Development of Validated Stability-indicating High Performance Thin Layer Chromatography Method for Estimation of Rabeprazole Sodium and Aceclofenac in Bulk Drug

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Authors' contributions

This work was carried out in collaboration among all authors. For this research work, author SS conceptualized and designed the experimental work. Author SS performed the actual experimental activities, data collection, interpretation of the results and drafting the paper. Author VU has provided editorial assistance and critically reviewed the manuscript for the intellectual inputs. Authors MS and AD have contributed for analysis of study data and review of manuscript. Author VB has helped in drafting the manuscript and collation of data. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Objective: To develop a validated stability-indicating high performance thin layer chromatography method for the estimation of Rabeprazole Sodium (RZL) and Aceclofenac (ACF) in bulk drugs.
Methods: A high performance thin layer chromatographic (HPTLC) method has been developed for the separation of RZL & ACF on plates precoated with aluminium back silica gel 60 F_{254}. Different mobile phases were used on trial and error basis for separation of two drugs. The final mobile phase selected for analysis was toluene: ethyl acetate: methanol: acetic acid: ammonia in the ration of 6:4:1:0.2:0.1 (v/v). Both the drugs showed maximum absorbance at 279 nm which was selected as the detection wavelength throughout the experimental work. Developed method was validated as per ICH guidelines. Forced degradation of drugs was carried out under various stress

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conditions and HPTLC method was used for analysing the stability of drugs.
Results: HPTLC method was successfully developed for separation of RZL and ACF with clear separation of bands of the drugs. Method validation after assessment of various parameters indicated low % RSD within an acceptable limit of < 2.0 and the stability studies indicated the satisfactory separation of both the drugs from that of degraded products with considerable % recovery profile.
Conclusion: The developed method is rapid, reliable, precise, and reproducible and demonstrates the suitability of the method for stability determination of rabeprazole and aceclofenac.

1. INTRODUCTION

ACF chemically is [(2,6-dichlorophenyl)amino] phenylacetoxyacetic acid [1]. It is used as an effective non-steroidal anti-inflammatory drug (NSAID). RZL is chemically known as 2-({[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl]methyl}sul phenyl)-1*H*-benzimidazole sodium [2-3]. It is a proton pump inhibitor that suppresses gastric acid secretion by specific inhibition of the gastric H+, K+-ATPase enzyme system at the secretory surface of the gastric parietal cell and used in the treatment of gastroesophageal reflux disease (GERD) and duodenal ulcers [4]. The combination of these two drugs has therapeutic indication in variety of painful conditions like rheumatoid arthritis, osteoarthritis and ankylosing spondylatis [5].

Thin layer chromatography (TLC) is a popular technique for the analysis of various organic and inorganic materials, because of its distinctive advantages such as minimal sample clean-up, wide choice of mobile phases and high sample loading capacity. TLC is a powerful tool for screening unknown materials in bulk drug and separation of probable components of the drug [6]. With advance technique, HPTLC emerged as an important instrument in drug analysis. Because of its simplicity and rapidity it is often used to check purity of products [7]. In HPTLC several samples can be run simultaneously. It is superior to other analytical techniques in terms of total cost and time [8]. Any developed analytical method, needs to be validated with respect to accuracy, precision, selectivity, sensitivity, linearity and range, robustness etc. to demonstrate its suitability for intended use [9-10]. It is required for assuring quality, achieving acceptance of products by the international agencies, mandatory requirement purposes for accreditation as per ISO 17025 guidelines and mandatory requirement for registration of any pharmaceutical product [11]. Developed and validated analytical method may be useful for various purposes including the forced degradation of drugs. It shows the chemical behaviour of the molecule which in turn helps in the development of formulation and package [12]. Analytical method is considered suitable for forced degradation, if it is able to separate the band of drug molecule from that of degraded products with acceptable recovery profile of drug for further study. Nevertheless, the regulatory guidance is very general and does not explain about the performance of forced degradation studies. However, the available ICH guidelines on stability testing of new drug substances and drug products and evaluation of stability data are helpful in this context [13-14].

Although, various analytical techniques has been developed for estimation of RZL and ACF individually or with other components in bulk drug and pharmaceutical dosage forms, the most suitable, efficient and cost effective HPTLC method has not yet been determined [15-23] Similarly, this technique has not been used for stability indication of drugs so far. It requires modification depending on the factors that predict the better resolution, validation within accepted limits and clear separation from degradation products. The present work was planned to develop and validates the HPTLC method and uses it for stability study of rabeprazole sodium and aceclofenac.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

RZL and ACF in pure form were provided as a gift research samples by Ranbaxy Laboratories Ltd. Maharashtra (India). It was used without further purification and certified to contain 99.54 % and 99.78 % (w/w) on dry weight basis, rabeprazole sodium and aceclofenac respectively. All chemicals and reagents of analytical grade were purchased from S. D. fine chemical Laboratories, Mumbai, India.

2.2 Instruments and chromatographic conditions

Chromatographic separation of drugs were performed on aluminium plates precoated with silica gel 60 F_{254} , (0.2 mm layer thickness) purchased from E-Merck, Darmstadt, Germany. Samples were applied on the plate as a band of 8 mm length using Camag 100 μl sample syringe (Hamilton, Switzerland) through a Automatic TLC sampler 4 (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber and a densitometry scanning was performed using Camag TLC scanner 3 in the range of 400-200 nm, operated by winCATS software (version 1.4.2, Camag). Chamber saturation time was 10 min. Migration distance was 80 mm, slit dimensions were 6.00 x 0.45 mm and Deuterium lamp was used as a radiation source.

2.3 Preparation of standard stock solution and working standards

Standard stock solution was prepared by dissolving 10 mg of RZL and 100 mg of ACF separately in methanol and volume was adjusted to 50 mL with the same. From these solutions 0.5 ml solution was taken each into 10 ml volumetric flask and volume was adjusted to 10 ml with methanol, to give a solution concentration of 10 ng/μL and 100 ng/μL of RZL and ACF respectively. As per the experimental procedure and requirement the working standards from standard stock solution were applied on pre-coated TLC plate. Plate was then developed in Camag development chamber.

