



Research Article

Stress Degradation Studies on Bimatoprost and Development of Validated Stability Indicating Densitometric RP-TLC and HPLC Methods

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Abstract

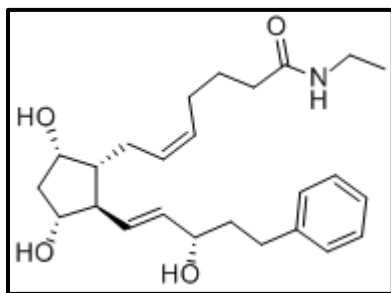
Simple, sensitive and accurate stability-indicating densitometric RP-TLC and RP HPLC-UV methods were developed and validated for analysis of Bimatoprost (BMT). Stress stability studies were performed using hydrolytic (acid & alkali) and oxidative degradation products and conformed using LC-MS. Structure elucidation and pathway of degradation were presented. Both methods were based on reversed phase thin-layer and liquid chromatographic separation of BMT from hydrolytic and oxidative degradation products. Acetonitrile, water and 33% ammonia (4:5:1, by volume) and acetonitrile –water (40:60, v/v) at 30°C were used as mobile phases for separation of BMT from degradation products using RP TLC and HPLC methods respectively. Quantification was achieved at 220 nm for both methods. The linear ranges were 0.5-6.0 µg/band and 5 – 100 µg /mL with mean recoveries ± RSD%, of 98.72 ± 0.31% and 99.25 ± 0.59% for the two methods respectively. The specificity of HPLC method was further assured by peak purity. The proposed methods are rapid with retention time less than 6 min. The methods met ICH regulatory requirements. The two methods were successfully applied for the quantification of BMT in drug substance and ophthalmic solution with acceptable accuracy and precisions; the label claim percentages were 93.145 ± 0.89 and 95.35 ± 0.65 for densitometric RP-TLC and RP HPLC-UV methods respectively. The research work has a great value for quality control and stability studies of BMT.

Keywords: Bimatoprost, BMT, Stress stability studies, RP-TLC, Densitometry, HPLC-UV, Validation, Ophthalmic solution.

Abbreviation: BM-Bimatoprost, LC-MS-Liquid Chromatograph-Mass spectroscopy, RP-TLC-Reversed Phase Thin Layer Chromatograph, RP HPLC-UV-Reversed Phase High Performance Liquid Chromatograph-Ultra Violet Detector, µm-Micro-Meter, µg-Micro-gram, v/v-Volume per Volume, mL-Milli-Liter, °C-Centigrade, LOD-Limit of Detection, LOQ-Limit of Quantification.

Introduction

Bimatoprost, (7-[3, 5-dihydroxy-2- (3-hydroxy-5-phenyl-pent-1-enyl)-cyclopentyl]-N-ethyl-hept-5-enamide), is antiglaucoma agent (ophthalmic) [1].



BMT is a prostaglandin analog/prodrug used topically (as eye drops) to control the progression of glaucoma and in the management of ocular hypertension [2-8]. Few methods were reported for determination of BMT. These included Ultra Performance Liquid Chromatography (UPLC-MS) for determination of the drug in presence of its impurity (methyl ester) [9], HPLC-UV methods for determination of BMT in bulk and ophthalmic solution [10], and HPLC-MS/MS for quantification of BMT, latanoprost and travoprost in Eyelash Enhancing Cosmetic Serums [11]. To the best of our knowledge, there is no reported reverse phase TLC or HPLC stability indicating methods for determination of BMT using stress conditions. In this study, we present rapid, selective chromatographic methods for determination of BMT in ophthalmic solution and in synthetic mixtures of the drug and hydrolytic and oxidative degradation products.

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Experimental

Instrumentation

TLC scanner three densitometer (Camag, Muttentz, Switzerland).

