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Development and Validation of Stability-Indicating HPTLC Method for Mirabegron with Identification, and Characterization of Degradant by ESI-MS

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ABSTRACT

The objective of the present study is to elucidate the structure of the degradants of Mirabegron by High-performance thin layer-Tendom mass spectrometry, advising its structure elucidation, identification of its pathways of degradation, and establishing a stability-indicating assay method. In the present study, comprehensive stress testing of Mirabegron was carried out according to ICH guideline Q2 (R2) and a stability-indicating assay method for the identification, quantification, and characterization of the drug Mirabegron and its degradants, and the founding of pathways of acidic and alkaline degradation. Stability studies were conducted using stress conditions such as hydrolytic, oxidative, thermal, and photolytic conditions and degradation products were investigated and characterized by HPTLC-MS as per ICH guideline Q2 (R2)]. The current study develops a novel analytical method for separating drugs from the degradation products that is formed under stress conditions by utilizing Aluminum plates precoated silica gel G $60F_{245}$ and *n*-Butanol: Methanol: Water: Ammonia (6:2:2:0.2 v/v/v/v) optimized mobile phase. Quantification was done by densitometric analysis at wavelength 257 nm. The method produced a resolved band for Mirabegron (R_f) (0.67 ± 0.014). A linearity study was done in the range of 100-700 ng/band and the linear relationship in the calibration curve with R^2= 0.997. The Limit of Detection and Limit of Quantification was 0.047 and 0.143 ng/band obtained, respectively. Developed and validated a new, specific, sensitive, and economical High-Performance Thin-Layer Chromatography method for Mirabegron a β 3-receptor agonist for the determination in bulk powder and tablet. Precision and recovery results as per as the International Conference on Harmonization guidelines were found to be in good range. The molecule is sufficiently stable under various stress tests. However, the degradation product formed in alkaline and oxidative hydrolytic stress has been used in LC-Q-TOF-MS/MS for characterization and the degradation pathway of the drug have been proposed.

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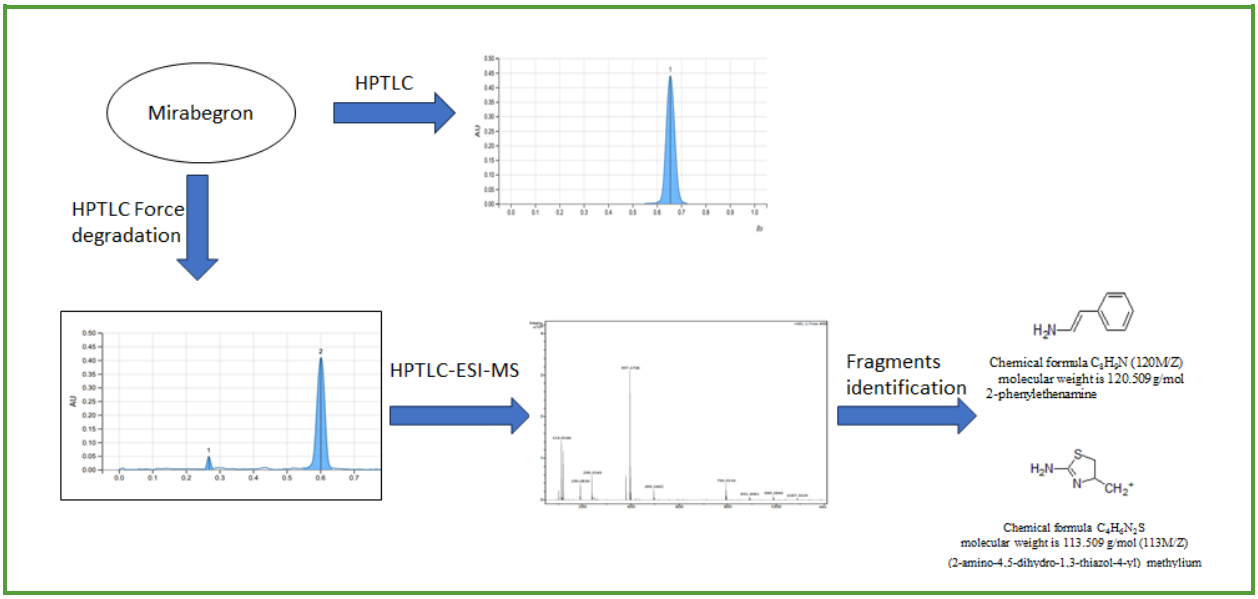
Graphical Abstract



Introduction

High-Performance Thin Layer Chromatography is a robust and highly valuable tool for small molecule separation and purity identification of chemicals, insecticides, steroids, water, and natural, packed food in quantitative and qualitative evaluation [1]. HPTLC method is extremely used for stability testing of bulk drugs and formulations, HPTLC-MS is a famous convenient sophisticated, and powerful analytical technique proved for the separation of individual components from the combination and identification of compounds and their degradants [2].

