room temperature (28°C ± 2) at relative humidity of 60% ± 5. Each chromatogram was developed over

a distance of 80 mm. Following the development, the TLC plates were dried in a stream of air with the help of hair dryer in a wooden chamber with adequate ventilation. Densitometric scanning was performed at 258 nm. The source of radiation used was deuterium lamp emitting a continuous UV spectrum between 200 and 400 nm. Evaluation was performed by linear regression of peak areas determined by UV absorption as a function of sample analysis.

Preparation of standard stock and working standard solutions

For preparation of standard stock and working standard solutions framycetin sulphate (10 mg) was weighed accurately and transferred into a 10 ml volumetric flask and dissolved in water. Then mixture was sonicated for 20 min. Volume was made up to the mark with water to give the concentration of 100 ng/spot.

Prewashing of plates

Densitometric estimation was carried out on (20 cm × 10 cm) pre-coated silica gel 60 F-254 plates from E. Merck. The plates were pre-washed with methanol, dried and activated for 30 min at 110°C.

Selection of solvent & stationary phase

Water was selected as a solvent for preparing drug solutions. Identification and separation of framycetin sulphate was carried out on (20 cm × 10 cm), pre-coated silica gel aluminium plates 60 F-254 (250 µm thickness E. Merck, Darmstadt, Germany).

Sample application

The standard and working standard solution of framycetin sulphate were spotted on pre-coated TLC plates in the form of narrow bands of length 10 mm at 10 mm from the bottom and left margin and 10 mm distance between two bands. Samples were applied under continuous drying stream of nitrogen gas at constant application rate of 150nl/s.

Selection of wavelength

Evaluation was performed by linear regression of peak areas determined by UV absorption as a function of sample analysis at 258 nm using water as a blank solution. The detection wavelength was selected at 258 nm and the spectrum of the drug is depicted in (fig.2).

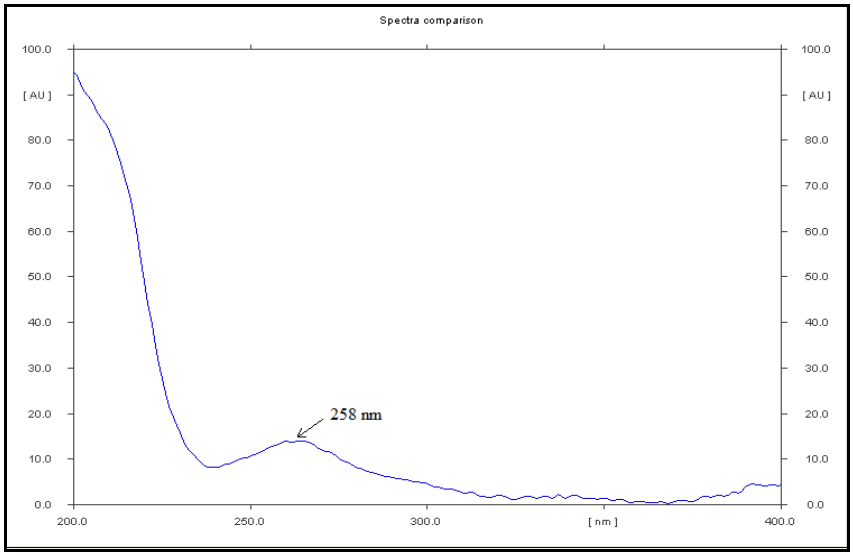

Fig. 2: Spectrum for selection of wavelength (258 nm).

Optimization of the mobile phase

Various solvent systems like mixture of (a) water: methanol (5: 5; v/v) (b) water: methanol (7: 3; v/v) (c) water: methanol: acetic acid (5: 5:5 v/v/v) and (d) toluene: methanol (7: 3; v/v) were tried to separate and resolve spot of framycetin sulphate from its impurities and other excipients of formulation. The mixture of water: methanol: acetonitrile (2:2:6; v/v) resolved framycetin sulphate but there was tailing in the peaks. To improve peak symmetry, the ratio was changed and spots were observed. Finally, the mixture of acetonitrile: water: methanol (7.5: 0.5: 2; v/v/v) showed well resolved peak with better peak shape. The drug was resolved with R_f value of 0.46±0.02. Pre-saturation of TLC chamber with mobile phase for 35 min assured better reproducibility in migration of framycetin sulphate and better resolution

Method validation

The developed HPTLC method was validated as per the ICH guidelines Q2 (R1) for linearity, precision, repeatability, accuracy, specificity, robustness, limit of detection (LOD), limit of quantification (LOQ).

