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Development and validation of stability-indicating HPTLC method for determination of Rutoside in bulk drug and pharmaceutical dosage form

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ABSTRACT

A simple, selective, precise and stability-indicating high-performance thin layer chromatography (HPTLC) method for the analysis of Rutoside both in bulk drug and pharmaceutical formulation has been developed and validated. The method employed HPTLC aluminium plates precoated with silica gel 60 F_{254} as the stationary phase. The solvent system consisted of Toluene: methanol: gacial acetic acid (4:1.5:0.5 *v/v/v*). The system was found to give compact spot for Rutoside (R_f value of 0.40 ± 0.02). Densitometric analysis of Rutoside was carried out in the absorbance mode at 366 nm. The linear regression analysis data for the calibration plots showed good linear relationship with $r^2 = 0.999 \pm 0.0015$ with respect to peak area in the concentration range 700–1200 ng per spot. The mean values ± SD of slope and intercept were 5.2599 ± 1.47 and 2899.5 ± 1.78, respectively, with respect to peak area. The method was validated for precision, recovery and robustness. The limits of detection and quantification were 27.99 and 84.83 ng per spot, respectively. Rutoside was subjected to acid and alkali hydrolysis, oxidation, light and thermal degradation. The drug undergoes degradation under acidic, basic and light exposure conditions. This indicates that the drug is susceptible to acid, base and light (Photo degradation). The degraded product was well resolved from the pure drug with significantly different R_f value. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of investigated drug. The proposed developed HPTLC method can be applied for the identification and quantitative determination of Rutoside in bulk drug and pharmaceutical formulation.

KEYWORDS

Rutoside;
HPTLC;
Validation;
Stability;
Degradation.

INTRODUCTION

Rutoside (3, 3, 4, 5, 7-pentahydrohyflavone-3-rhamnoglucoside) is a flavonoid of the flavonol type, consisting of the flavonol quercetin and disaccharide Rutosideose (rhamnose and glucose)[1-3]. It is found in many typical nutrimental plants (especially in buckwheat, apple and black tea) and is an important dietary constituent of food and plant-based beverages[4,5]. Like other flavonoid derivatives, which all display a remark-

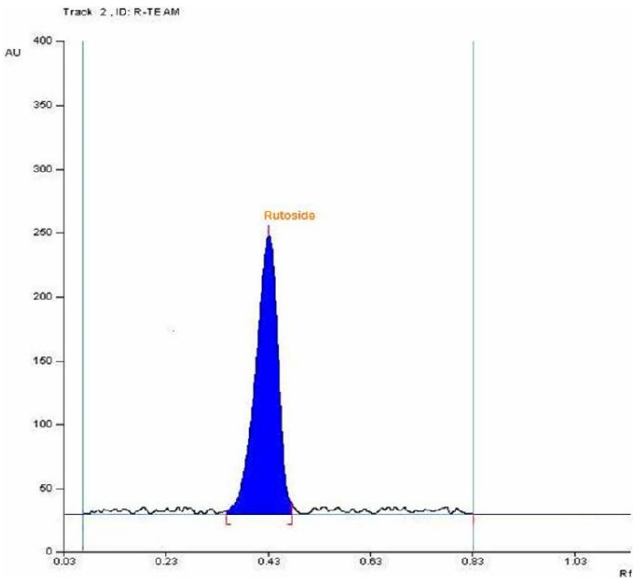

Figure 1 : Densitogram of standard Rutoside (R_f 0.40 ± 0.02), measured at 366 nm, mobile phase toluene: methanol: glacial acetic acid (4:1.5:0.5 *v/v/v*).

able array of biological and pharmacological activities, Rutoside exhibits antioxidant, anti-inflammatory, anticarcinogenic, antithrombic, cytoprotective and vasoprotective activities[6,7]. By increasing the strength of the capillaries and reducing their permeability, Rutoside helps preventing hemorrhages and ruptures in the capillaries and connective tissues, and is therefore often used to treat chronic venous insufficiency, hemorrhages and epitaxis[2-8].

