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ORIGINAL

Comparison of Two Stability-Indicating Chromatographic Methods for the Determination of Mirabegron in Presence of Its Degradation Product

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Abstract Mirabegron is a novel β_3-adrenoceptor agonist containing an amide group. It was subjected to stress conditions of acidic and alkaline hydrolyses. The hydrolytic degradation product was isolated and its structure was confirmed using mass and IR spectrometry. Two stability-indicating chromatographic methods have been proposed for the determination of mirabegron. TLC method was applied using silica gel as stationary phase and chloroform–methanol–ammonia (9.0:1.0:0.1 by volume) as the mobile phase, and chromatograms were scanned at 250 nm. Accurate determination of the drug was achieved over the concentration range of 2–12 μg per band. In addition, an isocratic HPLC method was developed on Agilent C18 column (150 mm × 4.5 mm I.D., particle size 5 μm) using ethanol-phosphate buffer pH 2.5 (30:70, by volume) as a mobile phase with flow rate of 1 mL min^{-1}.The intact drug was detected at 250 nm with running time less than 5 min. Mirabegron was determined accurately in a concentration range of 1–25 μg mL^{-1}. The proposed chromatographic methods were applied successfully for the assay of mirabegron in pharmaceutical dosage form and both methods were validated as per the International Conference on Harmonization guidelines and statistically compared with a reported gradient HPLC method.

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Introduction

Overactive bladder syndrome is popular in 10–15% of all men and women. This lower urinary tract symptom has a serious effect on quality of life and social functioning. Three subtypes of β adrenoceptors (β_1, β_2, and β_3) have been identified in both the detrusor muscle and the urothelium [1]. It has been proven that stimulation of human β_3 adrenoreceptors relaxes the detrusor smooth muscle contributing to urine storage [2].

Mirabegron (MIR) has been introduced recently as β_3 agonist [1]. It is chemically designed as 2-(2-amino-1, 3-thiazol-4-yl)-N-[4-(2-{[(2R)-2-hydroxy-2-phenylethyl] amino}ethyl)phenyl]acetamide. MIR is the first of a new class of compounds under development for the treatment of overactive bladder by potent and selective activation of human adrenoceptors [2].

As a new drug, forced degradation studies should be included, as per regulatory requirements for registration, to increase the chemical information about the degradation pathways of active pharmaceutical ingredients in pure and pharmaceutical forms [3].

Reviewing the literature revealed few chromatographic methods for the determination of MIR either in pharmaceutical dosage form [4, 5] or in plasma [6]. Concerning the stability of MIR, there was only one reported stress degradation study [7] which claimed formation one, two and four degradation products upon neutral, acidic and basic hydrolysis, respectively, while MIR was stable towards thermal and photo decompositions.

This research focused on the identification and characterization of the main MIR degradation product (DEG) produced upon complete hydrolysis. Mass and IR spectrometry were used to identify the chemical structure of possible degradation. Furthermore, our aim was to develop simple stability indicating TLC and HPLC methods for MIR determination. Planar chromatography has never been proposed in the literature for MIR determination besides the proposed columnar chromatographic method using an isocratic eco-friendly mobile phase was applied on different commercially available C18 columns for comparison. The proposed chromatographic methods were optimized for efficient separation of MIR and its degradation product. The developed methods were applied for commercial pharmaceutical dosage form analysis.

Experimental

Materials and Reagents

Mirabegron pure sample was purchased from Holypharm Biotech company (Hangzhou, China), Its purity was found to be 99.99% according to a reported HPLC method [4].

Betmiga prolonged-release tablets manufactured by Astellas Pharma Ltd, batch No.14G25/44 and labeled to contain 50 mg of mirabegron per tablet.

All chemicals used throughout the work were of analytical grade and solvents were of spectroscopic and HPLC grade. These included hydrochloric acid and sodium hydroxide (Merck Darmstadt, Germany), sodium dihydrogen phosphate (El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt), methanol, ethanol, chloroform (Sigma Aldrich, Darmstadt, Germany), ammonia solution 33% (Adwic,Cairo, Egypt) and double distilled deionized water (Otsuka, Cairo, Egypt). Phosphate buffer solution pH 2.5 was prepared by dissolving 7.8 g of sodium dihydrogen phosphate in 900 mL of double distilled deionized water, its pH was adjust with phosphoric acid to 2.5 and diluted to 1000 mL with the same solvent [8].