Linearity (Calibration curve)

A stock solution of framycetin sulphate 10 mg was prepared by dissolving it in water. 10 different concentrations of framycetin sulphate (100, 300, 500, 700, 900, 1100, 1300, 1500, 1700 and 1900 ng/spot) were applied on the TLC plate. The data obtained were treated by least-square regression analysis method.

The linearity range of framycetin sulphate was obtained by plotting the peak area of framycetin sulphate against its varied concentrations over a range (100–1900 ng/spot).

Precision

The intra and inter-day variations were determined using three different concentration levels 100, 300 and 500 ng/spot of framycetin sulphate (n = 3). The precision of the developed method was evaluated by performing repeatability of the sample application and peak area measurement in six replicates of the same spot. The results are expressed in terms of percent relative standard deviation (% RSD) and standard error (SE).

Repeatability

It is also termed as intra-assay precision. Repeatability of sample application was assessed by spotting 500 ng/spot of standard drug solution six times on TLC plate at different times on same day by sample applicator, followed by development of plate and recording of the peak areas for six spots.

Recovery and Specificity studies

Recovery studies were carried out to determine accuracy of the developed method at 80%, 100% and 120% levels. It was done by mixing known quantity of standard drug 300 ng/spot with the sample formulation and contents were analysed by the proposed method. The % recovery and % RSD were calculated respectively.

The specificity of the method was ascertained by analysing the R_f values and spectra pattern of reference marker and drug samples. The marketed formulation, Safrodex100 mg (Sanofi Aventes Co. Ltd) was sonicated (10 mg in 5 ml water) for 20 min. The volume was made up to 10 ml by adding water. The resulting solution was centrifuged and the supernatant was filtered. The amount of framycetin sulphate was determined by developing the chromatogram (300 ng/ spot) in triplicate by maintaining the chromatographic conditions. The spot for framycetin sulphate in formulation was confirmed by comparing the R_f and densitogram of the spot with that of standard.

Robustness

In this study, small changes in the composition, volume of mobile phase and development chamber saturation time were made and their effects on the results were examined. The study was done in triplicate at a concentration 300 ng/spot of framycetin sulphate. The results of peak area are expressed as % RSD and SE.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were experimentally determined by visual detection method recommended in ICH guidelines. To estimate the LOD and LOQ blank methanol was spotted six times. Spotting for LOD was done by taking different concentrations as 100, 300, 500, 700 and 900 ng/ spot and the values were considered with a signal-to-noise ratio of 3:1 and 10:1 respectively.

Accelerated degradation of framycetin sulphate

Accelerated degradation was carried by exposing the drug to different stressed conditions. A drug stock solution of framycetin sulphate (10 mg) was prepared in 10 ml water. This drug solution was used for forced degradation to provide an indication of the stability indicating property and specificity of the proposed method.

Acid and base induced degradation

To 5 ml solution of framycetin sulphate 5 ml 0.1 N HCl and 0.1 N NaOH were added separately. These mixtures were refluxed separately for 2 h and 6 h at 80°C respectively. One microliter 500 ng/ spot of resultant solutions were applied on TLC plate and developed.

Hydrogen peroxide induced degradation

To 5 ml solution of framycetin sulphate, 5 ml (H_2O_2) (3% v/v) was added. This solution was heated in boiling water bath for 20 min to remove completely the excess of hydrogen peroxide and refluxed for 4 h at 80°C.One micro litre 500 ng/spot of resultant solutions were applied on TLC plate and developed.

Dry heat induced degradation

Dry heat degradation of framycetin sulphate was carried out by placing the bulk drug into a hot air oven at 80ºC for 8 h. One micro litre 500 ng/spot of resultant solutions were applied on TLC plate and developed.