A detailed literature survey for Rutoside (Rutin) revealed that several analytical techniques have been described for Rutoside determination[9,10]. For example, Pharmacopoeia Helvetica recommends direct spectrophotometric detection based on the strong absorption of ultra-violet (UV) light by conjugated double bounds and hydroxyl groups[9] and indirect spectrophotometric methods based on the formation of colored chelating complexes with metal ions were proposed[10,11]. Several HPLC[12,13], LC-MS[14], HPTLC[15,16], RP-UFLC[17], RP-HPLC[18], UV induced Fluorescence[19] were developed for estimation of Rutoside simultaneously in herbs, herbal formulations and Pharmaceutical dosage forms.

However only few methods have been developed and validated for pharmaceutical dosage forms. Hence, our study reports a simple, precise and economic, stability-indicating HPTLC method for determination of Rutoside in Tablet formulation. The method was validated according to ICH guidelines[20].

EXPERIMENTAL

Material and reagents

Rutoside bulk drug and tablet Rutoside (250 mg) were obtained from Macleods Pharmaceuticals (Daman, India). Hydrochloric acid and sodium hydroxide pellets were obtained from Merck Laboratories Ltd., India. Methanol, Hydrogen peroxide, toluene and glacial acetic acid are also obtained from Merck Specialties Private Ltd., India. All chemicals used are of HPLC grade. Milli-Q Water was used throughout the experiment.

Instrumentation and chromatographic conditions

The samples were spotted in the form of bands of 6 mm width with a Camag microliter syringe on precoated silica gel aluminium plates 60 F_{254} (20 × 20 cm with 250 mm thickness, E. Merck), using a Camag Linomat 5 applicator. The plates were prewashed with methanol and activated at 60 ^{0}C for 5 min prior to chromatography. The slit dimension was kept at 6.00 × 0.45 mm (micro) and 20 mm/s scanning speed was employed. The mobile phase consisted of toluene: methanol: glacial acetic acid (4: 1.5: 0.5 *v/v/v*), and 10 ml of mobile phase was used. Linear ascending development was carried out in a 20 × 20 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 20 min at room temperature. The length of the chromatogram run was approximately 8 cm. Subsequent to development; the TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on a Camag TLC scanner 3 and was operated by Win-CATS software. Finally, the mobile phase consisting of toluene: methanol: glacial acetic acid (4: 1.5: 0.5 *v/v/v*) gave a sharp and well-defined peak at R_f value of 0.40 (Figure 1). Finalized chromatographic conditions were expressed in TABLE 1.

Preparation of standard stock solutions

An accurately weighed quantity of 10 mg Rutoside was transferred to 10 mL volumetric flask and dissolved in methanol, and volume was made up to mark with the

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TABLE 1 : Finalized chromatographic conditions

Parameters	Specifications
Stationary phase	Aluminum backed silica gel 60 F-254 TLC plates, (10 cm × 10 cm, layer thickness 0.2 mm, E-Merck, Darmstadt, Germany) prewashed with methanol
Mobile phase	toluene: methanol: glacial acetic acid (4:1.5:0.5 *v/v/v*)
Chamber saturation	20 minutes
Migration distance	80 mm
Activation of prewashed plate	10 min
Band width	6 mm
Slit dimensions	6.00 x 0.45 mm
Radiation source	Deuterium lamp
Scanning wavelength	366 nm
Distance between bands	15.0 mm

same solvent to obtain concentration of 1000 ng/ìL. Aliquots of standard solutions 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2 ìL of Rutoside was applied on TLC plates with the help of microliter syringe, using Linomat 5 sample applicator to obtain the concentration of 700, 800, 900, 1000, 1100 and 1200 ng per spot.