Instruments

Camag® flat bottom chamber for TLC with scanning system consisted of a Camag® Linomat auto sampler (Muttenzl, Switzerland) and a Camag® 35/N/30319 TLC scanner with winCATS software; an ultraviolet (UV) lamp with a short wavelength at 254 nm (Desaga, Wiesloch, Germany); and TLC plates pre-coated with silica gel G.F254 10 × 20 cm, 0.25 mm thickness (Merck, Darmstadt, Germany).

HPLC system consisted of an Agilent pump with different flow rates (model 1100 series; Agilent, Santa Clara, CA, USA) equipped with a variable wavelength detector and a 20 µL injection loop. Agilent C18 column (150 mm × 4.5 mm I.D., particle size 5 µm), Agilent C18 column (250 mm × 4.5 mm I.D., particle size 5 µm), Kinetex® C18 column (250 mm × 4.5 mm I.D., particle size 5 µm) with coreshell technology and XBridge® C18 column (250 mm × 4.5 mm I.D., particle size 5 µm) were used as stationary phases. The samples were injected with a 50 µL Hamilton analytical syringe. The detector was operating at 250 nm and the elution was isocratic with a flow rate of 1 mL min^{-1}. The output signal was monitored and processed using Hewlett-Packard Chemstation software for LC 3D systems; Rev. B.04.03 (16) Copyright© Agilent Technologies 2001–2010.

Jenway digital ion analyser 3330 with Jenway pH glass electrode (Essex, UK) was used for pH adjustments.

API 4000 Q-Trap mass spectrometer (AB Sciex, Toronto, Canada) equipped with an atmospheric pressure chemical ionization (APCI) interface using positive ion mode.

IR spectrophotometer Shimadzu 435 (Shimadzu, Kyoto, Japan).

Preparation of Degradation Product

Mirabegron degradation product (DEG) was prepared by reflux 50 mg of pure MIR with 50 mL 1 N HCl at 100 °C for 9 h, or with 1 N NaOH at 100 °C for 7.5 h. Complete degradation was confirmed by TLC by applying neutralized aliquots on silica gel 60 as stationary phase and using chloroform–methanol–ammonia (9.0:1.0:0.1 by volume) as a mobile phase. The degraded sample solutions were neutralized and evaporated to dryness. The solid residue was extracted in methanol and evaporated to dryness. The residue was used for further identification and characterization by mass and IR spectrometry.

Standard Solutions

Stock Standard Solution

Stock standard solution (10 mg mL^{-1}) of MIR was prepared by accurately weighing 250 mg of its bulk powder into 25-mL volumetric flasks. About 10 mL of ethanol was added, sonicated for few minutes, and diluted to the volume with ethanol.

Working Standard Solution

Working standard solutions (100 µg mL^{-1}) of MIR were prepared by accurately transferring 0.5 mL from its stock

standard solution into separate 50-mL volumetric flasks and diluted to the volume with ethanol.

Procedures

Construction of Calibration Curve for TLC

Aliquots equivalent to 2–12 mg of MIR were transferred from its stock standard solution into series of 10-mL volumetric flasks; the volumes were completed to the mark with ethanol. A 10-µL aliquot of each solution was applied on a TLC plate using applicator with 50 µL Hamilton syringe. Bands of 6 mm width were spaced 1 cm from the bottom edge of the plate. The plate was developed in a chromatographic tank, previously saturated for at least 1 h with the developing mobile phase; to a distance of about 8 cm by the ascending technique using chloroform–methanol–ammonia (9.0:1.0:0.1 by volume) as the mobile phase. The plate was removed and air-dried. The bands were scanned at 250 nm. A calibration curve representing the relationship between the recorded area under the peak and the corresponding concentrations of MIR in µg per band were plotted and the regression equation was computed.