Photochemical degradation

10 ml solution of framycetin sulphate was studied by exposing to direct sunlight for 48 h. One micro litre 500 ng/spot of resultant solutions were applied on TLC plate and developed.

Results and Discussion

Development of TLC procedure

The TLC procedure was developed and optimized with a view to quantify the framycetin sulphate content in standard and in test samples. The mobile phase acetonitrile: methanol: water (7.5: 0.5: 2 v/v/v) was optimized and selected by trial and error method on the basis of resolution with a sharp and well defined peak at R_f = 0.46±0.02 (fig. 3).

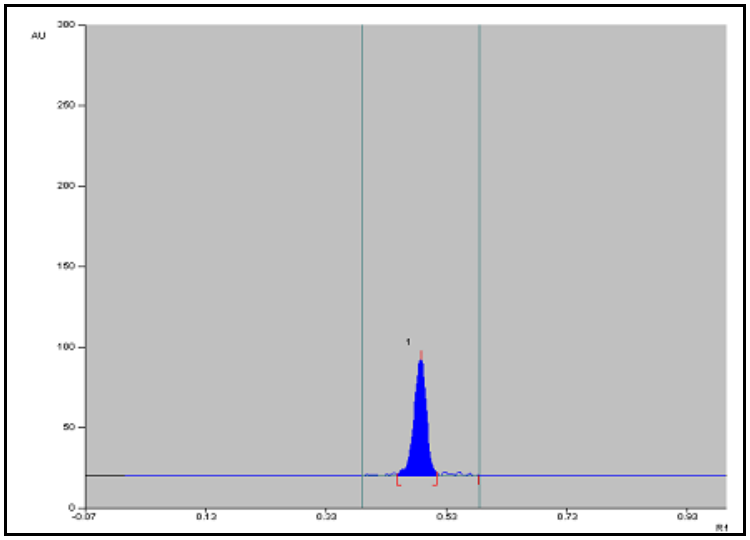

Fig. 3: Chromatogram of framycetin sulphate (R_f = 0.46)

Calibration curve

The developed HPTLC method for estimation of framycetin sulphate showed a correlation coefficient (r^2= 0.998) with SD 18.51 and intercept 380.39 in the concentration range of 100–1900 ng/spot (Table 1) with respect to the peak area, (fig. 4) displays the calibration curve of framycetin sulphate at 258 nm. The linearity of calibration graphs and adherence of the system to Beer's law was validated by correlation coefficient. No significant difference was observed in the slopes and standard curves (ANOVA, $p < 0.05$).

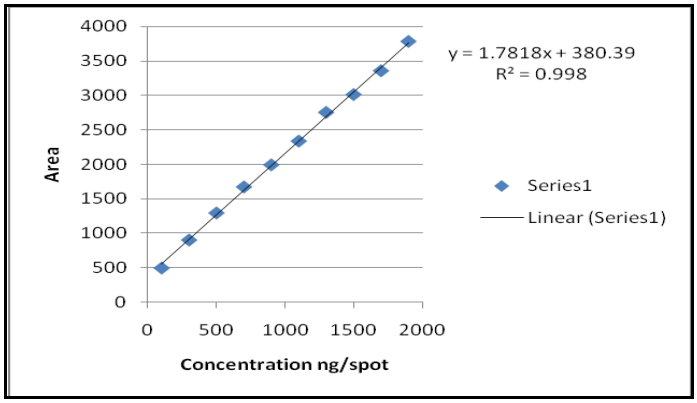

Fig. 4: Calibration curve of framycetin sulphate

Table 1: Summary of linear regression and validation data.

Parameters	HPTLC
Linearity range (ng/ spot)	100-1900
Correlation coefficient (r^2)	0.998
Intra-day precision (n=3)	7.06 ± 0.94
Inter-day precision (n=3)	7.00 ± 0.90
Limit of detection (ng/ spot)	100
Limit of quantification (ng/ spot)	300
Recovery (n=3) (%)	98.12%
Robustness	Robust
Specificity	Specific

Validation of the method

Precision

The results obtained from intermediate precision (inter-day) also indicated a good method precision. All the data were within the acceptance criteria which indicated that the method was precise. In precision studies on intra and inter-days, the resultant peak area for framycetin sulphate determined at three different concentration levels 100, 300, 500 ng/spot showed % RSD (<1.8%) for inter- and intra-day variations which suggested an excellent precision of the method (Table 2).