METHOD VALIDATION

Linearity and range

Aliquots of standard stock solutions 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2 ìL of Rutoside was applied on TLC plates with the help of microliter syringe, using Linomat 5 sample applicator to obtain the concentration of 700, 800, 900, 1000, 1100 and 1200 ng per spot. The standard curves were evaluated for within day and day-to-day reproducibility. Each experiment was repeated 6 times. The calibration curve was plotted by considering the peak areas versus corresponding concentration.

Sensitivity

In present study the sensitivity parameter was evaluated by determining the LOD and LOQ of the drug. The LOD and LOQ parameter was evaluated by using the slope of line and standard deviation obtained from calibration curve studies. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using equations LOD = $3.3 \times N/B$ and LOQ = $10 \times N/B$, where N is the standard deviation of the peak areas of the drugs (n = 3), taken as a measure of noise, and B is the slope of the corresponding calibration curve.

Specificity

Method specificity was evaluated for interference of closely related impurities and excipients in the analysis of drug solution. The specificity of the method was ascertained by analyzing the standard drug and sample. The spot for Rutoside in sample was confirmed by comparing the R_f values and spectra of the spot with that of the standard. The peak purity of Rutoside was assessed by comparing the spectra at three different levels, i.e. peak start (S), peak apex (M) and peak end (E) positions of the spot.

Accuracy

To check the accuracy of the method, recovery studies were carried out at three different levels 80, 100 and 120 %. Base level concentrations of analytes in tablet formulation used were 900 ng/spot for Rutoside.

Precision

Repeatability of sample application and measurement of peak area were carried out using six replicates of the same spot (900 ng per spot of Rutoside). The intra and inter-day variation for the determination of Rutoside was carried out at three different concentration levels of 800, 900 and 1000 ng per spot.

Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions of toluene: methanol: glacial acetic acid (4: 1.8: 0.5 and 3.9: 1.2: 0.5 *v/v/v*) were tried, and chromatograms were run. The volume of mobile phase and saturation time

TABLE 2 : Linearity study of Rutoside

Sr. No	Concentration in [ng/spot]	Peak area mean ± S.D.	% R.S.D.
1	700	6512.69 ± 28.55	0.43
2	800	7091.39 ± 24.22	0.34
3	900	7556.67 ± 6.16	0.79
4	1000	8114.63 ± 56.45	0.69
5	1100	8587.40 ± 27.51	0.32
6	1200	9185.42 ± 30.36	0.33

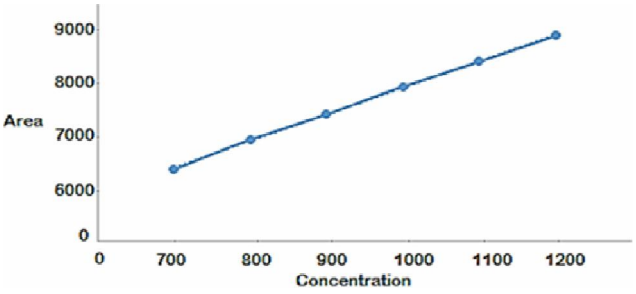

Figure 2 : Calibration curve of Rutoside; Y = 5.2599 X + 2844.5; Where, Correlation coefficient = 0.999, Slope = 5.2599, Intercept = 2844.5

was varied in the range of ± 5%. Time from spotting to chromatography and from chromatography to scanning was varied from 5, 20 and 40 min.

Ruggedness

Ruggedness of the method was performed by spotting 900 ng of Rutoside by two different analysts, keeping same experimental and environmental conditions.

Application of proposed method for estimation of drug in tablet formulation

Twenty tablets were weighed and finely powdered. Amount of tablet powder equivalent to 10 mg of Rutoside was weighed accurately, transferred to 100 ml volumetric flask and shaken with 50 ml methanol for 15 min. Volume was made up to 100 mL with methanol and ultrasonicated for 15 min. Solution was then filtered through Whatmann filter paper No. 41. The solution was suitably diluted with methanol to get concentration 100 ng/iL of Rutoside. 9 iL of each drug was applied in the form of bands on the TLC plate in order to get 900 ng/spot concentration. The plate was developed using mobile phase, containing toluene: methanol: glacial acetic acid (4: 1.5: 0.5 *v/v/v*).