Construction of Calibration Curve for HPLC

Accurately measured volumes of MIR working solution (100 µg mL^{-1}) were transferred into 10-mL measuring flasks, diluted to the volume with the mobile phase, ethanol–phosphate buffer pH 2.5 (30:70 by volume), to get the final concentration range from 1 to 25 µg mL^{-1}. Twenty microliters of these solutions were injected in triplicate into the HPLC system utilizing Agilent C18 column (150 mm × 4.5 mm I.D., particle size 5 µm) as the stationary phase. The chromatograms were recorded at 250 nm and a calibration curve for MIR was plotted and the corresponding regression equation was calculated.

Assay of Pharmaceutical Formulation

The contents of twenty Betmiga prolonged-release tablets were weighed and carefully ground and mixed. An accurate amount of the powdered tablet equivalent to 10 mg of MIR was weighed, transferred into 100-mL beaker then 20.0 mL ethanol was added and sonicated for 30 min. The solution was filtered into 50-mL volumetric flask. The residue was washed three times each using 5 mL ethanol then the solution was completed to the mark with the same solvent.

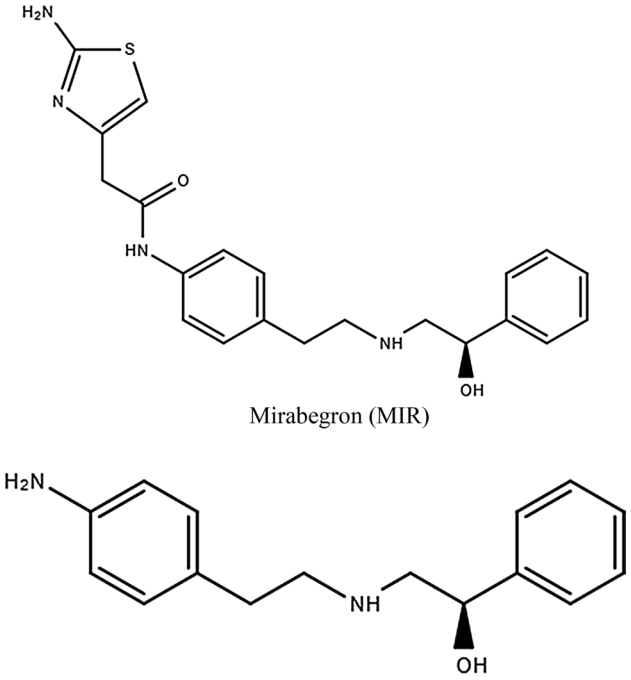

Fig. 1 Chemical structures of mirabegron (MIR) and its hydrolytic degradation product (DEG)

Direct application of 20 µL aliquot from the pharmaceutical solution was done for the TLC assay. Whereas 0.5 mL aliquot was transferred from the pharmaceutical solution into 10-mL volumetric flask and the volume was completed with ethanol for HPLC analysis. The descried procedures under the calibration of each method were followed for the determination of MIR concentrations.

Results and Discussion

Many pharmaceutical compounds undergo degradation during storage or even during the different processes of their manufacture [9]. Amides and N-substituted amides undergo hydrolysis upon heating with aqueous acid or base [10]. MIR has an amide linkage which is liable to hydrolysis, Fig. 1. To achieve complete degradation of the drug and find the final degraded form, our study was carried out using 1 N HCl and 1 N NaOH. Meanwhile the reflux took place; TLC was used to monitor the degradation process. MIR spot disappeared and complete degradation was achieved after 9 and