Table 2: Precision study (n= 3).

Precision	Conc. (ng/spot)	R_f	SD	SE	%RSD
Intra-day	100	0.46	7.06	2.35	1.40
	300	0.46	13.18	4.39	1.43
	500	0.46	16.71	5.57	1.30
Inter-day	100	0.46	7.50	2.5	1.45
	300	0.46	14.84	4.94	1.66
	500	0.46	15.50	5.01	1.24

SD = standard deviation; RSD = relative standard deviation; SE=standard error

Repeatability

The % RSD for repeatability of the drug was found to be (<2). The measurement of peak areas at three different concentration levels showed low value of % R.S.D. (<2) (Table 3). Hence the proposed method for estimation is proved to be repeatable in nature.

Table 3: Repeatability study (n=3).

Conc. (ng /spot)	Area	SD	SE	%RSD
500	1216	17.15	5.71	1.14
500	1228	20.31	6.77	1.65
500	1298	15.94	5.31	1.24

SD = standard deviation; RSD = relative standard deviation; SE=standard error

Recovery and Specificity studies

Results of the recovery study showed high efficiency of framycetin sulphate from the samples. The proposed method afforded recovery in the range of 89.79-98.12 % (Table 4). This confirms that the proposed method can be used for the determination of framycetin sulphate in formulations at different concentration levels.

The peak purity of framycetin sulphate was assessed by comparing their respective densitograms at peak start, peak apex and peak end positions of the spot i.e., r (start, middle) = (0.32 -0.4) and r (middle, end) = (0.4 -0.48).Good correlation was obtained between standard and sample densitograms of framycetin sulphate.

Table 4: Recovery study (n=3).

Levels	Concentration added (ng/ spot)	Concentration found (ng/ spot)	%Recovery
80	181.26	101.47	89.79
100	200.91	101.39	98.12
120	221.14	101.50	95.21

Robustness of method

The % R.S.D. and SE of the peak areas was calculated for change in mobile phase composition, mobile phase volume, temperature, time from spotting to chromatography and time from chromatography to scanning in triplicate at concentration level of 300 ng/spot of framycetin sulphate. The deviation obtained by deliberate changes in various parameters % R.S.D (<2) (Table 5) which indicated that the developed HPTLC method was robust.

Table 5: Results of robustness testing.

Parameters	SD	SE	%RSD
Mobile phase composition (±) 0.1ml	7.54	2.51	1.40
Amount of mobile phase (±) 0.1ml	13.12	4.37	1.43
Temperature (±) 5ºC	16.71	5.57	1.28
Relative humidity ± 5%	13.03	4.34	1.15
Time from spotting to chromatography (±) 5 min	20.14	6.71	1.65
Time from chromatography to scanning (±) 5 min	18.26	6.08	1.32

SD = standard deviation; RSD = relative standard deviation, SE=standard error

LOD and LOQ

Detection limit and limit of quantification were found to be 100 and 300 ng/spot respectively, which indicate adequate sensitivity of the method. The stronger pronounced effective diffusion of the framycetin sulphate spot at higher R_f value 0.46 ± 0.02 as against 0.42 ± 0.02 in our proposed method (Table 1).

Stability indicating property

The number of degradation products with their $R_f$values at % recovery of framycetin sulphate was calculated and listed in (Table 6).

Table 6: Summary of forced degradation study.