2.4 Method development

Chromatographic separation studies were carried out using the mixture of working standard solution containing 10 ng/μL of RZL and 100 ng/μL of ACF. Selection of mobile phase for development of chromatogram was derived from experiment and observations. Various compositions of solvent system were tried for separation of drugs.

2.5 Selection of detection wavelength

After chromatographic development, bands of drugs were scanned using TLC scanner over the range of 200-400 nm and from that absorption maxima and the overlain absorption spectra were obtained.

2.6 Assay of marketed formulation

Fixed dose combination capsule Altraday (Ranbaxy Laboratories Ltd. India B.No. I000414, Mfg. dt.: Feb.2014, Exp. dt: Jul 2015) was assayed. Each capsule contains; Enteric coated RZL (IP)…20 mg, Sustained released ACF (IP)…200 mg. The 20 Altraday capsule with and without granules were accurately weighed, from that the average weight of granules was determined and they were finely powdered. Solution containing 10 mg of RZL and 100 mg of ACF was obtained by dissolving the required quantity of powdered material in methanol, which was sonicated for 10 min. The solution was filtered and volume was made up with methanol. The known theoretical concentration was applied on a plate and actual concentration was determined. From that, the % purity of drugs was determined.

2.7 Method Validation

2.7.1 Linearity and range

The linearity was studied over the increasing drug concentration and plotting the graph of peak area vs. concentration in ng.

2.7.2 Precision

Precision of the system and method was evaluated intraday and interday by analyzing independent sample preparations obtained from homogenous sample. In the intraday study, the concentrations of two drugs were calculated on the same day at an interval of 2 hrs. In the interday study, the concentrations of drug contents were calculated on three different days. Repeatability was also determined using homogenous sample.

2.7.3 Accuracy

To ensure the accuracy of method, recovery studies were performed by standard addition method at 80 %, 100 % and 120 % concentration levels of the label claim, to the pre-analyzed samples and their contents were re-analyzed, using the proposed method. Percentage recovery for both the drugs was then determined.

2.7.4 Sensitivity

The sensitivity of HPTLC method was estimated by determining the limit of detection (LOD) and limit of quantitation (LOQ) with suitable precision and accuracy.

2.7.5 Specificity

The specificity of the method was assessed by comparing the chromatogram obtained from standard drugs with that obtained from capsule solution, mobile phase and diluent.

2.7.6 Robustness

Robustness is checked by making slight deliberate change in mobile phase composition by 0.2 ml and increase in time from spotting to chromatography by 10 min.

2.8 Stress Degradation Studies of Drug

Stress degradation studies were carried under the influence of acid, base, neutral, oxidative, photochemical and dry heat conditions. For each study, two samples were prepared viz; the blank and the drug solution. The blank subjected to stress in the same manner as the drug solution of RZL and ACF was subjected to stress condition. Dry heat and photolytic degradation were carried out in solid state.

2.8.1 Acid induced degradation

10 ml of standard RZL and ACF was mix with 5 ml of 0.1 M HCL separately. Each solution was diluted with 10 ml of methanol. Aliquots of 2 ml were prepared in Eppendorf tubes and then heated for 1 and half hr at 65^0 C using Eppendorf heating mantle. After heating, the samples were withdrawn and neutralized with 0.5 ml of 0.1 M NaOH. Resultant solution was applied on TLC plate and the chromatogram was developed.

2.8.2 Base induced degradation

10 ml of standard RZL and ACF was mix with 5 ml of 0.1 M NaOH separately. The solution was diluted with 10 ml of methanol. Aliquots of 2 ml were prepared in Eppendorf tubes and then heated for 1 and half hr at 65^0 C using Eppendorf heating mantle. After heating, the samples were withdrawn and neutralized with 0.5 ml of 0.1 M HCL. Resultant solution was applied on TLC plate and the chromatogram was developed.

2.8.3 Neutral degradation

10 ml of standard drugs solution were mix with 5 ml of water individually. The solution was diluted with 10 ml of methanol. Aliquots of 2 ml were prepared in Eppendorf tubes and then heated for 2 hrs 30 min. at 65^0 C using Eppendorf heating mantle. Resultant solution was applied on TLC plate and the chromatogram was developed.

2.8.4 Hydrogen peroxide induced degradation

10 ml of standard RZL and ACF was mixed with 5 ml of 3 % H_2O_2 separately. The solution was diluted with 10 ml of methanol. Aliquots of 2 ml were prepared in Eppendorf tubes then heated for 1 hr at 65^0 C using Eppendorf heating mantle. Resultant solution was applied on TLC plate and the chromatogram was developed.

2.8.5 Photochemical degradation

Photolytic studies were carried out by exposure of both the drugs to UV light up to 200 watt hours/square meter. Resultant solutions were applied on TLC plate and the chromatogram was developed.

2.8.6 Dry heat degradation

To study the effect of temperature, RZL and ACF in powder form were exposed to dry heat in hot air oven at 105^0 C for 18 hrs, the sample was removed and placed on the bench top to attain the laboratory temperature. The resultant solutions were applied on TLC plate and the chromatogram was developed.

3. RESULTS

3.1 Optimization of HPTLC Method

The 2 μL solution of standard RZL and ACF were spotted on TLC plates and plate was developed in different solvent systems. First two methods led to formation of rigid bands but it didn't separate the drugs from each other. Third method caused difficulty in band visibility. Best suited system was found to be toluene-ethyl acetate-methanol-acetic acid-ammonia (6:4:1: 0.2:0.1 v/v). In this method, the TLC plate was developed with first two bands of RZL, then mixture of RZL and ACF which indicates the clear separation of two drugs, followed by two bands of ACF Fig. 1. First and last two tracks of individual drug solution helped to identify the bands of drugs in mixture.

3.2 Determination of absorption maximum & Isosbestic point

The λmax of drugs were determined by scanning the TLC plate. The absorption maximum of rabeprazole and aceclofenac was found to be

281 and 277 nm (reported are 282 and 275 nm in official pharmacopoeia) respectively. Both the drugs showed maximum absorbance at 279 nm i.e. isosbestic point and it was selected as the detection wavelength for further experimental work.