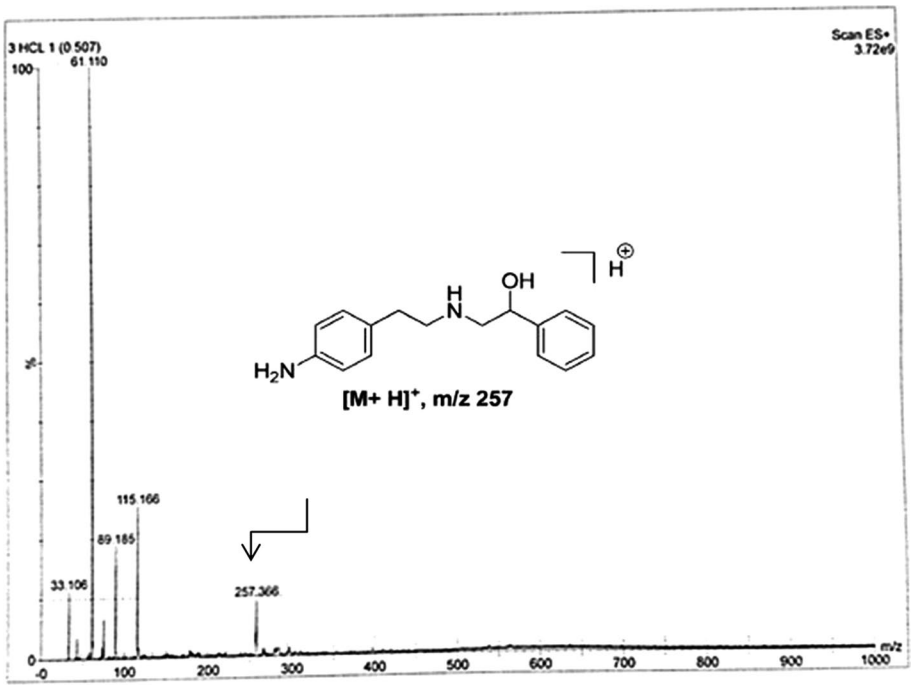

Fig. 2 Mass spectrum of the hydrolytic degradation product

7.5 h by the action of acidic and alkaline hydrolysis, respectively.

TLC chromatogram showed only one degraded spot of MIR in both acidic and alkaline hydrolyses having the same R_f values. Visualizing the TLC plates using iodine vapor revealed no more spot. Furthermore, one hydrolytic degradation product was evidenced by HPLC where the detected peak purity factors were 999.995 and 999.162 for MIR and DEG, respectively, confirming the absence of other overlapped peaks and only one degradation product was detected.

The structure of isolated DEG, Fig. 1, was elucidated and confirmed by mass and IR spectrometry. LC–MS chart showed parent peak at $m/z = 257$ corresponding to molecular weight of the proposed DEG, Fig. 2. On the other hand, carbonyl sharp peak at 1651 cm^{-1} and the characteristic vibrational spectra of thiazole ring in the range from 600 to 1400 cm^{-1} [11] were found in the intact drug IR spectrum, Fig. 3a. These characteristic bands were absent in the IR spectrum of DEG, Fig. 3b. Regarding the remaining part of the intact MIR (2-(2-amino-1,3-thiazol-4-yl)acetic acid), it may be photo degraded by visible light that lead to opening and destruction of the thiazole containing structure [12, 13], hence it could not be detected.

It is worth mentioning that DEG was reported as one of MIR metabolites in human body as the major metabolic routes of MIR in humans predicted to be amide hydrolysis [6]. This also justifies the importance of our proposed chromatographic separation.

Our results were compared to previously reported stress degradation study [7] where less drastic conditions were used and complete degradation of MIR was not achieved. The same degradation product was produced by neutral hydrolysis. However, some additional degradation products were reported in acidic and alkaline hydrolysis that did not appear in our result. These products may be intermediates formed during the hydrolytic reaction.

TLC Method

Densitometry offers a simple way of quantifying directly on a TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing them to a standard curve from reference materials chromatographed simultaneously under the same condition [14]. The proposed TLC method was conducted for separation and determination of the studied drug and in the presence of its degradation product depending on the difference in R_f values.

Several developing systems were applied for the separation of the proposed components. Sufficient separation was achieved upon using chloroform–methanol as a developing system; however, MIR showed broad band. Adding ammonia showed better result and optimum separation was achieved when chloroform–methanol–ammonia (9:1:0.1 by volume) was used as a developing system. Two well resolved bands of MIR and DEG were obtained having