The following requirements are taken into consideration:

- Slit dimensions: 6 x 0.3 mm
- Scanning speed: 20 mm/s
- Spraying rate: 10 μ L/s
- Data resolution: 100 μ m/step
- Band width: 3 mm
- Result output: chromatogram and integrated peak area, Linomat IV with 100 μ L syringe (Camag), Sonix TV ss-series ultrasonicator (USA, Newtown, CT)
- UV lamp with short wave length 254 nm. (Desaga, German).
- RP-TLC plates (20 x 20 cm) coated with silica gel 60 F254 (1.05554.0001) (Merck, Germany).
- Hamilton micro syringe, (5 μ L & 25 μ L).
- LC-MS-QQQ Mass Spectrometer 6420 TripleQuad (Agilent technology).
- The pH was measured with Jenway pH meter 3510 (UK).
- HPLC (Agilent technologies 1260 series), USA consist of quaternary pump, equipped with a variable wavelength detector and injector valve with 20 μ L constant loop and vacuum degasser.
- Membrane filters with pore size 0.45 μ m and 0.47 μ m diameter (Alltech Associated, USA).

Material and Dosage Form

Authentic sample

BMT was kindly supplied by Chemipharm Pharmaceutical Industries S.A.E 6th October Egypt, Batch No: 20170504000. The purity of the sample was found to be 100.29% according to certificate from the company.

Dosage form

BMT ophthalmic solution (Lumigen®) was labeled to contain 0.03% BMT, El-Sofiko pharm Co., Egypt; Batch No. 85543 was purchased from the local market.

Chemicals and reagents

Acetonitrile, HPLC grad was from Lab-Scan (Eire), hydrochloric acid from Fischer Scientific (UK), and 33% ammonia, 30% hydrogen peroxide, and sodium hydroxide were from Adwic Co., Egypt. Water for HPLC was prepared by double glass distillation and filtration through a 0.47 μ m membrane filter (Alltech Associates, USA).

Standard Solutions

Standard stock solution of bmt (1mg/ml): Stock standard solution of BMT (1 mg/mL) was prepared by accurately weighing 100 mg of drug in 100 mL volumetric flask and dissolved in acetonitrile. Then the volume was completed with the same solvent.

Standard working solutions of BMT

Working standard solutions of BMT were prepared in concentration range of 5.0 - 100.0 μ g/mL in mobile phase for RP-HPLC method.

Chromatographic methods

Densitometric RP TLC method

Stationary phase: Aluminium sheet TLC silica gel 60 F254 S plates.

Mobile phase: acetonitrile–water–33% ammonia (4:5:1, by volume).

Chamber saturation: 10 min.

Sample applicator: CAMAGE Linomat 5.

Band: 3 mm.

Densitometric scanner: CAMAGE Linomat 3 TLC scanner.

Wavelength: 220 nm.

Isocratic RP HPLC UV method: Column: Waters corporation, USA, μ Bondapak™ C18 RP – Column (150 mm, 5 μ m, 3.9 mm i.d.) was used for separation and quantification.

- Hamilton syringe 50 μ L capacity.
- Mobile Phase: consisting of acetonitrile - water (40:60, v/v)
- UV detector wavelength: at 220 nm.
- Flow rate: 1.0 mL/min.
- Temperature: 30°C.
- Injection volume: 20 μ L aliquots of each solution were injected in triplicate and eluted with the aforementioned mobile phase. The average peak areas were calculated using chemstation software.

Procedures

Calibration curves

Densitometric RP -TLC method: Serial volumes, equivalent to concentration ranges (0.5 – 6 μ g/ band), were applied automatically to the RP-TLC plates from stock standard solution (1 mg/ mL in acetonitrile), and developed under the specified conditions mentioned under the chromatographic conditions (2.5.1). The peak areas were recorded and calibration curve was constructed by plotting the integrated peak area versus the corresponding concentration of BMT. The regression equation was computed for the studied drug and it was used for determination of unknown samples containing BMT.

RP-HPLC method: Aliquots equivalent to 5 – 100 μ g/ mL BMT standard working solutions were injected in triplicate and chromatographed under the conditions described before at (2.5.2). The obtained chromatograms were recorded at 220 nm. The calibration curve representing the relationship between peak area and concentration was plotted and regression equation was computed.