Mirabegron is a beta-3 adrenergic agonist clinically used in overactive bladder (OAB) conditions. It relaxes the bladder's smooth muscles by stimulating beta-3 adrenergic receptors. It promotes bladder filling and increases urine storage capacity [3]. Chemically, It is 2-(2-aminothiazol-4-yl)-N-[4-(2-{[(2R)-2-hydroxyphenylethyl]-amino}ethyl)phenyl]acetamide (Figure 1) and the 396.506 g/mol^{-1} is the molecular weight (Figure 1) [4]. The drug is primarily used to relieve symptoms without disrupting the empty phase of the micturition cycle [5, 6].

Numerous methods have been used for the quantitative and qualitative identification of Mirabegron in the formulation and matrix. UPLC-MS/MS method for Mirabegron assay in rat plasma [7], UPLC-QTOF-MS/MS method, *in silico* toxicity prediction of its all-degradants [8], LC-MS/MS resolve of Mirabegron in human plasma along with separation of metabolites [9], and HPTLC method for Mirabegron and solifenacin succinate by simultaneous estimation [10]. Since no HPTLC-ESI-MS method for the analysis of Mirabegron was stated, the current work reports the development of a novel, accurate, sensitive, and precise HPTLC-ESI-MS technique for the quantification of Mirabegron in bulk and formulation. In a given study, the degradation products characterized and elucidated the degradation pathway.

Experimental

Material and Methods

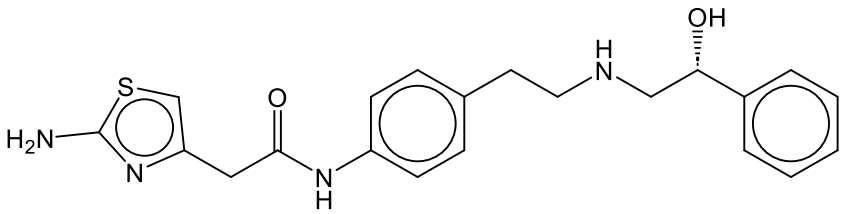


Figure 1. Chemical structure of Mirabegron

Mirabegron was supplied as a gift sample by Zim Laboratories Ltd. (Mumbai, India) with 99.87% purity. All needed chemicals and reagents used were procured from Merck Pvt. Ltd. (Mumbai, India).

Chromatographic parameter

Methanol was used to prewash HPTLC plates and dried at 100 °C for 5 min. With a Camag microliter syringe, the drug standard and sample bands of 6 mm width were applied on precoated silica gel aluminum plates 60 F254 (20×10 cm and 200 mm), using a Camag Linomat 5 applicator. 6 × 0.45 mm as the slit dimension and 20 mm/s scanning speed set. The optimized mobile phase was n-Butanol: Methanol: Water: Ammonia (6:2:2:0.2 v/v/v/v) and. Separation was carried out in a 20×10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) which was saturated with the optimized ratio of the mobile phase. Chamber saturation time was 30 min at room temperature (25 ± 2 °C). Approximately 70 mm was the distance of the densitogram run. HPTLC plates were dried with a dryer. For densitometric scanning, Camag TLC scanner 3 was used and was operated by the winCATS software (Version 3.1.21109.3) Mode. The degradation product was identified using the Impact II UHR-TOF Mass Spectrometer System, Mass range: 100 to 3500 m/z with Ionizer Electrospray Ionization (ESI) and Mass resolution: of 50,000.

Method validation

After analytical method development validation, it is important to enhance proficiency in chromatographic separation, accuracy, and recovery. As per ICH guidelines, developed and validated the HPTLC study for various parameters [10].

Selectivity

The selectivity was studied by analyzing Mirabegron samples. The densitogram of Mirabegron in Figure 2 did not show any interfering compound.

Specificity

The specificity was resolute by analyzing the stress degradation action of Mirabegron in different stress conditions. The degradants were well resolved. Peak purity calculation was carried out on the stressed samples of Mirabegron and this parameter was also established by subjecting the degradant to HPTLC-MS analysis. HPTLC-MS analysis showed a good mass purity for Mirabegron and its degradants, which also presented the specificity of the method. The densitogram of the Mirabegron sample is shown in Figure 2.