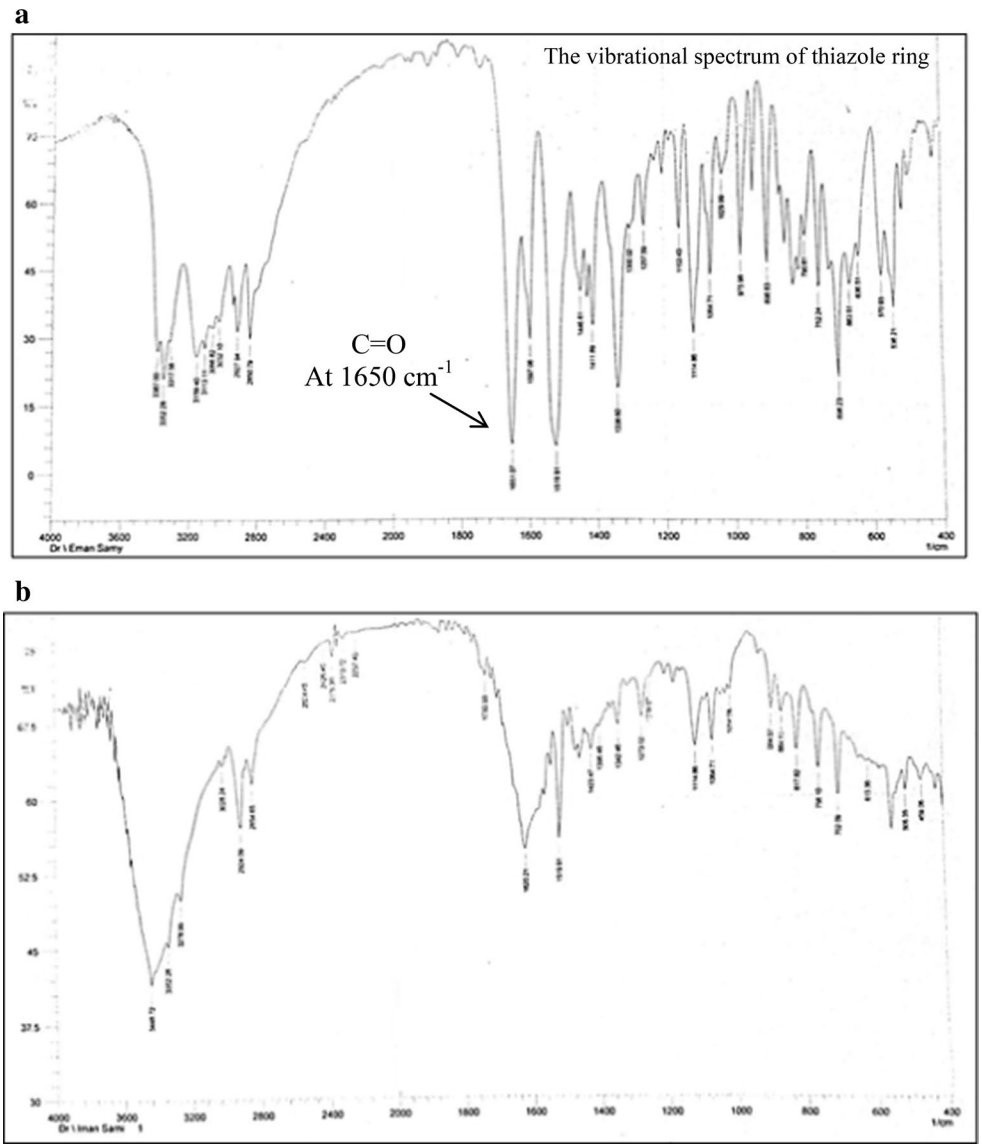

Fig. 3 IR spectra of **a** intact MIR and **b** its hydrolytic degradation product (DEG)

R_f values of 0.20 and 0.32, respectively, Fig. 4. A polynomial relationship was obtained between the integrated area under the peak of the separated bands at the selected wavelength (250 nm) and the corresponding concentration of MIR (2–12 µg per band), Fig. 5.

HPLC Method

Seeking eco-friendly mobile phase, ethanol was used as an organic modifier in the proposed method, since the previously reported LC methods [4, 7] used acetonitrile in mobile phase. Usually ethanol replaces acetonitrile in many chromatographic methods [15], as the latter is ranked by the US Environmental Protection Agency as a hazardous solvent [16]. Optimization of mobile phase was carried out using different ratios of ethanol to buffer solution using Agilent C18 (150 mm × 4.5 mm I.D., particle size 5 µm) column. The optimum chromatographic separation was achieved using ethanol–phosphate buffer pH 2.5 (30:70, by volume) as a mobile phase with flow rate of 1 mL min^{-1}. UV detection was carried out at 250 nm for better sensitivity for the studied drug and its degradation product, Fig. 6.