Application of the proposed methods for determination of BMT in the drug product

Three bottles (Lumigen 0.03%) were accurately transferred and mixed in stopper conical flask (15 mL). Each milliliter was equivalent to 0.3 mg of BMT (stock solution of drug product).

Densitometric RP-TLC method: One mL of above stock solution was transferred to 10 mL volumetric flask, and diluted to volume with acetonitrile. The obtained solution claimed to contain 0.03 mg/ mL of BMT. Then the procedure described under (2.5.1) was followed.

RP- HPLC method: One mL of stock solution of drug product was transferred to 10 mL volumetric flask, and diluted to volume with mobile phase. The obtained solution claimed to contain 0.03 mg/ mL of BMT was analyzed by the proposed HPLC method described under, 2.5.2 The concentration of the drug was calculated from regression equation.

Stress degradation study for BMT

Hydrolytic degradation conditions (1 mg/mL): Accurately weighed amount about 50.0 mg BMT was placed in a round-bottomed flask containing 50.0 mL of 2 M HCl or 50 mL 2 M NaOH each was added separately and left for 2 hrs in water bath at 90°C. After the specific

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time the solution was neutralized with 2 M NaOH, or 2 M HCl, respectively and evaporated on water bath. The residue of each solution was dissolved in 20.0 mL acetonitrile and the volume was completed with acetonitrile. The obtained solutions were labeled to contain the acid or alkaline degradation product derived from 1 mg/ mL of BMT.

Oxidative degradation conditions (1 mg/mL): Accurately weighed amount 50.0 mg BMT was placed in a round-bottomed flask containing 50.0 mL H₂O₂ (30%) and left for 2 h at room temperature. Then the solution was evaporated on water bath at 40°C. The residue was dissolved in 20.0 mL acetonitrile and the volume was completed with acetonitrile to obtain a solution labeled to contain the oxidative degradation derived from 1 mg/mL of BMT.

Results and Discussion

Method development

The densitometric RP TLC and RP HPLC techniques were developed for the quantification of BMT, in ophthalmic solution and in laboratory prepared mixtures (hydrolytic and oxidative). The methods were based on difference in R_f / R_t values of BMT and its degradation products. Forced degradation of BMT had been studied through acid, alkaline and oxidative stress conditions. Complete degradation was achieved under acid and H₂O₂ stress conditions.

Separation and identification of hydrolytic and oxidative degradation products

Densitometric RP TLC based method: Different parameters (mobile phase, scan mode, and wavelength) affecting separation were optimized to provide selective, accurate, and precise results for analysis of bimatoprost in synthetic mixtures of acid and oxidative degradation products. Complete resolution was achieved by use of developing system consisting of acetonitrile-water- 33% ammonia (4:5:1, by volume). The R_f of BMT was 0.25. The R_f of acid and oxidative degradation products were, 0.71 and 0.63 respectively (Figure 1).

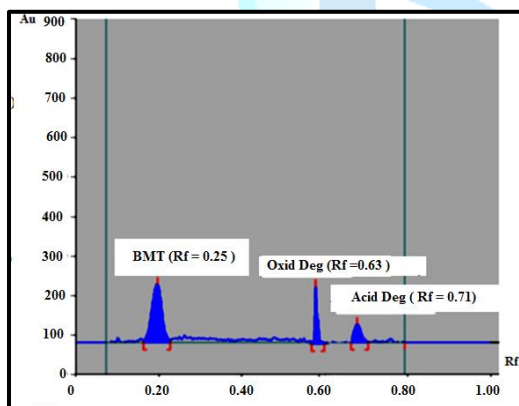


Figure 1: RP-TLC Chromatogram of bimatoprost and its oxidative and acid degradants developing system, acetonitrile-water-ammonia (4:5:1 by volume), at ambient temperature.

Isocratic RP HPLC UV based method: Several mobile phase compositions, wavelengths and temperatures were tried. Complete separation was achieved by using, acetonitrile-water (40: 60, v/v), C18 column, 150 mm, 5 µm, 3.9 mm i.d., flow rate 1.5 mL /min and controlled temperature (30°C). Quantification was achieved with UV detection at 220 nm. The retention time was 2.763, 5.769, 1.445 for BMT and acid and oxidative degradation products respectively. The specificity of HPLC method was illustrated in Figure 2.