3.3 Densitogram of drugs and Retention factor finding

The solution of 20ng/band and 200ng/band was applied six times on TLC plate with the help of Hamilton syringe (100µL) and automatic TLC sampler 4 and the plate was developed in selected mobile phase. After development, the plate was dried and scanned over 80 mm distance at 279 nm. Densitogram of drugs were obtained using winCATS software. It has resulted in formation of two symmetric peaks without any interference and good resolution at R_f 0.33 ± 0.01 and 0.45 ± 0.008 for RZL and ACF respectively. This satisfactory result has given confirmation for use of HPLTC method for further analysis. Other chromatographic conditions like sample application volume and sample application positions, were enhance to give reproducible R_f value and symmetrical drug peak.

3.4 Assay of drugs in formulation

The percentage purity obtained after assay of drugs in capsule formulation was 97.65 for RZL and 99.30 for ACF and the percentage RSD was < 2.0. (Table 1) As per IP, the formulation contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of RZL and ACF. It has been evident in this assay. Similarly, the relative standard deviation was within acceptable limit.

3.5 Method Validation

#### 3.5.1 Assessment of linearity & calculation of range

As concentration of both the drugs increased, peak area increased proportionately indicating the linear relationship. The linearity in the proposed HPTLC method for determination of RZL and ACF was found in the concentration range of 10-80 ng/band and 100-800 ng/band respectively with regression coefficient (r^2) value > 0.99. The linear range of detectability obeyed Beer's Law and it was well within higher and lower linear concentration of drugs. Fig. 2.
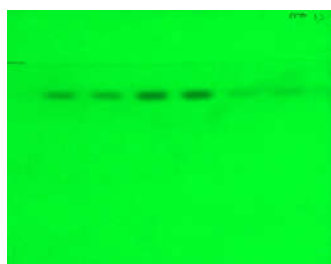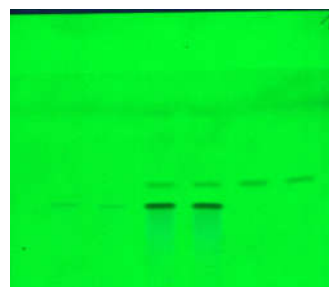

(a): Toluene-chloroform-methanol- ethyl acetate (6 :2 :2 :1 v/v)
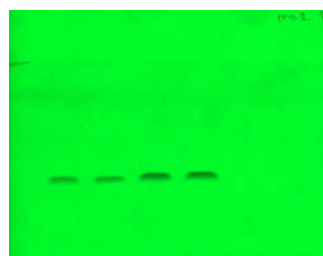
(b): Toluene-isopropyl alcohol- ammonia (8:7:1 v/v)
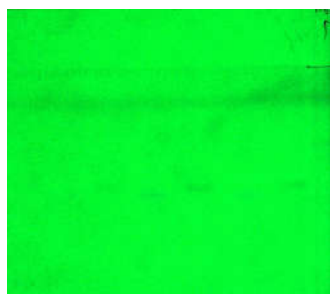
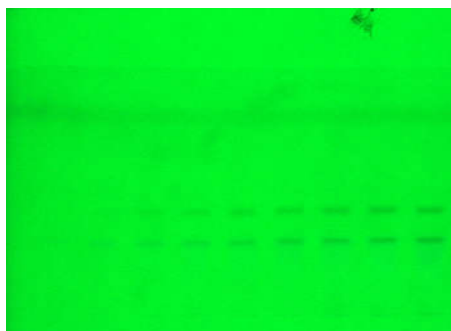(c): Acetonitrile-toluene-methanol-acetic acid (6:4:2:1 v/v)

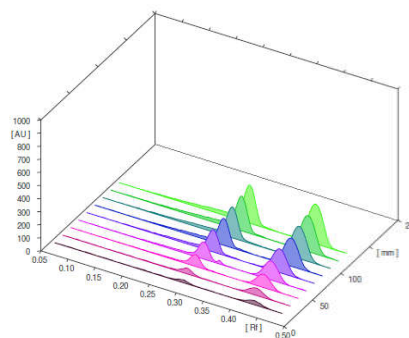(d) Toluene-ethyl acetate-methanol-acetic acid-ammonia (6:4:1:0.2:0.1 v/v)

Fig.1. Methods development for separation of RZL & ACF

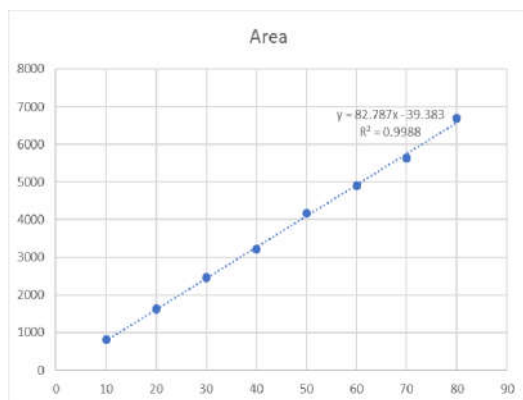**Table 1. Assay of RZL & ACF using commercial formulation (n=3)**

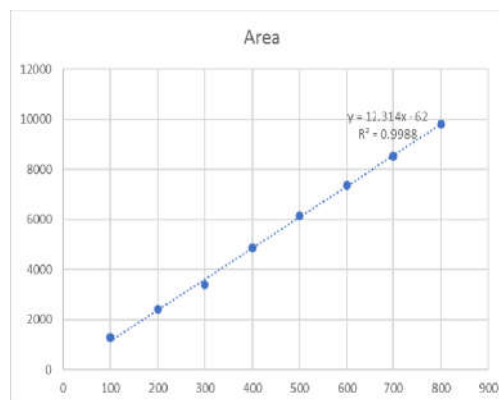| Component	Label claim (mg)	Amount found (mg)	Mean ± SD	% RSD	% Purity
RZL	20	19.51	19.53	0.98	97.65
		19.73	± 0.190		
		19.35			
ACF	200	198.44	198.60	0.36	99.30
		199.54	± 0.711		
		197.82			

(a) Developed TLC plate of linearity study

(b) 3D image of drugs peaks of linearity Study

(c) Linear curve of RZL

(d) Linear curve of ACF

Track no.	Concentration (ng/band)	Rf Value	Area	Track no.	Concentration (ng/band)	Rf Value	Area
1	10	0.29	806.35	1	100	0.41	1292.79
2	20	0.29	1622.39	2	200	0.41	2416.92
3	30	0.29	2458.65	3	300	0.41	3400.82
4	40	0.29	3206.22	4	400	0.41	4859.37
5	50	0.30	4163.93	5	500	0.42	6149.97
6	60	0.30	4906.52	6	600	0.42	7360.13
7	70	0.30	5636.71	7	700	0.42	8540.83
8	80	0.30	6687.53	8	800	0.42	9814.44

Fig. 2. Linearity studies of RZL & ACF

3.5.2 Precision

The developed method was precise for quantitative study because the precision study was found statistically significant with % RSD <2.0 for intra. However, slight variation was observed during inter-day precision with % RSD value >2.0 but it may have occurred because of experimental error Table 2.