The proposed chromatographic system allows complete baseline separation with optimum resolution and high

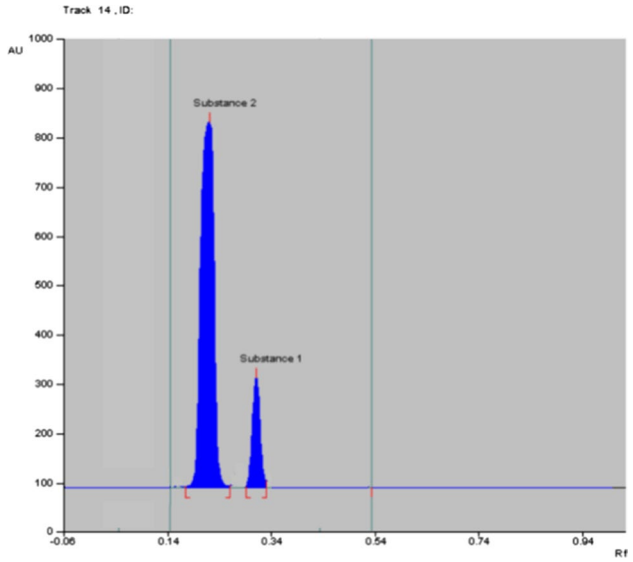

Fig. 4 TLC chromatogram of MIR and DEG where R_f values were 0.20 for MIR and 0.32 for DEG using mobile phase of chloroform: methanol: ammonia (9:1:0.1 by volume) and detection at 250 nm

efficiency within short run time (~3.5 min). Linear relation was obtained between peak area and the concentration of MIR in the range of 1–25 µg mL^{-1}.

The robustness of the proposed HPLC method and its ability to remain unaffected by small changes in chromatographic conditions were tested. Effect of changing flow rate (±0.1 mL min^{-1}), pH (±0.2 unit) and mobile phase ratio (±10%) on MIR peak area was calculated as RSD%. About 7.5% change in peak area was calculated upon changing flow rate or pH value. Lower effect of mobile phase ratio was observed as the calculate RSD% was 3.4%.

The suggested mobile phase was further tried on different commercial C18 columns; System suitability parameters were calculated [17] in Table 1. The obtained Capacity factor (k'), selectivity (α), resolution (R_S) and tailing factor (T). Number of Theoretical Plates per Meter (N/m) was used to compare efficiencies of different length columns. Although MIR showed higher capacity factor (~2) on Agilent (250 mm) column and optimum tailing factor (1 ± 0.05) on Agilent (250 mm) and Kinetex columns, both columns had lower efficiency in separation, whereas XBridge and Agilent (150 mm) have the highest column efficiency. Obviously, using Agilent (150 mm) as stationary phase reduced the run time by half compared to other columns. Thus, Agilent (150 mm) column was selected to develop the proposed HPLC method.

Eco-scale [18] is an additional calculated parameter in our results to provide a comprehensive evaluation of mobile phase amount and hazards, energy and waste for the chromatographic runs. Accordingly, penalty points are calculated for the analytical procedure and subtracted from