Linearity

The stock standard solution was 1000 µg.mL^{-1} concentration prepared. Aliquots of standard solutions 0.1-0.7 mL of Mirabegron were shifted into 10 mL volumetric flasks, and volumes were made with methanol. A volume of 1µL was loaded with a microliter syringe, using a Linomat

5 applicator on an aluminum silica gel plate to get 100-700 ng/band concentrations. The curves were evaluated for intra-day and inter-day precision. The procedure was repeated six times.

Precision

Six replicates (100 ng/band) were used for repeatability and measurement of peak area. The intermediate precision for the intra-day variation experiments was studied using 3 different concentrations within a day. The inter-day changes in the methods were evaluated by analyzing 3 concentrations for three different days. The intra-day and inter-day change for the identification was considered at levels of 400, 500, and 600 ng/band.

Limit of detection and limit of quantification

Mirabegron concentrations in the lower part of the calibration curve were used for the Limit of detection and quantification determination. Mirabegron of 100-700 ng/band concentration was spotted on a silica gel plate.

Robustness

Mobile phases having various compositions like *n*-Butanol: Methanol: Water: Ammonia (5.9:2.1:2:0.1 v/v/v/v), *n*-Butanol: Methanol: Water: Ammonia (6:2:2:0.1v/v/v/v) were tried and Chamber saturation time was changed 20 and 30 min, and densitograms were recorded. Result of robustness is indicated in Table 1.

Analysis of a marketed formulation

Weighed twenty tablets on Metlar toledo balance (ME 204), and finely powdered. taken weight of the powder equivalent to 100 mg of Mirabegron in 50 mL of methanol and kept for sonication for 30 min, and finally 100 mL volume make up with methanol. The final solution for 5 min centrifuged at 3000 rpm, and then filtered using a 0.41 µm filter (Millifilter, Milford, MA). The final solution was to get a sample solution of 100 µg.mL^{-1} further diluted. 2 mL was diluted to 10 mL with methanol and 1 µL was loaded to the HPTLC plate.

Forced degradation study

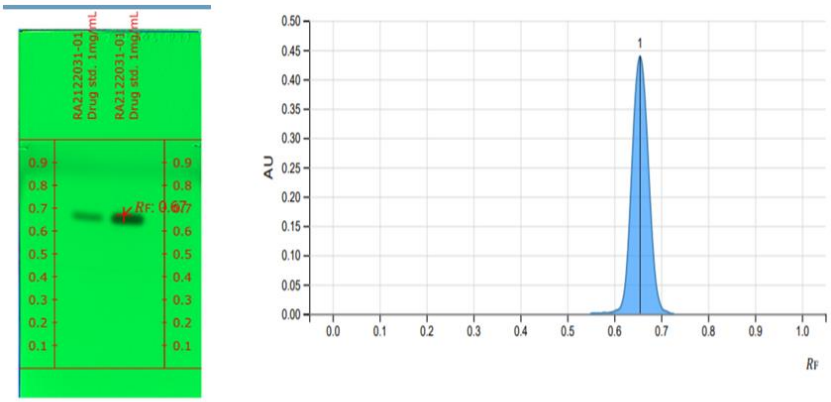
A stock solution containing (1000 µg. mL^{-1}) Mirabegron was prepared. This solution was used for forced degradation to indicate the stability-indicating property and specificity of the proposed method. In all degradation studies, the average peak area of Mirabegron after application (100 ng/band) of replicates was obtained. The stress study was conducted under different stressor conditions such as acidic, basic, oxidative, photolytic, thermal, and hydrolytic. Result of robustness is indicated in Table 2.

Acidic and basic condition

10 mg of Mirabegron was dissolved in a 2 mL of 0.1 N HCl, and 0.1 N NaOH. These solutions were kept for a day in the dark. 1 mL of the resulting solution was taken separately and neutralized, then with methanol volume made up to 10 mL. The final solution was applied on a TLC plate (100 ng/band).

Oxidative condition

10 mg Mirabegron was dissolved in 5 ml of methanolic H_2O_2 solution (3% v/v). The solution was kept for a day at room temperature. The final solution (100 ng/band) was spotted on a TLC plate.