3.5.3 Determination of drug recovery through standard addition method

The results of the accuracy study are reported in Table 3. From the recovery study, it was clear that the method is very accurate for quantitative estimation of rabeprazole sodium and aceclofenac in capsule dosage form because all the statistical results were within the acceptance range (i.e., % RSD <2.0) and % drug recovery profile was also within the established limits.

3.5.4 LOD and LOQ

The detection limit for RZL was found to be 3.02 ng/band and for ACF it was 29.78 ng/band. Similarly, the limit of quantitation was 9.15 ng/band and 90.26 ng/band approximately, three times that of detection limit. These can be correlates to linearity study of both the drugs where lower concentration of drugs detected was 10 and 100 ng/band respectively. LOQ for both drugs was below the minimum amount of drug quantitated throughout the experimental work.

3.5.5 Assessment of specificity

The chromatogram obtained from standard drugs and drugs in formulation shows bands while the chromatogram obtained from mobile phase and diluent didn't show appearance of any band on TLC plate. The retention factor of standard drugs and the retention factor of two drugs in formulation was also the same. No interference of excipients from formulation, diluent or mobile phase was found. Fig. 3.
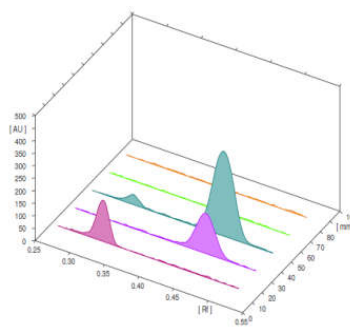
Method was also validated for robustness where no significant changes were seen in R_f , peak areas and concentration obtained with % RSD < 2.0. The summary of results of HPTLC method validation is depicted in Table 4.

3.6 Assessment of Degradation Pathways

Two different TLC plates were developed for RZL and ACF degradation. Fig. 4. The acid and hydrogen peroxide induced degradation samples of both the drugs show the colour change to naked eyes; indicating the formation of degraded product. In case of other degradation, no such colour formation or any other change was observed by initial visual observation which made it difficult, to point out the degradation. However, the development of chromatogram indicated the formation of degradation product. These degradation products with different R_f value were well separated from each other and also from the band of original drugs molecule. The identification of band of drug molecule was done by comparing each band formed during the degradation with the band of standard drug; run at the start of degradation chromatogram and by determining the R_f value. The % drug recovery was calculated based on how much degradation of the standard drug occurred.
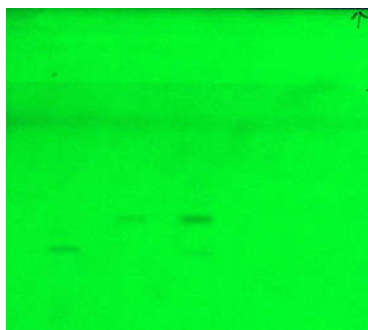
(a) Developed TLC plate for sensitivity study   (b) 3D image for sensitivity study

Fig. 3. Sensitivity study

Table 2. Precision studies (n=3)

Drug	Conc. (ng/band)	Intra-day precision		Inter-day precision	
		Mean conc. obtained ± SD	% RSD	Mean conc. obtained ± SD	% RSD
RZL	20	19.30 ± 0.179	0.93	19.11 ± 0.315	1.65
	30	29.05 ± 0.135	0.46	29.10 ± 0.607	2.09
	40	38.71 ± 0.531	1.37	38.00 ± 1.925	5.07
ACF	200	198.88 ± 0.975	0.49	197.35 ± 2.279	1.15
	300	296.46 ± 5.586	1.88	298.05 ± 2.314	0.77
	400	396.73 ± 4.03	1.01	397.44 ± 2.786	0.70

Table 3. Recovery studies (n=3)

Drug	Label claim (mg)	Amount added (mg)	Total amount (mg)	Amount recovered (Mean ± SD)	% RSD	% Recovery
RZL	20	16 (80%)	36	35.35 ± 0.401	1.13	98.19
	20	20 (100%)	40	39.46 ± 0.383	0.97	98.65
	20	24 (120%)	44	43.04 ± 0.917	2.13	97.81
ACF	200	160 (80%)	360	358.71 ± 1.289	0.35	99.64
	200	200 (100%)	400	399.76 ± 0.220	0.05	99.94
	200	240 (120%)	440	440.20 ± 0.346	0.07	100.04

Table 4. Summary of results of validation of HPTLC method

Sr. No.	Validation Parameter	Result	
		Rabeprazole sodium	**Aceclofenac**
1.	Linearity		
	• Regression equation	82.787x - 39.383	12.314x - 62
	• Regression coefficient (r^2)	0.998	0.998
2.	Range	10-80 ng/band	100-800 ng/band
3.	Precision	(% RSD)	(% RSD)
	• Repeatability	1.64	0.81
	• Intra-day precision	0.92	1.13
	• Inter-day precision	2.93	0.87
4.	Accuracy	% Recovery	% Recovery
	• 80%	98.19	99.64
	• 100%	98.65	99.94
	• 120%	97.81	100.04
5.	LOD	3.02 ng/band	29.78 ng/band
6.	LOQ	9.15 ng/band	90.26 ng/band
7.	Specificity	Specific	Specific
8.	Robustness	(% RSD)	(% RSD)
	• Change in MP composition	1.97	1.42
	• Time from spotting to chromatography	1.38	1.51

3.6.1 Stability analysis of RZL

Acid degradation of RZL has led to formation of two degradation products with % recovery of 88.88. Base degradation formed two products with recovery of 93.05%. Similarly, two degradation products are formed each during the water and hydrogen peroxide induced degradation with % recovery of 79.81 and 80.55 respectively. In case of photolytic degradation, drug showed more stability with no degradation and formation of single peak. RZL is unstable in excess heat according to storage conditions specified in IP and it has been evident in dry heat degradation where two degradation products formed with 78.24% recovery.