FORCED DEGRADATION STUDIES

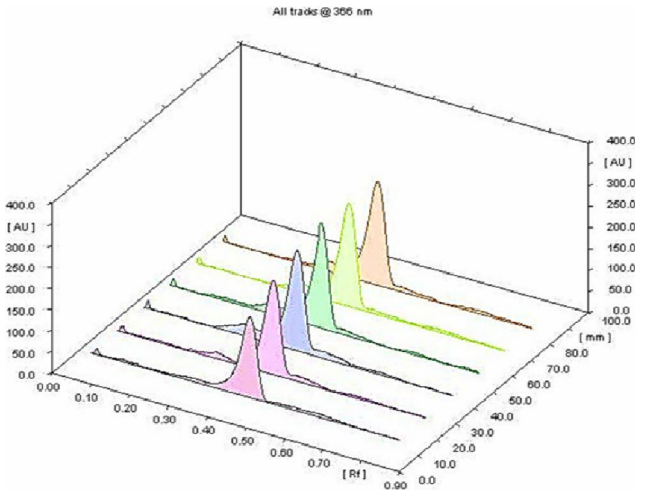

Figure 3 : The 3D linearity spectra of Rutoside standard drug solution.

The drugs were subjected to stress conditions of acid hydrolysis, alkali hydrolysis, oxidation, thermal degradation and light degradation. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and intrinsic stability of the molecule. Specificity is the ability of the method to measure the analyte response in presence of its potential impurities.

Acid- and base-induced degradation

Ten milligrams of Rutoside was separately dissolved in 10 mL of methanolic solution of 0.1 N HCl and 0.01 N NaOH. These solutions were kept for 6 hrs. and 12 hrs. respectively at room temperature in the dark in order to exclude the possible degradative effect of light. One millilitre from the above solutions was taken and neutralized and then diluted up to 10 mL with methanol. The resultant solutions were applied on TLC plate in triplicate (9 iL each, i.e. 900 ng per spot).

Hydrogen peroxide-induced degradation

Ten milligrams of Rutoside was separately dissolved in 10 mL of methanolic solution of hydrogen peroxide (10 %, *v/v*). The solution was kept for 48 hrs. at room temperature in the dark in order to exclude the possible degradative effect of light. The resultant solution was applied on TLC plate in triplicate (0.9 µL each, i.e. 900 ng per spot).

Photochemical degradation

The photochemical stability of the drug was also studied by exposing the stock solution to direct sunlight

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TABLE 3: Recovery studies

Drug	Initial Amount [ng/spot]	Amount added (%)	Amount recovered ± S.D.[ng/spot] [n=3]	% Recovered	% RSD
Rutoside	800	80	1438.17± 11.07	99.87	0.77
	800	100	1595.42± 8.05	99.71	0.50
	800	120	1755.67± 11.05	99.75	0.63

TABLE 4 : Precision studies (Intra- day and inter- day)

Drug	Conc.[ng/spot]	Intra day % Amount found		Inter day % Amount found	
		Mean ± SD [n= 3]	% RSD	Mean ± SD [n= 3]	% RSD
Rutoside	800	100.52 ± 0.65	0.64	100.69 ± 0.63	0.62
	900	99.49 ± 0.40	0.40	99.38 ± 0.50	0.51
	1000	99.82 ± 0.40	0.40	100.11 ± 0.54	0.54