Sr. No.	Stressed Conditions	Framycetin sulphate		Degradation Product		Figure No.
		%	R_f	%	R_f	
1	Acid, 10 ml (0.1 N HCl reflux for 6 h at 80ºC)	96.36%	0.46	3.64%	0.02, 0.38	fig.5
2	Base, 10 ml (0.1 N NaOH reflux for 8 h at 80ºC)	91%	0.46	9%	0.23, 0.36	fig.6
3	Hydrogen peroxide, 10 ml, (3% v/v H_2O_2 reflux for 1 h at 80ºC)	87.63%	0.46	12.37 %	0.25,0.60, 0.79	fig.7
4	Dry heat (8 h at 80ºC)	91.79%	0.46	8.21%	0.09, 0.60	fig.8
5	Photochemical stability (Daylight, 48 h)	100%	0.46	----	Not detected	----

Acid and Base induced degradation

The chromatograms of acid and base degraded products showed additional peaks at R_f value 0.02 and 0.46 in acid induced degradation and 0.23 and 0.36 in base induced degradation respectively. The % recoveries of framycetin sulphate at the level of 96.36% in acid and 91.00% in basic condition which suggested that framycetin sulphate undergoes degradation under both (fig. 5) the conditions (fig. 6).

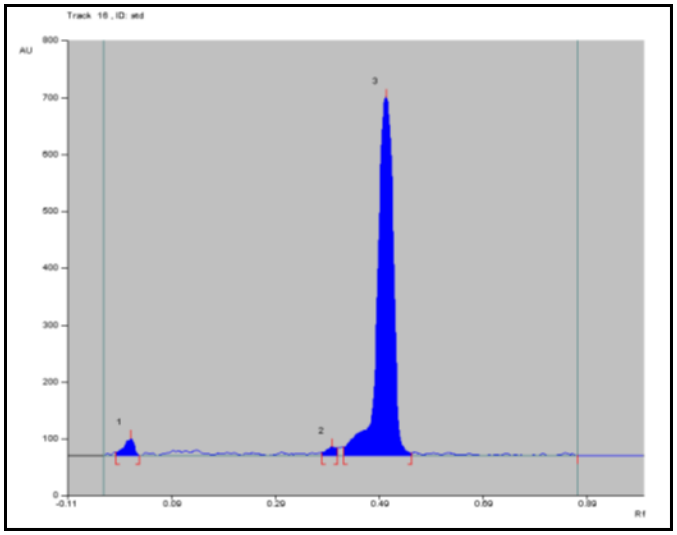

Fig. 5: Chromatogram of acid [0.1N HCl (reflux for 6h at 80ºC)] treated sample.
Peak 1- degradant [R_f=0.02], Peak 2degradant –[R_f=0.38], Peak 3 – Framycetin sulphate[R_f= 0.46]

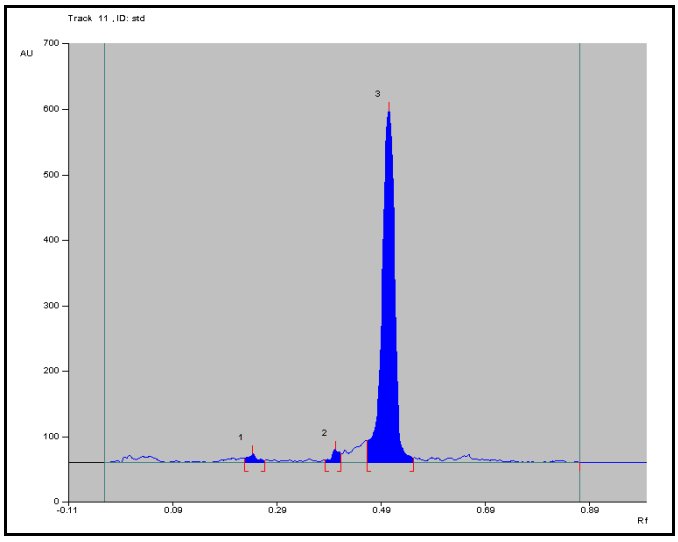

Fig. 6: Chromatogram of base [0.1 N NaOH (reflux for 8 h at 80ºC)] treated sample.
Peak 1- degradant [R_f=0.23], Peak 2-degradant [R_f=0.36], Peak 3- Framycetin sulphate [R_f= 0.46]

Hydrogen peroxide induced degradation

The chromatogram of hydrogen peroxide showed three additional peaks at R_f value 0.25, 0.60 and 0.79 (fig. 7) other than the standard peak of framycetin sulphate. The % recovery of framycetin sulphate at the level of 87.63% suggested that it showed degradation under oxidative condition.