3.6.2 Stability analysis of ACF

Stability study of Aceclofenac indicated more loss of drug with less drug recovery. The acid, base, and water induced degradation of ACF showed the % recovery of barely 21.72, 21.24 and 23.17 with formation of five, three and five degradation product respectively. In contrast, the photolytic degradation and dry heat degradation demonstrated 88.77 and 96.58% recovery. ACF needs to be protected from light because of instability and it has substantiated during its photolytic degradation. Result of stability analysis of RZL and ACF are illustrated and summarized in Figs. 5,6,7,8 and Table 5.
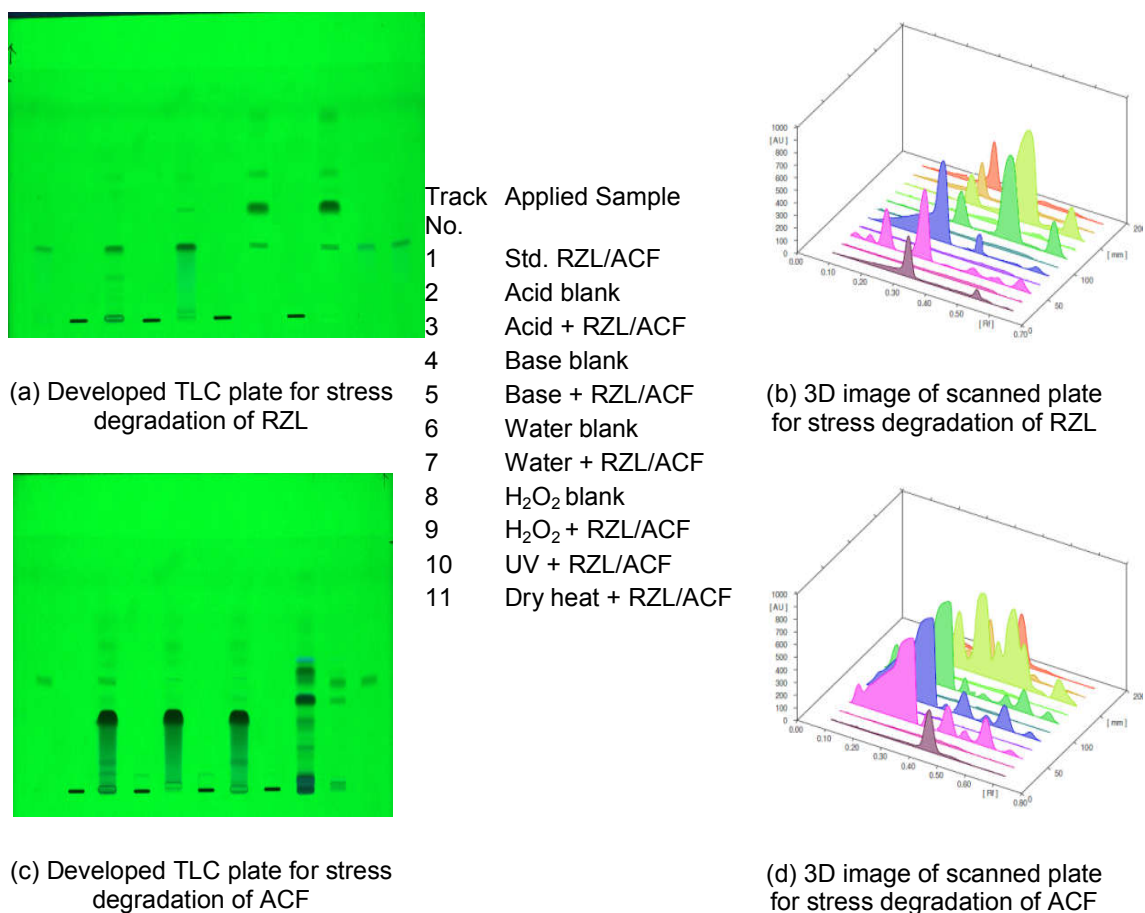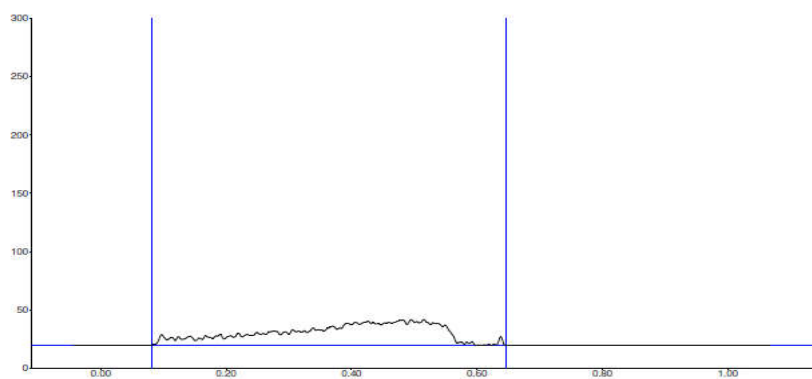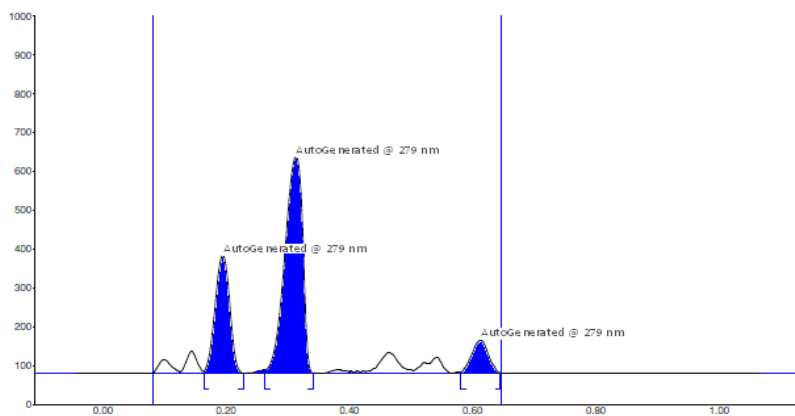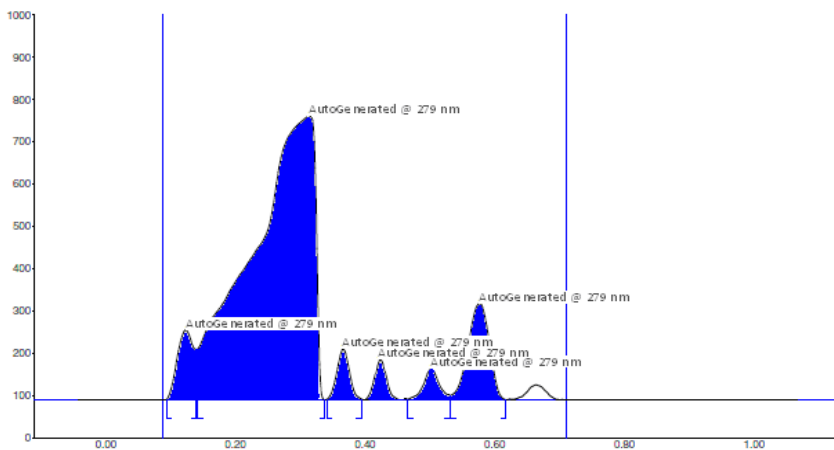