TABLE 5 : Results of repeatability studies

	Amount Taken (ng/spot)	Amount Found (ng)	Amount Found %
Rutoside	900	894.34	99.37
	900	897.22	99.69
	900	905.15	100.57
	900	901.38	100.15
	900	899.28	99.92
	900	906.71	100.74
	Mean ± SD	900.68± 4.71	100.07 ± 0.52
	%RSD	0.53	0.52

TABLE 6 : Results of robustness studies

Parameters	± SD of peak area	% RSD
Mobile phase composition		
toluene : methanol: glacial acetic acid (4:1.8:0.5 *v/v/v*)	8.00	0.10
toluene : methanol: glacial acetic acid (3.9:1.2:0.5 *v/v/v*)	8.39	0.11
Mobile Phase volume		
5 mL	8.90	0.11
10 mL	10.39	0.13
Saturation Time		
15 Min.	24.59	0.32
25 Min.	21.44	0.28
Time from spotting to chromatography	21.16	0.50
Time from chromatography to scanning	23.34	0.43

for 48 hrs. The resultant solution (0.9 µL, i.e. 900 ng per spot) was applied on a TLC plate.

Dry heat degradation

The powdered drug stored at 70°C for 48 hrs. under dry heat condition showed no significant degradation. In all degradation studies, the average peak areas of Rutoside after application (900 ng per spot) of three replicates were obtained.

RESULTS AND DISCUSSIONS

Validation of the method

Linearity and range

The linear regression data for the calibration curves showed good linear relationship over the concentration range 0.7 - 1.2 ng/µL for Rutoside (n = 6). Typically, the regression equations for the calibration curve was found to be $y = 5.2599x + 2844.5$ ($r^2 = 0.999$) for Rutoside. The calibration curve was plotted by considering the peak areas versus corresponding concentration (Figure 2). The results were expressed in TABLE

TABLE 7 : Results of ruggedness

	Analyst –I		Analyst –II	
Drug	% Amount found ± SD [n = 3]	% RSD	% Amount found ± SD [n = 3]	% RSD
Rutoside	100.05± 0.52	0.64	99.33 ± 0.13	0.12

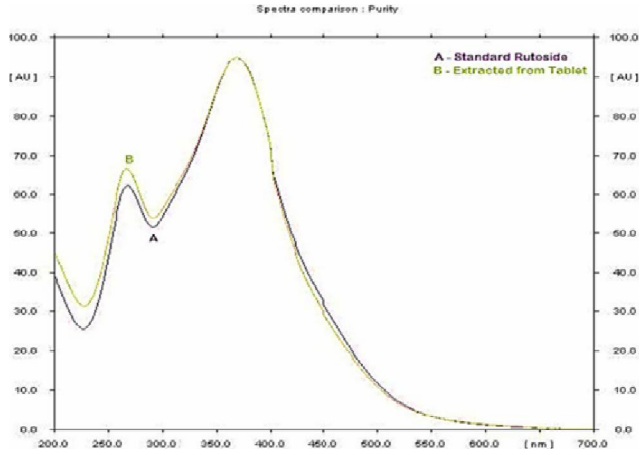

Figure 4 : Peak purity spectra of standard Rutoside (A), sample (B) extracted from a Rutoside tablet, scanned at the peak-start, peak-apex, and peak-end