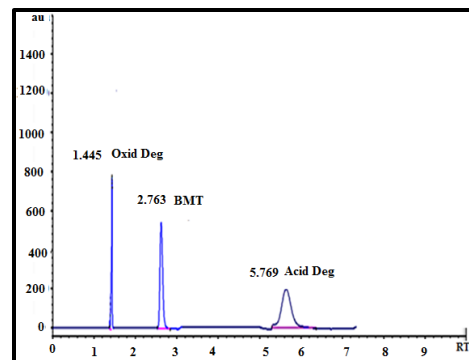
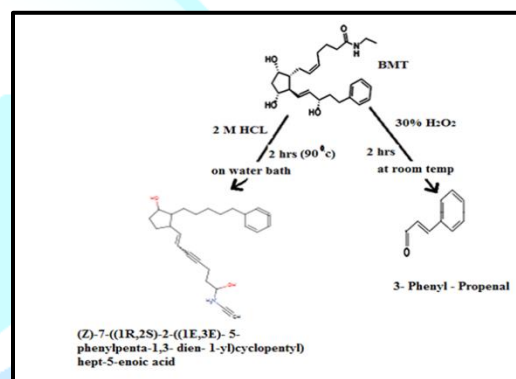


Figure 2: HPLC Chromatogram of bimatoprost and its oxidative and acid degradants, Mobile phase, acetonitrile - water (60:40 v/v) and detection at 220 nm at controlled temperature (30°C).

For confirmation of degradations products (acid & oxidative) LC- MS was used. The suggested scheme of the degradation pathways was postulated Scheme 1. One degradation product was obtained by each acid and oxidative stress condition.



Scheme 1: Suggested scheme of degradation of bimatoprost.

Validation of the proposed methods

Linearity, linear relationship was found to exist between the peak areas of the separated bands/ separated peaks and drug concentrations over the range of 0.5–6 µg/ band and 5 – 100 µg/ mL), for densitometric RP TLC and RP HPLC methods, respectively [12,13] (Figure 3 and Table 1).

Parameters	Obtained values		
	Intact	Acid deg	Oxid deg
Retardation factor (R _f)	0.25	0.71	0.63
Injection repeatability	0.06%		RSD of R _f for 5 injections
	1.43%		RSD of PA for 5 injections
Resolution		4.03	3.411
Symmetry factor	0.88	0.81	0.79
K (capacity factor)	2.57	9.142	8
α (Selectivity)		2.84	2.52

Table 1: The system suitability test results of the proposed densitometric RP-TLC method for the determination of bimatoprost.

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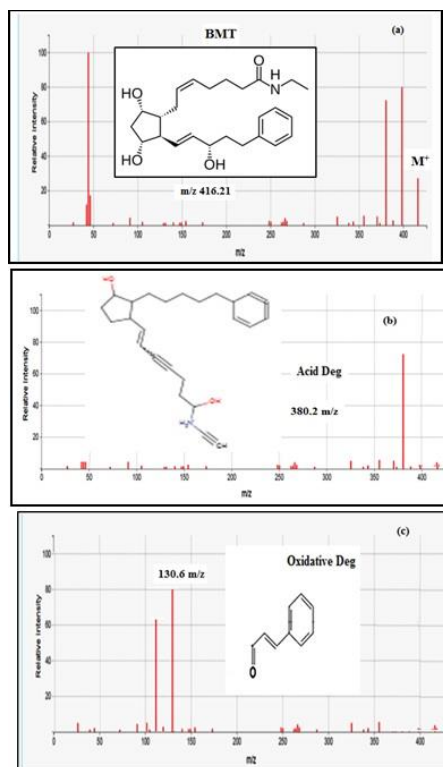


Figure 3: Mass spectra of a) BMT, (b) acid degradation, and (c) oxidative degradation of bimatoprost.