(a) (b)

Figure 2. Photo documentation plate (a) Densitogram of standard Mirabegron 1000 ng/band R_f (0.67 ± 0.014) in *n*-Butanol: Methanol: Water: Ammonia (6:2:2:0.2 v/v/v/v) mobile phase set at 254 nm (b)

Table 1. Results of robustness evaluation

Parameter	Amount of drug spotted (ng/spot)	Area	%RSD
Mobile phase composition n-Butanol: Methanol: Water: Ammonia (5.9:2.1:2:0.1 v/v/v/v)	100	0.00255+0.00002	0.393701
Mobile phase ratio n-Butanol: Methanol: Water: Ammonia (6:2:2:0.1 v/v/v/v)	100	0.00247+0.00002	0.404858
Chamber saturation time:20 min	100	0.00250+0.00005	1.4
Chamber saturation time: 30 min	100	0.00241+0.00006	1.7

Table 2. Result of assay evaluation

Sr. No.	Concentration (ng/spot)	Area	Percentage	SD	RSD
1	300	0.0056	98.178939	1.04377016	1.05322107
2	300	0.00564	99.0657165		
3	300	0.00562	98.6223278		
4	300	0.00565	99.2874109		
5	300	0.00561	98.4006334		
6	300	0.00573	101.060966		

Table 3. Degradation condition for Mirabegron

Sr. No.	Stress condition	Exposure condition
1.	Acidic hydrolysis	0.1 N HCl, 24 h at room temperature
2.	Alkaline hydrolysis	0.1 N NaOH, 24 h at room temperature
3.	Thermal	60 °C for 16 h
4.	Photolytic	Exposure to sunlight, 24 h
5.	Oxidation	3% H_2O_2, 24 h at room temperature

Photolytic condition

In a photolytic study, about 10 mg of Mirabegron was exposed to (60,000-70,000 lx) for 24 h. The intensity of sunlight was measured by a calibrated lux meter. 1 mL of the stock solution was taken separately and diluted up to 10 mL with methanol. The final solution (100 ng/band) was spotted on a plate.

Hydrolytic condition

10 mg Mirabegron was dissolved in water and kept at room temperature for 24 h. About 1 mL of the solution was separately taken and volume was made up with methanol. The final solution (100 ng/band) was spotted on a plate.

Thermal condition

In the thermolytic study, about 10 mg drug was kept to heat in an oven at 60 °C for 16 h, and then the drug was dissolved in methanol and added to the mark with the same solvent (100 ng/band). The resultant solution was loaded on a TLC plate. All the above silica gel plates were developed and scanned as per the optimized chromatographic conditions, as indicated in Table 3.

Characterization of degradations

In alkaline and oxidative stressor, from stock solution 1 µL was taken and loaded on the TLC plate. The plates were kept in a saturation chamber for separation. Densitogram of alkaline and oxidative degradation product is shown in Figures 3 and 4 and results are given in Table 4. ESI-MS studies provide fragments of degradation products. MS helps in the identification and characterization of each separated fragment.

Results and Discussion

Development of optimized mobile phase

Various mobile phase compositions for HPTLC were tried to attain highly resolved and reproducible peaks. In the n-Butanol: Methanol: Water: Ammonia (6:2:2:0.2 v/v/v/v) mobile phase drug showed good sharpness when scanned at 257 nm.

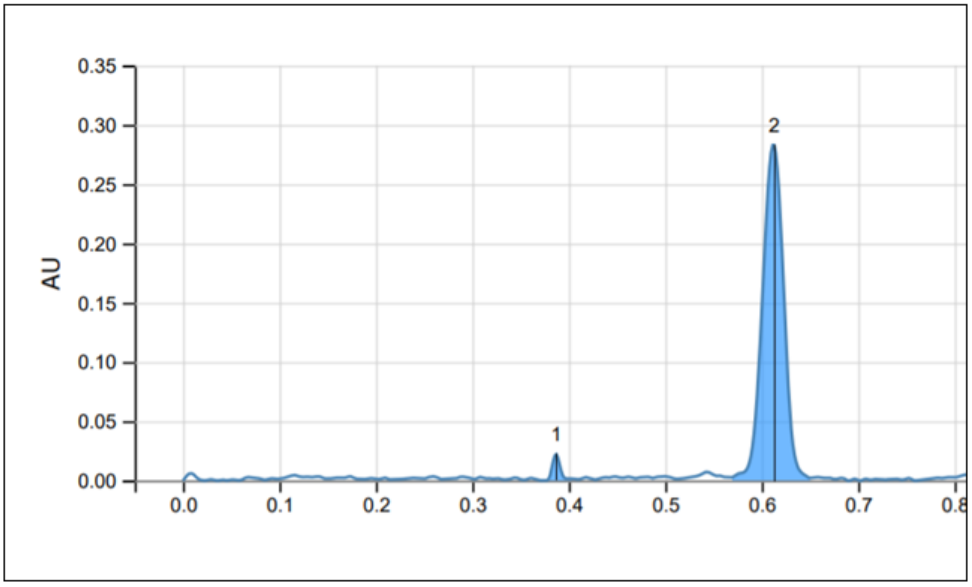


Figure 3. Densitogram of Mirabegron standard drug subjected to base degradation for 24 h room temperaure (Mirabegron Rf=0.61, $DP1$ Rf=0.38). 0.1 N NaOH