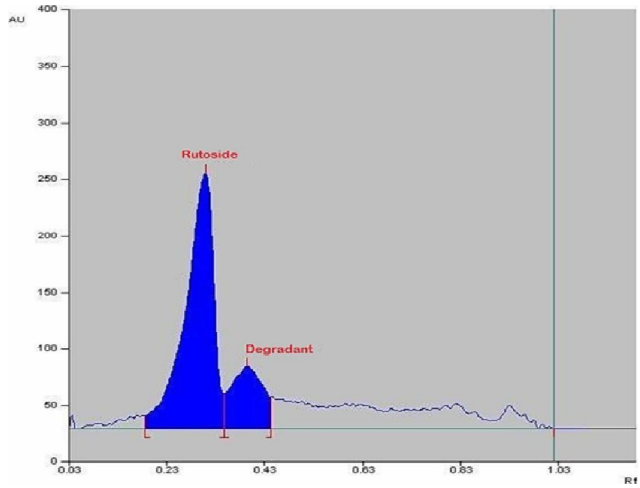

Figure 5 : Chromatogram of Rutoside subjected to acid hydrolysis

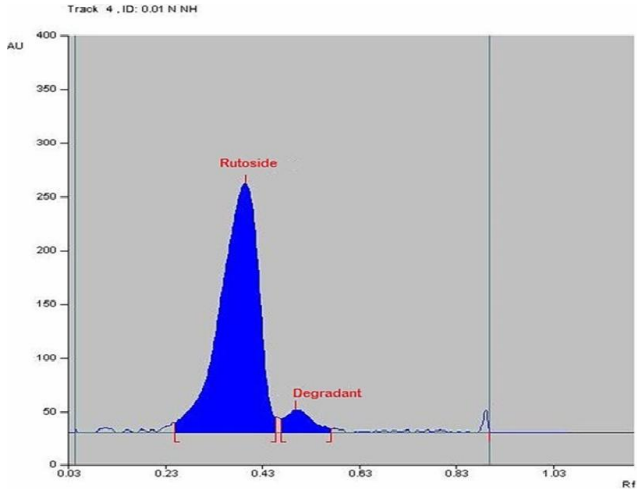

Figure 6 : Chromatogram of Rutoside subjected to alkaline hydrolysis

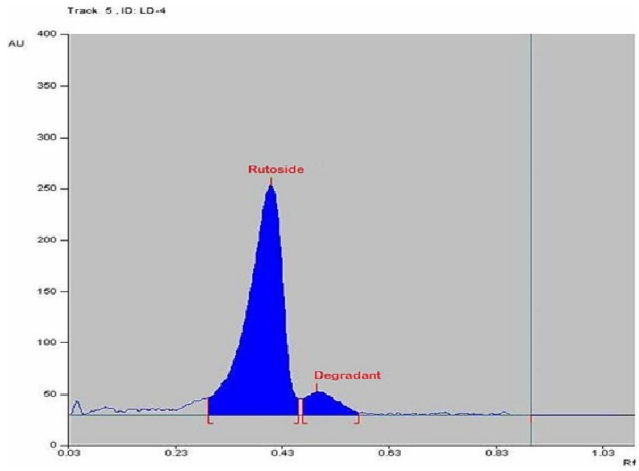

Figure 7 : Chromatogram of Rutoside subjected to light degradation

2 and 3D linearity spectra of Rutoside shown in Figure 3.

Sensitivity

The linearity equation was found to be Y = 1.9615 X + 5396. The Limit of Quantification and Limit of Detection for Rutoside were found to be 84.83 ng and 27.99 ng, respectively. [Where, N = 16.64, B = 1.9615]. This indicates the adequate sensitivity of the method.

Specificity

The peak purity of Rutoside was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e. r^2 (S, M) = 0.99893 and r^2 (M, E) = 0.999. Good correlation (r^2 = 0.9989) was also obtained between standard and sample spectra of Rutoside (Figure 4).

Accuracy

The proposed method when used for subsequent estimation of Rutoside from the pharmaceutical dosage formed after over spotting with 80%, 100% and 120% of additional drug afforded good recovery of Rutoside.