Accuracy, the previously mentioned procedures under linearity was repeated three times for five different concentrations within the linearity range. The concentrations were calculated from the corresponding regression equations. The mean percentage recoveries were 98.72 ± 0.30 , and 99.25 ± 0.59 , for RP- TLC and RP-HPLC, respectively, as presented in **Table 2**.

Parameters	HPLC			Comments
	Intact	acid deg	oxid deg	
Retention time	2.763 ± 0.01911	$5.769 \pm .21$ $1.445 \pm .33$		\pm Standard deviation
Injection repeatability	0.0086% 0.636%			RSD of Rt for 5 injections RSD of PA for 5 injections
K'	3.605	2.615	1.475	Capacity factor
Selectivity factor (α)	2.087	1.86		Calculated by K'/K'
Resolution	4.28	2.05		Calculated by $2(t_2 - t_1)/w_1 + w_2$
Tailing factor	0.94	0.82	0.76	Calculated to 5 % of peak height
Theoretical plates (N)	2436			Column efficiency plates/column
HETP	0.006			Height equivalent theoretical plates

Table 2: The system suitability test results of the proposed HPLC method for the determination of Bimatoprost.

Statistical comparison between the proposed and manufacturer methods was performed. The results indicated no significant difference (**Table 3**).

Parameters	Densitometry- RP TLC method	RF-HPLC method
Linearity range	0.50 - 6.0 $\mu\text{g/ band}$	5.0 - 100.0 $\mu\text{g/mL}$
LOD	0.08 $\mu\text{g/ band}$	6.2×10^{-2} $\mu\text{g/mL}$
LOQ	0.26 $\mu\text{g/ band}$	20.6×10^{-2} $\mu\text{g/mL}$
Accuracy*		
Mean \pm RSD%	98.72 ± 0.31	99.25 ± 0.59
Regression		
Slope	734.75	7.04
SE of slope	7.58	0.05
Confidence limit of slope**	715.26–754.22	6.92–7.16
Intercept	293.03	-4.97
SE of intercept	27.35	3.15
Confidence limit of intercept**	222.69–363.35	-12.08–2.15
Co-relation coefficient	0.9995	0.9994
SE of estimation	38.05	5.46

n= 5, **95% Confidence limit.

Table 3: Results of assay validation obtained by applying the proposed densitometric-RP TLC and RP- HPLC methods.

Precision, repeatability was assessed by analyzing three concentration levels (0.5, 2, 4 $\mu\text{g/ band}$ for RP TLC, and 5, 25, 50 $\mu\text{g/ mL}$ for HPLC) in triplicates of each sample in a single assay run. The intermediate precision was assessed by analyzing the same concentrations in triplicate, in three separate assay runs. The assays gave satisfactory results (**Table 4**).

RP- TLC method		
Conc. add ($\mu\text{g/ band}$)	Conc. found ($\mu\text{g/ band}$)	Recovery % *
1.5	1.48	98.66
2.5	2.48	99.12
3.5	346.00%	98.94
4.5	443.00%	98.43
5.5	5.41	98.45
Mean \pm RSD%		98.72 ± 0.30
RP-HPLC method		
Conc. add ($\mu\text{g/ mL}$)	Conc. found ($\mu\text{g/ mL}$)	Recovery % *
15	15.04	100.26
20	19.6	98
30	30.3	101
45	44.2	98.22
50	49.4	98.8
Mean \pm RSD		99.25 ± 0.59

Average of three different determinations.

Table 4: Accuracy of the proposed methods for the determination of bimatoprost in drug substance.

Limits of detection and quantification, LOD

The Limits of Detection (LOD) and Quantification (LOQ) LOD and LOQ were calculated based on Standard Deviation of response and Slope of calibration curve according to ICH guidelines (ICH, 2005). LOD and LOQ were calculated from the formula (ICH, 2005).

$$\text{LOD} = 3.3\sigma/S$$

$$\text{LOQ} = 10\sigma/S$$

Where,

σ = Standard deviation of the y-intercept of the regression line

S = Slope of the calibration curve

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LOD was calculated and found to be 0.08 µg/band, and 6.2 X10⁻² µg/mL for RP-TLC, and HPLC respectively. LOQ was calculated and found to be 0.26 µg/band, and 20.6 X 10⁻² µg/ mL for RP-TLC and HPLC respectively, as shown in **Table 1**.