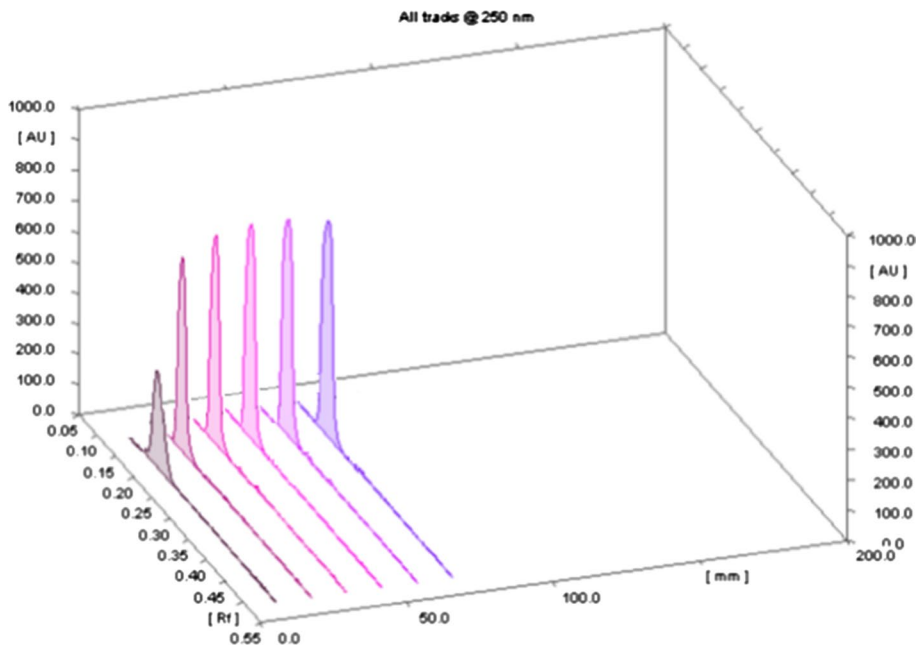

Fig. 5 Scanning profiles for MIR (2–12 µg per band) using mobile phase of chloroform: methanol: ammonia (9:1:0.1 by volume) and detection at 250 nm

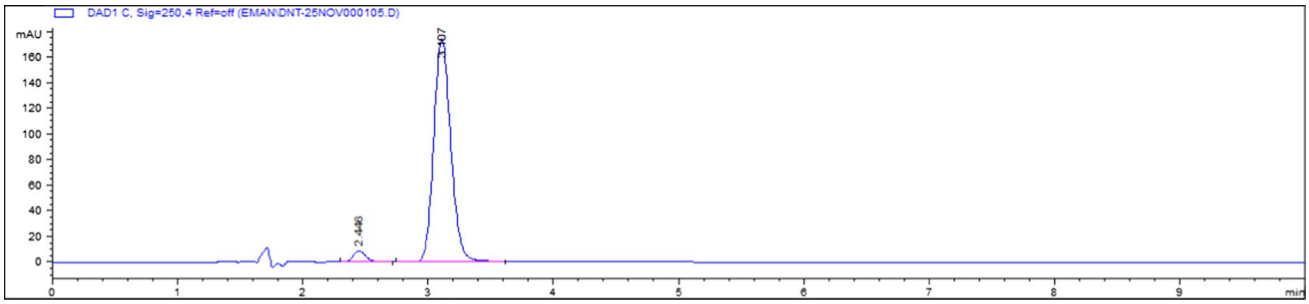

Fig. 6 HPLC chromatogram of MIR and DEG with retention times 3.11 and 2.45 min, respectively, using ethanol-phosphate buffer pH 2.5 (30:70, by volume) as a mobile phase on Agilent C18 column (150 mm × 4.5 mm I.D., particle size 5 µm) with flow rate 1 mL min^{-1} and detection at 250 nm

Table 1 System suitability parameters calculated for MIR separation different HPLC columns

Parameter	Agilent C18 (250 mm)	Kinetex® C18	XBridge® C18	Agilent C18 (150 mm)	Reference value [17]
Retention time (min)	7.66	6.23	6.94	3.11	
Capacity factor (k\)	2.06	1.88	1.76	1.04	1–10 acceptable
Selectivity (α)	1.73	1.69	1.61	1.27	>1
Resolution (R_S)	8.26	7.24	8.99	3.12	>1.5
Tailing factor (T)	0.95	1.05	0.88	0.84	$T = 1$ for a typical symmetric peak
Column efficiency (N/m)	14872	8768	20816	17620	Increases with efficiency of the separation

Table 2 Validation parameters for the proposed chromatographic methods

Parameters	TLC	HPLC
Linearity		
Range	2–12 µg per band	1–25 µg mL^{-1}
Slope (X coefficient)	2009.2	78.7
SE of slope (X coefficient)	0.0037	0.4377
Slope (X^2 coefficient)	−74.571	–
SE of slope (X^2 coefficient)	0.0002	–
Intercept	6322.5	75.594
SE of intercept	0.0113	6.6295
Correlation coefficient (*r*)	0.9994	0.9999
Accuracy (Mean ± RSD%)	100.21	100.10
	±1.469	±0.747
Precision (RSD%)		
Repeatability[a]	0.253	0.346
Reproducibility[b]	0.548	0.610
LOD (µg mL^{-1})	0.15	0.29
LOQ (µg mL^{-1})	0.46	0.89