(a) Developed TLC plate for stress degradation of RZL

(c) Developed TLC plate for stress degradation of ACF

Track No.	Applied Sample
1	Std. RZL/ACF
2	Acid blank
3	Acid + RZL/ACF
4	Base blank
5	Base + RZL/ACF
6	Water blank
7	Water + RZL/ACF
8	H_2O_2 blank
9	H_2O_2 + RZL/ACF
10	UV + RZL/ACF
11	Dry heat + RZL/ACF

(b) 3D image of scanned plate for stress degradation of RZL

(d) 3D image of scanned plate for stress degradation of ACF

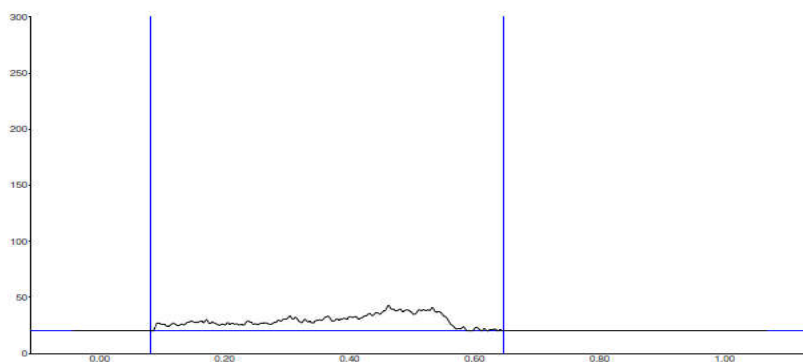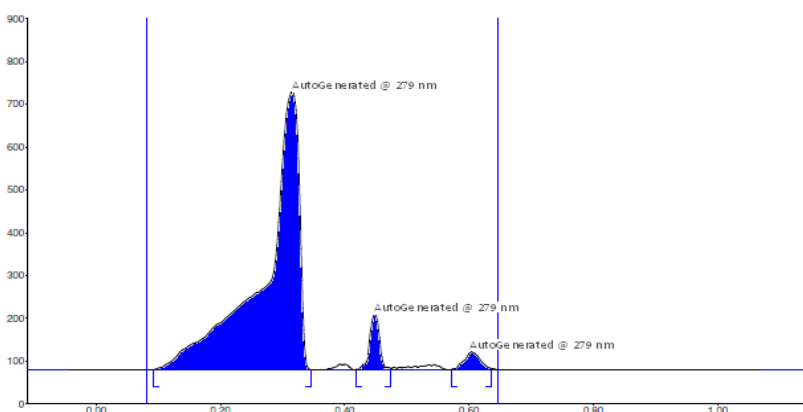**Fig. 4. Stress degradation studies of RZL & ACF**
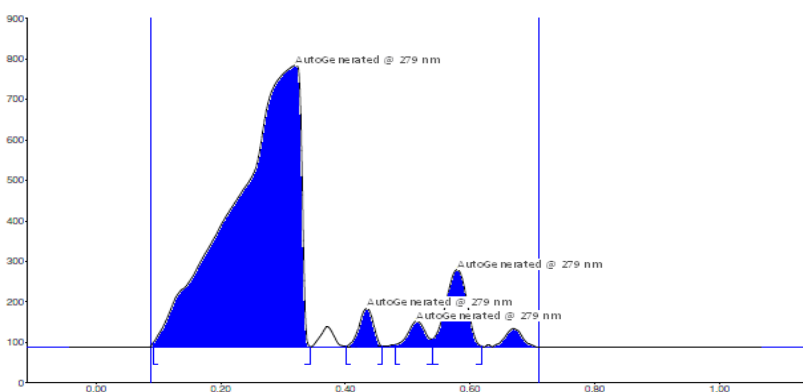
(a)

(b)

(c)