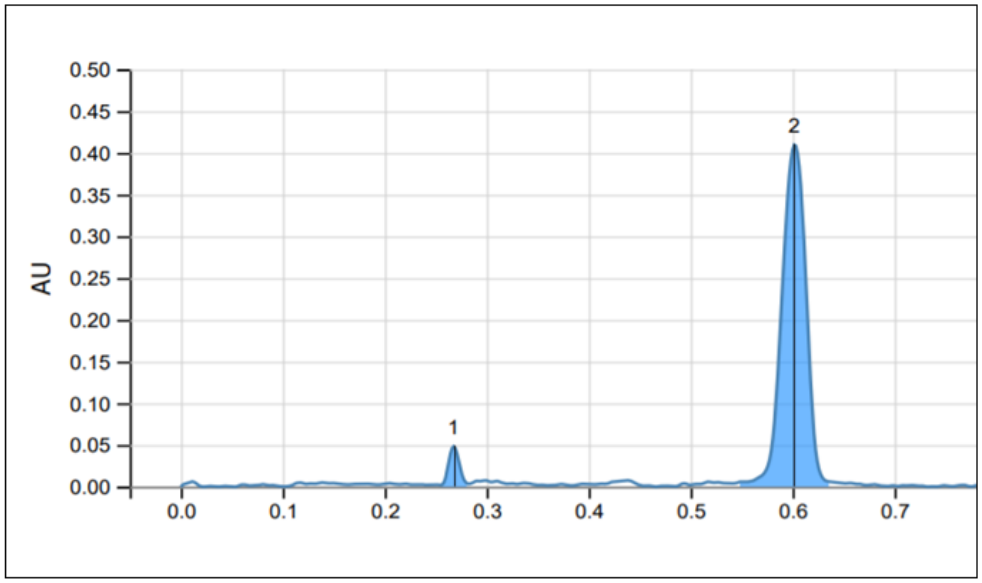
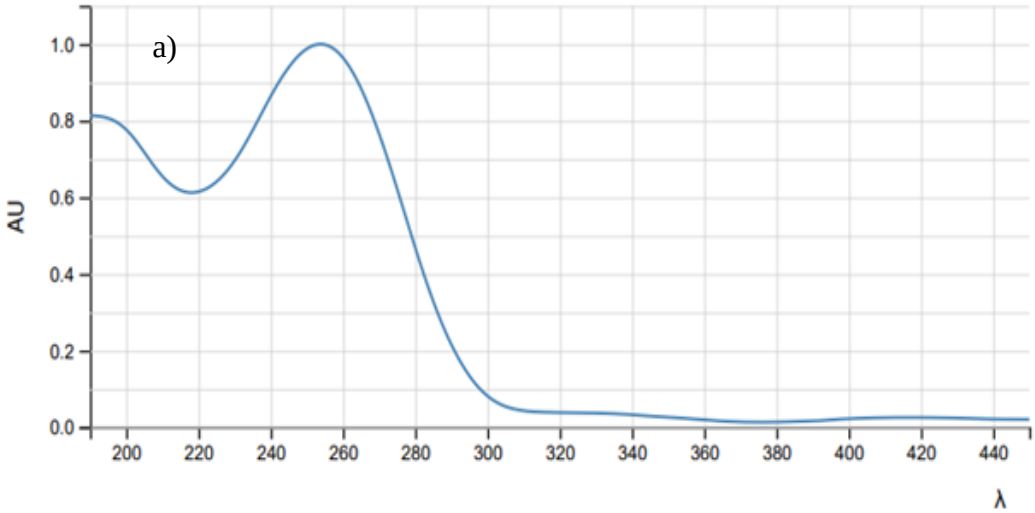


Figure 4. Densitogram of Mirabegron standard drug subjected to 3% H_2O_2 degradation for 24 h room temperature (Mirabegron Rf = 0.63)

Table 4. Results of degradation studies of Mirabegron

Stress condition	Exposure Conditions	degradation product (%)
Acid hydrolysis	0.1N NaOH, for 24 h, room temperature	6.81
Alkaline hydrolysis	0.1 N HCl, for 24 h, room temperature	5.82
Neutral hydrolysis	H_2O, 24 h	4.50
Oxidation	3% H_2O_2, for 24 h, room temperature	10.54
Thermal	60 ◦C, 24 h	4.50
Photolytic	Exposure to UV light, 30 days	0.0