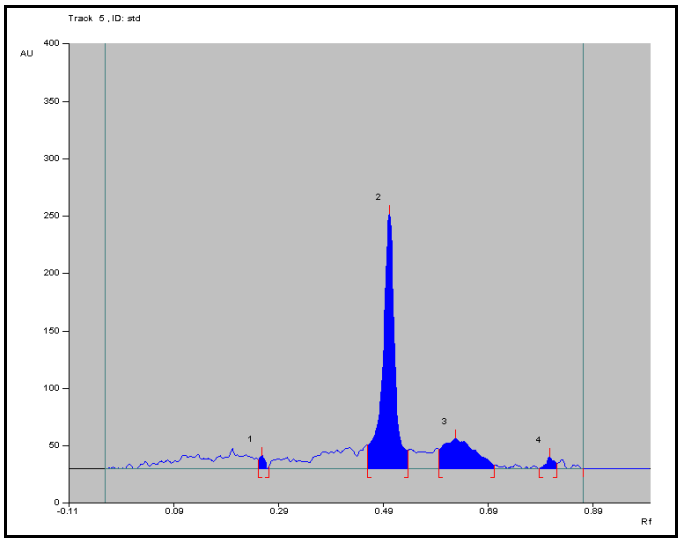

Fig.7: Chromatogram of hydrogen peroxide [3% v/v H_2O_2 (reflux for 1h at 80ºC)] treated sample
Peak 1- degradant [R_f=0.25], Peak 2-Framycetin sulphate [R_f=0.46], Peak 3- degradant [R_f=0.60], Peak 4-degradant[R_f= 0.79]

Dry heat degradation

The sample degraded under dry heat showed two additional peaks at R_f value 0.09 and 0.60 (fig. 8) other than the standard peak of framycetin sulphate. The % recovery of framycetin sulphate at the level of 91.79% suggested that framycetin sulphate undergoes degradation under dry heat condition.

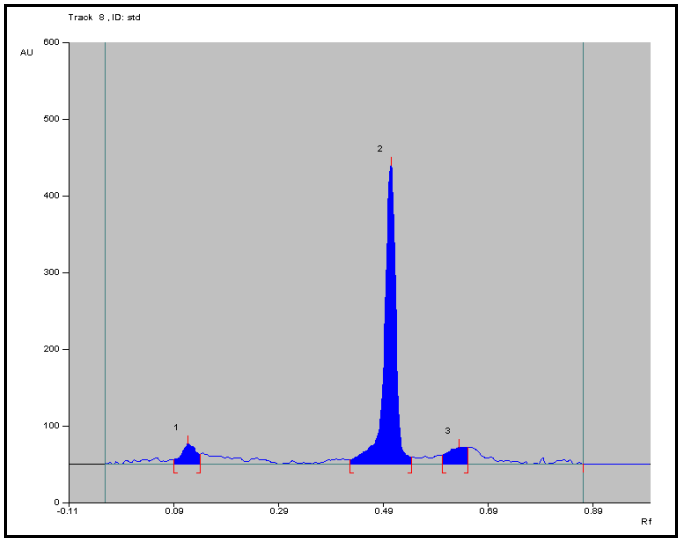

Fig. 8: Chromatogram of dry heat [4h at 60ºC] treated sample.
Peak 1- degradant [R_f=0.09], Peak 2- Framycetin sulphate [R_f=0.46], Peak 3- degradant [R_f=0.60]

Photochemical degradation

The sample exposed to photochemical degradation showed no additional peak other than framycetin sulphate which suggests that framycetin sulphate is stable towards photochemical degradation.

Conclusion

The developed HPTLC method enables accurate, precise, specific and stability indicating TLC method for determination of framycetin sulphate. Statistical analysis proves that the method is reproducible for routine

analysis of framycetin sulphate in pharmaceutical dosage form without interference from excipients.The developed method was able to separate the drug from its degradants hence can be successfully applied as a stability indicating one.

Acknowledgement

Authors are thankful to Encube Ethicals Life Pvt Ltd., Ponda, Goa, India for providing the gift sample of framycetin sulphate. The authors would also like to thank Dr. K.R. Mahadik, Principal, Poona College of Pharmacy, Pune, Maharashtra, India for providing necessary facilities to carry out the work.

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