[a] RSD% three concentrations (2, 8, and 10 µg mL^{-1}) for TLC and (5, 10, and 15 µg mL^{-1}) for HPLC, repeated three times within the day ($n = 9$)

[b] RSD% three concentrations (2, 8, and 10 µg mL^{-1}) for TLC and (5, 10, and 15 µg mL^{-1}) for HPLC, repeated in three successive days ($n = 9$)

eco-scale value of 100. Excellent green analysis is obtained with score >75 Eco-Scale. Our proposed HPLC conditions are compared favorably with the reported HPLC [4] and UPLC [7]. High eco-scale (91) calculated for the proposed method is apparently due to using ethanol in mobile phase and short analysis time, therefore, low mobile phase consumption and waste production.

Validation of the proposed methods was performed according to ICH guidelines [19]. Table 2 showed the calculated validation parameters. Mean and RSD% values were satisfactory to evaluate selectivity and accuracy of the proposed methods. Linearity parameters along with LOD and LOQ were calculated using residual standard deviation of regression line (σ) and the slope (S). Where LOD = 3.3 σ/S, while LOQ = 10 σ/S. Low RSD% values for repeatability and reproducibility assessed the high precision of the proposed methods. The determination of MIR in Betmiga prolonged-release tablets showed good recoveries, 100.31 ± 1.090 and 99.22 ± 0.468 for TLC and HPLC, respectively. The validity of the suggested procedures was further assessed by applying the standard addition technique, Table 3.

A statistical comparison of the results obtained by the proposed methods and a reported HPLC method [4] for MIR determination is shown in Table 4. The calculated *t*

Table 3 Application of standard addition technique for the analysis of MIR in its pharmaceutical dosage form by the proposed methods

TLC			HPLC		
Taken concentration ($\mu g\ mL^{-1}$)	Added concentration ($\mu g\ mL^{-1}$)	Recovery%	Taken concentration ($\mu g\ mL^{-1}$)	Added concentration ($\mu g\ mL^{-1}$)	Recovery%
4	2	100.09	10	5	101.04
	4	100.50		10	100.70
	8	100.34		15	99.82
Mean ± RSD%		100.31	Mean ± RSD%		100.52
		0.202			0.625

Table 4 Statistical comparison between the proposed methods and a reported HPLC method for the determination of MIR in bulk powder

Value	TLC	HPLC	Reported method [4][a]
Mean	100.21	100.10	99.99
SD	1.472	0.747	1.451
RSD %	1.469	0.747	1.451
n	6	6	6
V (Variance)	2.169	0.559	2.106
Student's *t* test (2.228)[b]	0.265	0.161	–
F (5.05)[c]	1.02	3.76	–

[a] HPLC method using Waters ODS C18 column (150 mm × 3.9 mm; 5 µm) and buffer pH 5- acetonitrile (50:50 v/v) as a mobile phase mixture at flow rate 1 mL min^{-1} and UV detection at 249 nm

[b] The corresponding theoretical values of t at ($P = 0.05$)

[c] The corresponding theoretical values of F at ($P = 0.05$)

and *F* values were less than the corresponding tabulated ones, which revealed that there was no significant difference with respect to accuracy and precision between the proposed methods and the reported one.

Conclusion

The present work emphasized two simple chromatographic stability-indicating methods for the determination of MIR in the presence of its degradation product. The proposed TLC method is very simple, rapid and use minimal volume of solvents compared with other separation techniques; furthermore, an extremely large numbers of samples can be analyzed at the same time without compromising accuracy. On the other hand, the proposed HPLC method gave a good resolution between MIR and its degradation product within a short time, good sensitivity, simple mobile phase and a dynamic range. Finally, the proposed methods were successfully applied and validated for determination of MIR in pure powder form and in pharmaceutical formulation and these methods can be used as stability-indicating procedures in quality control laboratories where economy and time are essential.

Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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