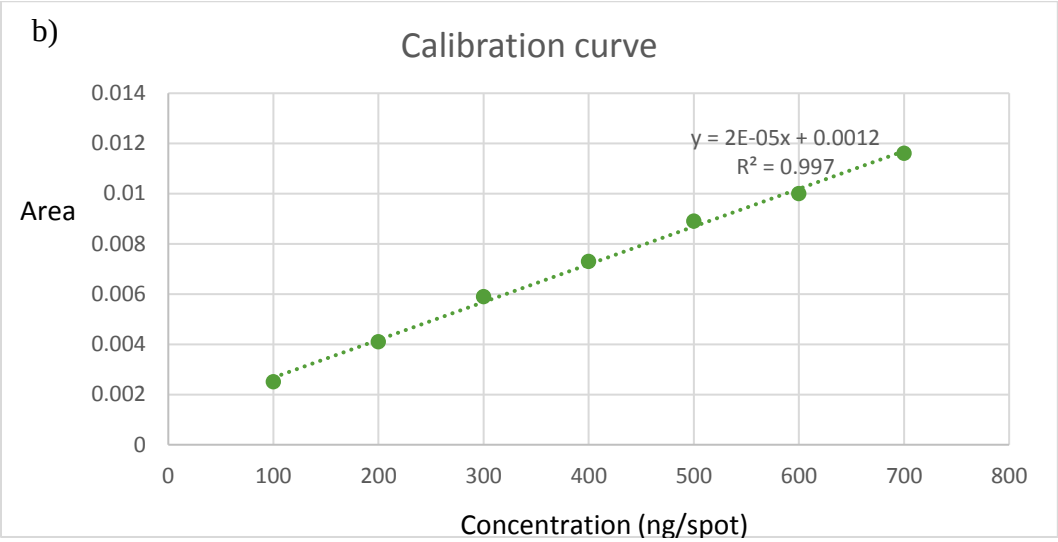


Figure 5. a) UV spectrum of standard Mirabegron (100 mg/ml) in methanol and b) calibration curve

Table 5. Result of calibration evaluation: linearity and range of MIR

Sr. No.	Concentration (ng/spot)	Area (AU)*
1	100	0.0025
2	200	0.0041
3	300	0.0059
4	400	0.0073
5	500	0.0089
6	600	0.0100
7	700	0.0116

**n=3*

Sharp peaks for the Mirabegron were obtained at R_f (0.67 ± 0.014). Chamber saturation with mobile phase time was 25 min at room temperature. The UV spectrum of a Mirabegron is revealed in Figure 5a while the Photographic plate and densitogram of a standard Mirabegron are shown in Figure 2a and b.

Calibration curve

The calibration curves showed a good linearity over the range of 100-700 ng/band. A linear regression equation was noted Y=0.0002x+0.0012. The regression coefficient (R^2 = 0.997). Data of calibration curve is given in Table 5 and calibration curve shown in Figure 5b.

Precision

The % RSD value for repeatability of the sample and Mirabegron was noted >2. The results show the high precision of the method, as listed in Table 6.

LOD and LOQ

The LOD and LOQ were noted as 0.047 and 0.143 ng/band, respectively. It shows the sensitivity of the method is acceptable. The results of LOD and LOQ is given in Table 7.

Robustness

For each parameter, SD of peak area was calculated and % RSD was noted > 2%. The low

value of % RSD indicates the consistency present Table 1.

Recovery

The % mean recovery was found to be 97.67% for MIR which indicates the planned method is accurate for the assessment of a drug in the formulation. The amount of spiked drug was resolute and the percentage recovery is presented in Table 8.

Specificity

It was found that excipients of formulation not interfere with the peak of Mirabegron R_f (0.67 ± 0.014).

LOD and LOQ

The % drug content of Mirabegron was 97.67%.

Table 6. Linear regression data of MIR

Parameter	MIR
Detection Wavelength (nm)	253 nm
Concentration range (ng/band)	100-700 ng/band
Correlation Coefficient (r2)	0.997
Linear Equation (y = mx + c)	Y=0.0002x+0.0012
	R2=0.997
Slope (m)	0.0002
Intercept (c)	0.0012

**n=5*

Table 7. Result of LOD and LOQ evaluation

LOD	0.047 ng/ band
LOQ	0.143 ng/band

Table 8. Result of recovery evaluation

Drug	Amount taken (ng/band)	Amount spiked (ng/band)	Final amount (ng/band)	% Recovery*	SD*	% RSD
MIR	500	400	900	96.09	1.93	1.97
	500	500	100	98.73		
	500	600	1100	98.17		
		Avg.		97.67		

Table 9. Optimized conditions

Parameter	Conditions
Mobile Phase	n-Butanol: Methanol: Water: Ammonia (6:2:2:0.2 v/v/v/v)
Spot application volume	100ng/band
Wavelength	257 nm
Retention factor	0.67
Chamber saturation time	20 min