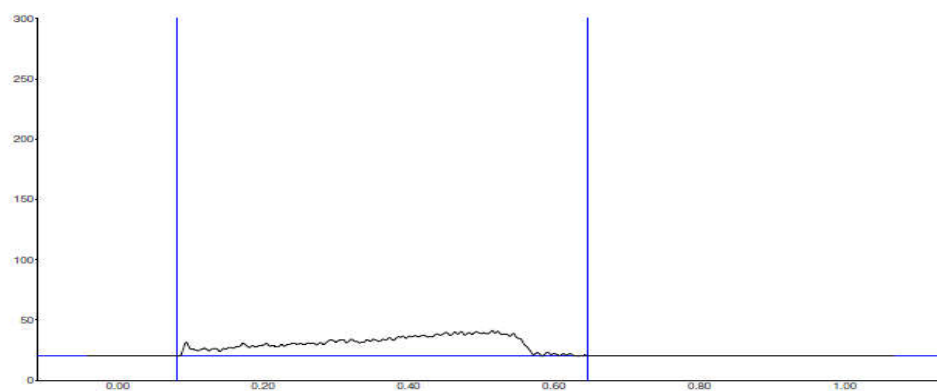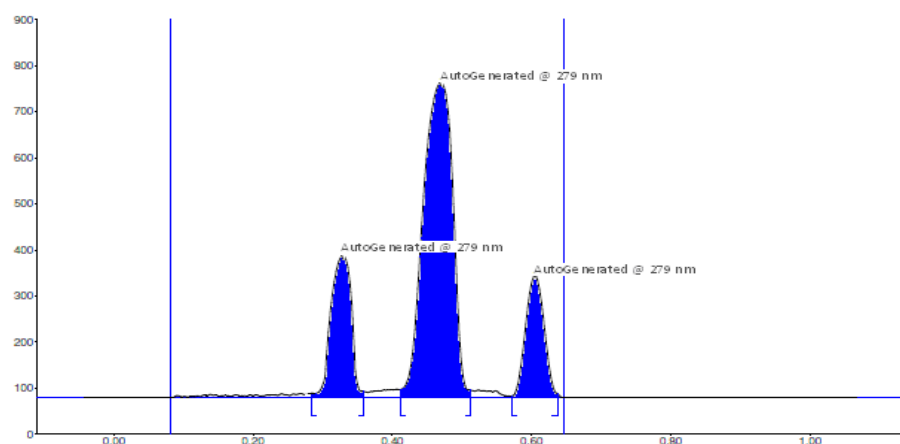**Fig. 5. a) Acid blank b) Acid hydrolysis of RZL C) Acid hydrolysis of ACF**
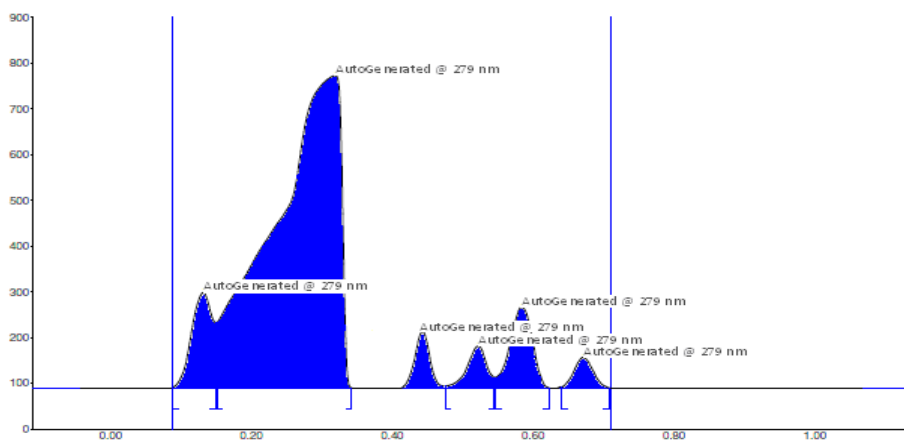
(a)

(b)

(c)

Fig. 6. a) Base blank b) Base hydrolysis of RZL C) Base hydrolysis of ACF
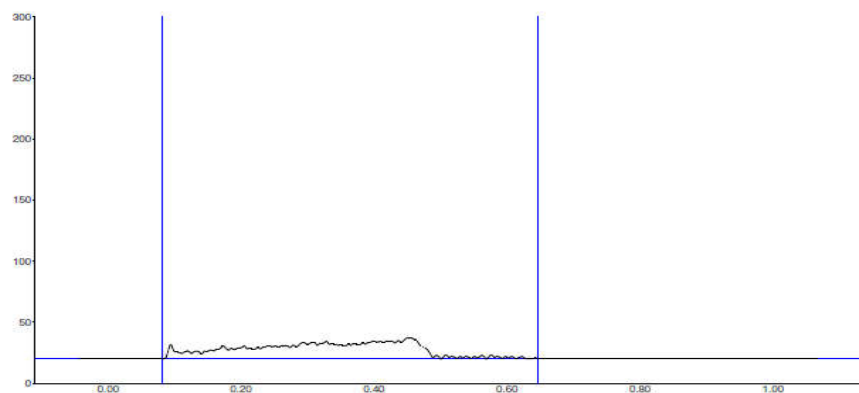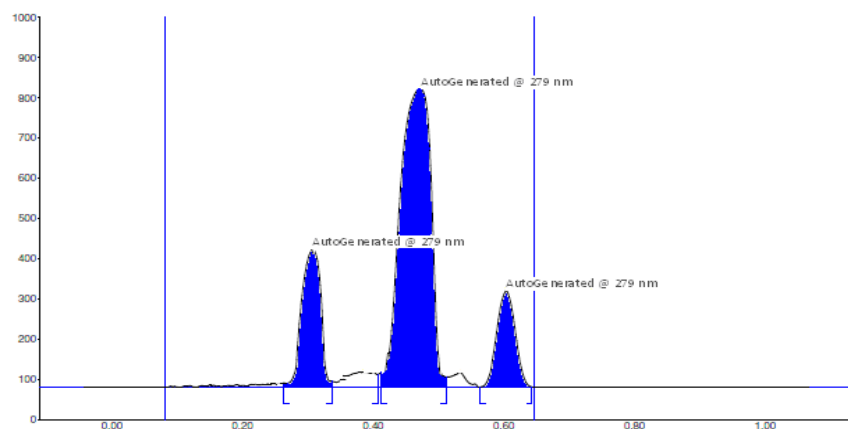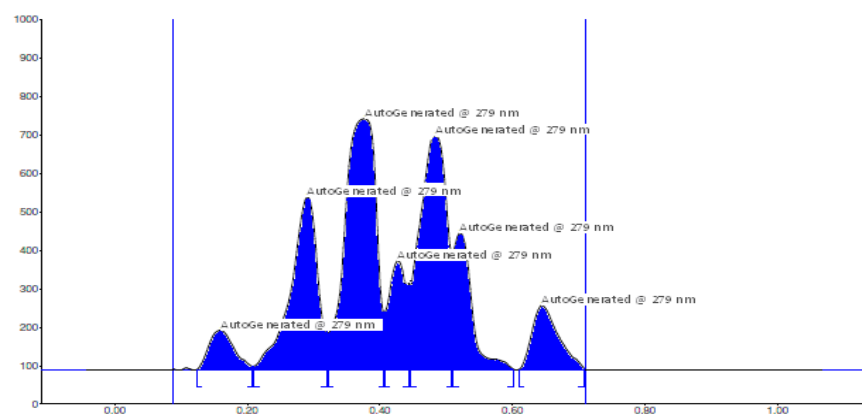
179