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TABLE 8: Analysis of marketed tablet formulation; Brand Name: ENZOMAC Mfg. By: Macleods Pharmaceuticals; Batch No.: BKT1004HB Average weight: 453.15 mg

Drug	Amount Taken (ng/spot)	Amount Found (ng)	Amount Found %
Rutoside	900	883.58	98.17
	900	883.89	98.21
	900	884.83	98.31
	900	886.32	98.48
	900	885.11	98.34
	900	885.96	98.44
	Mean ± SD	884.95± 1.08	98.32 ± 0.12
	%RSD	0.12	0.12

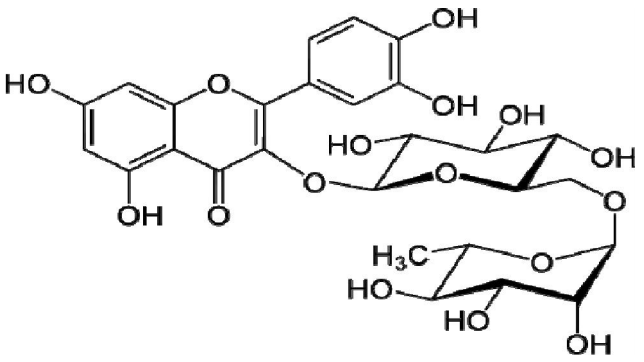

Figure 8 : Structure of Rutoside

TABLE 9 : Forced degradation studies

Agent	Exposure time	Condition	Degradants peak	Degradant R_f	% Degradation
0.1 N HCl	6 hrs.	Room temp.	Peak Found	0.42	20%
0.01N NaOH	12 hrs.	Room temp.	Peak Found	0.48	18.52%
10 % H_2O_2	48 hrs.	Room temp.	Not found	-	-
Dry Heat	48 hrs.	70^0 C	Not Found	-	-
Light	48 hrs.	Sunlight	Peak Found	0.51	14.69%

The amounts of drug added and determined and the percentage recovery are listed in TABLE 3, which showed that the % amount found was between 99.71 % and 99.87 % with % R.S.D. >2.

Precision

The precision of the developed HPTLC method was expressed in terms of percent relative standard deviation (% RSD). The results, presented in TABLE 4 and results of repeatability expressed in TABLE 5. % R.S.D. values found to be less than 2, revealed high precision of the method.

Robustness

The standard deviation of peak areas was calculated for each parameter, and % RSD was found to be < 2. The low values of % RSD values, shown in TABLE 6, indicated the robustness of the method.

Ruggedness

Peak area was measured for same concentration solutions, six times by two analyst. The results are given in TABLE 7, showed that the % R.S.D. was less than 2 and % amount found was between 99.33 - 100.05 %.

Analysis of the marketed formulation

A single spot at R_f 0.40 was observed in the chromatogram of the drug samples extracted from tablets. There was no interference from the excipients commonly present in the tablet. The low % RSD value indicated the suitability of this method for the routine analysis of Rutoside in pharmaceutical dosage forms. The results expressed in TABLE 8.

Force degradation

The chromatogram of the acid-degraded samples for Rutoside showed additional peak at R_f value 0.42 (Figure 5), base-degraded drug showed 0.48 (Figure 6) and light degraded drug showed additional peak at R_f value 0.51 (Figure 7). The spot of the degraded product was well resolved from the Rutoside (Figure 8) spot. In each cases, the concentration of the drug was changing from the initial concentration, indicating that Rutoside undergoes degradation under acidic, basic and light exposure conditions. This indicates that the drug is susceptible to acid–base hydrolysis and light degradation. The chromatograms of hydrogen peroxide and dry heat samples of Rutoside showed only the spots of the pure drug. The higher R_f values of degraded components indicated that they were more polar than the analyte itself. The results are listed in TABLE 9.

CONCLUSIONS

The modalities adopted in experiment were suc-

cessfully validated as per ICH guidelines. The developed HPTLC method was found to be accurate, simple, precise, specific and stability-indicating and can be conveniently applied for quality control analysis in industry and is having short run time which significantly reduces the analysis time and cost. Statistical analysis proves that the method is repeatable and selective for the analysis of Rutoside as bulk drug and in pharmaceutical formulations. The method can be used to determine the purity of the drug available from the various sources by detecting the related impurities. As the method separates the drug from its degradation products, it can be employed as a stability-indicating one.

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