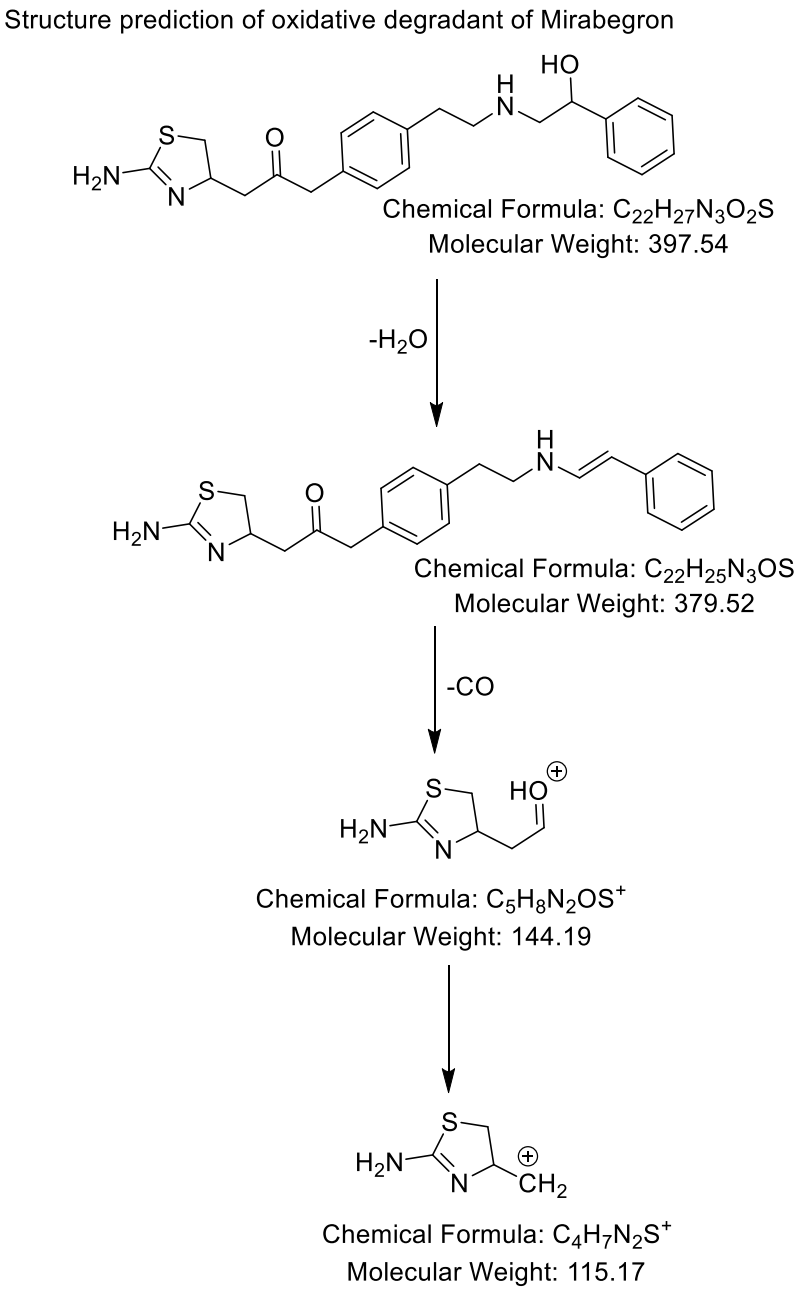


Figure 6. Degradation pathway of Mirabegron in oxidative stress condition

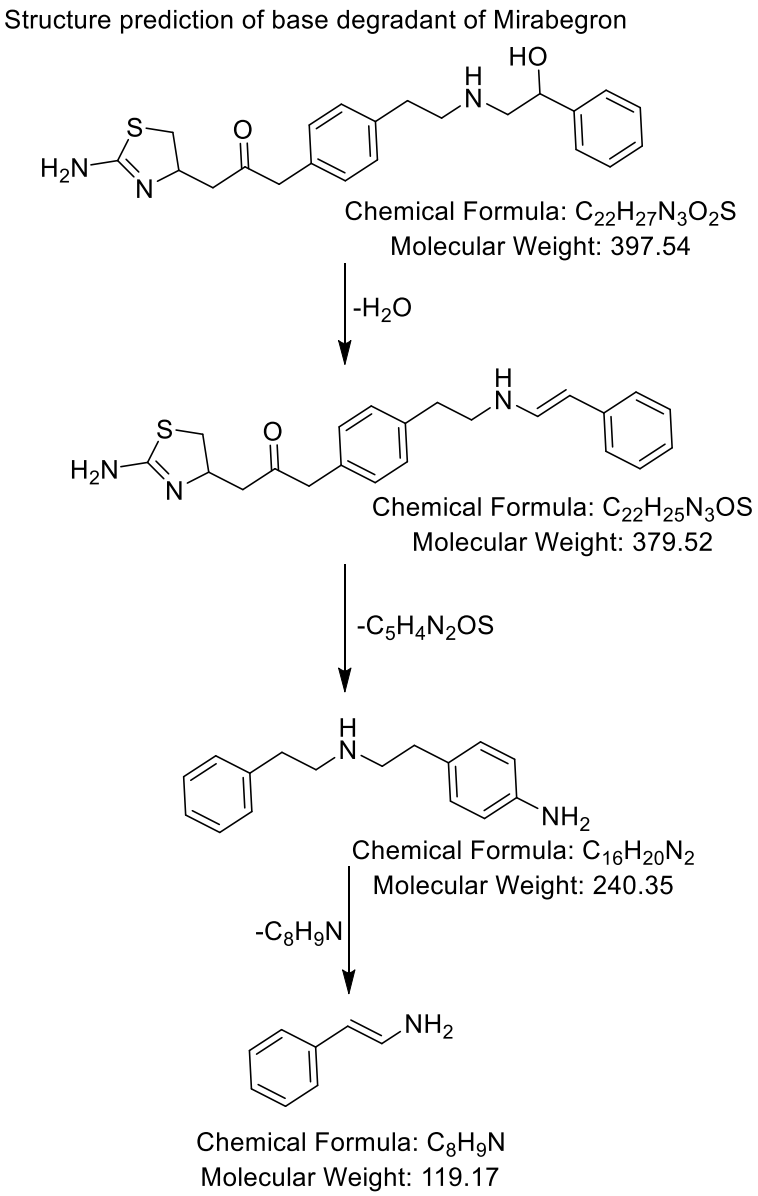


Figure 7. Degradation pathway of Mirabegron in alkaline stress condition

Optimized conditions

The chromatographic conditions for the developed method are as shown in Table 9. The densitogram of Mirabegron in the optimized condition is depicted in Figure 2.

ESI-MS interpretation of degradation product

The drug was analyzed by ESI-MS/ MS combined with precise mass studies to find its degradants.

ESI-MS spectra of oxidative degradation

The spectrum of MIR (m/z 397) protonated displays the product ions at m/z 379 (loss of H_2O from m/z 397), m/z 141 (loss of $C_{16}H_{18}N_2$ from m/z 379), and m/z 113 (loss of CO from m/z 141).

The base peak at m/z 113, which was also noted in the MS/MS of a protonated drug, specifies the presence of (2-amino-4,5-dihydro-

1,3-thiazol-4-yl) methylium as DP-1 (Figure S1, supporting information).

ESI-MS spectra of alkaline degradation

The range of protonated MIR (m/z 397) displays the product ions at m/z 379 (loss of H_2O from m/z 397), m/z 239 (loss of $C_5H_4N_2OS$ from m/z 379), and m/z 120 (loss of C_8H_9N from m/z 239). The base peak at m/z 120, which shown in the MS/MS of a protonated Mirabegron, give idea of the occurren (Figure S2, supporting information).

Degradation pathway of MIR

Degradation pathway of Mirabegron in oxidative and alkaline stress condition indicated in Figure 6 and 7.

Conclusion

A new, sensitive, and selective HPTLC method was developed for Mirabegron according to ICH guidelines. The method proved to be simple, accurate, precise, specific, and robust. It provided valued data regarding the degradation behavior of Mirabegron in different stress conditions. Characterization of alkaline and oxidative degradation products was studied by the ESI-MS method. The degradation product was identified as 2-phenylethenamine, (2-amino-4,5-dihydro-1,3-thiazol-4-yl) methylium derivative of Mirabegron. The proposed method is time-saving, efficient, and may be more beneficial for routine analysis of drugs in marketed formulation. The research described above can look for important information. The main separation of the degradation products of Mirabegron, its products, and degradation substances obtained in oxidative stress research is now more information for the development of research.

List of Abbreviations

HPTLC: High-Performance Thin Liquid Chromatography
MIR: Mirabegron
DP: Degradation product
ESI: Electro Spray Ionization
ICH: International Council for Harmonization
LC-MS: Liquid Chromatography-mass Spectroscopy
m/z: Mass to Charge
RSD: Relative Standard Deviation
TOF: Time of Flight

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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Supporting Information

Copy of HR-MS for (2-amino-4,5-dihydro-1,3-thiazol-4-yl) methylium and HR-MS spectra for 2-phenylethenamine.

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