(a)

(b)

(c)

Fig. 7. a) Water blank b) Water hydrolysis of RZL C) Water hydrolysis of ACF

(a)

(b)

(c)

Fig. 8: a) H$_2$O$_2$ blank b) Oxidation of RZL by H$_2$O$_2$ C) Oxidation of ACF by H$_2$O$_2$

Table 5. Summary of result of stress degradation study

Sr. No.	Stress degradation condition	Rabeprazole sodium			Aceclofenac		
		Drug Peak area	No. of degraded products with R_f value	% drug recovered	Drug Peak area	No. of degraded products with R_f value	% drug recovered
1	Standard drug	8018.4	-	-	8925.6	-	-
2	Acid blank	-	-	-	-	-	-
3	Acid degradation	7126.9	Two 0.20, 0.61	88.88	1938.9	Five 0.12, 0.32, 0.37, 0.50, 0.58	21.72
4	Base blank	-	-	-	-	-	-
5	Base degradation	7461.2	Two 0.43, 0.61	93.05	1896.3	Three 0.32, 0.52, 0.58	21.24
6	Neutral blank	-	-	-	-	-	-
7	Neutral degradation	6399.5	Two 0.47, 0.61	79.81	2068.0	Five 0.13, 0.32, 0.52, 0.58, 0.67	23.17
8	H_2O_2 blank	-	-	-	-	-	-
9	H_2O_2 degradation	6459.2	Two 0.47, 0.62	80.55	3698.1	Six 0.16, 0.29, 0.38, 0.49, 0.52, 0.65	41.43
10	UV degradation	6222.6	-	77.60	7923.7	Two 0.29, 0.37	88.77
11	Dry heat degradation	6273.8	-	78.24	8620.5	-	96.58

4. DISCUSSION AND OVERALL CONCLUSION

The control of drug substance stability is a serious subject to the pharmaceutical industry. Stability may get influenced by variety of environmental factors affecting the safety and efficacy of bulk products. The stability gives idea about degradation pathways and degradation products which may help during formulation development. Therefore, it is necessary to firmly manage the safety of products and to determine the stability at all stages of production from raw material to finished product. The chromatography plays important role in stability evaluation. The applications of chromatography have grown extensively in last fifty years, owing not only to the development of several new types of chromatographic techniques introduced or available in the market, but also to growing need by scientists for better methods for characterizing complex molecules. Indeed, the utmost advantage of the chromatographic method over any other analytical procedure is the ability to separate specific analyte, a feature that appeals to all branches of science, which enables to discover and analyze unknown elements and chemical compounds. HPTLC is a fast separation technique and flexible enough to analyze a wide variety of drugs. It evaluates the entire chromatogram with a variety of parameters without time limits. Moreover, there is simultaneous but independent development of multiple samples and standards on each plate, leading to an increased reliability of results.

Current research was based on the development and validation of HPTLC method for stability indication of standard drug molecules. For experimental work, advance HPTLC instrumentation techniques by Camag was used with less manual activities and more automation which has helped to reduce the errors. For example, sample application was carried out by using automatic TLC sample 4. It had led to the application of exact quantity of volume on plate and thus more accurate interpretation of results. The stationary phase consisted of silica gel 60 F_{254}. Silica gel 60 is a unique polymeric binder that results in uniform and hard surface that will not easily crack or blister. It was polar in nature for separation of polar compounds. The smooth and extremely dense plate coating ensures narrow bands and maximum separation efficiency with the lowest background noise. Layer thickness was 0.2 mm supported with flexible aluminium plates that can easily be cut to match individual separation requirements. F_{254} was an inorganic green fluorescent indicator for UV detection of colorless substances chemically bonded to stationary phase.

Each compound travels different distances up on the plate depending on the solvent. A good solvent system is one that moves all components of mixture off the baseline, but does not put anything on the solvent front i.e. R_f values between 0.15 to 0.85. This is not always possible, but should be tried out. According to these specifications, the suitable selected solvent in this study was methanol because both the drugs show significant solubility in methanol. It is also useful to elute the samples quickly with good retention. However, the combination of different solvents was used for better resolution and separation. The mobile phase used, consisted of toluene and ethyl acetate as a carrier solvent for the movement of polar compound, methanol for better interaction with stationary phase, acetic acid forms more dense band on plate while ammonia work as mobile phase modifier to improve peak shape and increase sample load tolerance. The validation of HPTLC method indicated that the method is simple, precise, robust, sensitive, and reproducible for estimation of drugs in combination. The literature for development and validation of HPTLC method for estimation of RZL & ACF is available [23]. However, the results obtained in this study are better in terms of separation and validation indicating the superiority of developed method in present work.

Stability study of RZL and ACF demonstrated satisfactory results. The type of degraded product formed, R_f value and the percent drug recovery variation showed the differences from the results reported in literature for drug degradation by other analytical methods [17,19-20] because of the nature, methodology and extent of degradation. The study activity was limited till the formation of chromatogram of degraded drug sample, its approximate interpretation, nevertheless there is a scope for the separation, quantification and analysis of the degraded products. By observing the retention factor of the degraded products formed, it has been found that few of the degraded products are having approximately same R_f value and peak shapes for different degradation pathway, indicating the structural similarity between the degraded products but it was based on assumptions only. For determination of types of degraded products, the further structural

identification analysis such as IR, NMR, Mass Spectrometry etc. needed to be carried out. However, these limitations cannot reject the fact that, the HPTLC method is simple, accurate, rapid and useful for stability indication of RZL and ACF molecules.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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