Specificity was assessed by analyzing synthetic mixtures of bimatoprost in presence of different concentrations of degradation products within the quantification ranges. The percentage recoveries were calculated (**Table 5**). The specificity was illustrated by high resolution of the studied compound (**Figure 2**).

Values	RP-TLC-densitometric method	RP-HPLC method	Manufacturer method*
Mean	98.72	99.25	100.29
Standard Deviation (SD)	0.302	1.31	1.17
Variance (S ²)	0.091	1.72	1.36
N	5	5	5
F (6.39)**	2.43	2.102	
t(1.860)**	1.496	0.943	

* Manufacturer HPLC method supplied from Chemipharm Company.

**Values between parentheses represents the tabulated t and F values at P=95%.

Table 5: Statistical analysis of the results obtained by the proposed methods and manufacturer method for the determination of bimatoprost in drug substance.

The specificity of the proposed HPLC method was further demonstrated by testing Peak purity. The peak purity of the cited drug

Drug substance	Conc.		Precision* RSD%				Accuracy* RE%			
			Intra		Inter		Intra		Inter	
	RP-TLC µg/band	RP-HPLC µg/mL	RP-TLC	RP-HPLC method	RP-TLC	RP-HPLC method	RP-TLC	RP-HPLC method	RP-TLC	RP-HPLC method
Bimatoprost	2	20	0.27	0.9	0.319	0.62	0.319	0.73	-0.467	-0.92
	3	30	0.21	0.396	0.132	0.54	0.132	1.55	-0.211	0.033
	4	50	0.65	0.085	0.264	0.409	0.264	-0.54	0.567	0.036

* n = 9.

Table 6: Intra and inter-day precision and accuracy of the proposed densitometric RP-TLC and RP-HPLC methods for determination of bimatoprost in drug substance.

Ratios of bimatoprost and its degradation products			Recovery* % of intact drug
Intact Mg	Acid degradation µg	Oxidative degradation µg	
4.9	0.1	0.1	100.5
4.5	0.5	0.5	98.87
4	1	1	1
Mean ± RSD%			99.123 ± 0.42

*Average of three different experiments.

Table 7: Specificity of the proposed densitometry-RP-TLC method for the determination of bimatoprost in laboratory prepared mixtures with its degradants.

System suitability tests (capacity factor “K”, selectivity “α”, resolution “RS”, tailing factor “T” and theoretical plates number “N”) were calculated to ensure that the system was working correctly during analysis. The results were meeting the reference criteria (**Tables 8 and 9**).

Application of the proposed methods

Analysis of BMT in ophthalmic preparation and application of standard addition technique by the proposed densitometric RP TLC and RP HPLC-UV methods were applied for the determination of BMT in pharmaceutical preparation. The percentage recoveries were obtained in the range of 97.67 ± 1.260 and 99.39 ± 1.95 (**Table 10**).

in the pharmaceutical preparation matrix spiked with its different degradation products using DAD was illustrated in **Figure 4** [14].

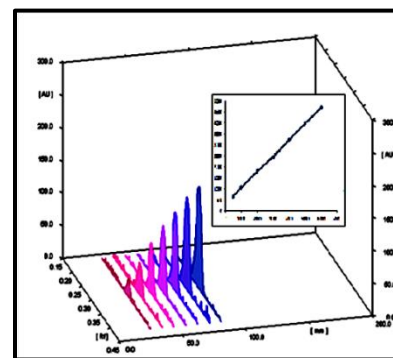


Figure 4: Linearity in concentration range 0.5 – 6.0 µg/band via densitometric RP-TLC method of bimatoprost, developing system, acetonitrile-water: conc ammonia (4:5:1 by volume), at ambient temperature.

Robustness was assessed by evaluating the influence of small variation of experimental variables as developing system composition, saturation time and temperature on reliability of the method. The results indicated that the ability of the method to remain unaffected by small changes in these parameters. The RSD was less than 2% for both methods. As presented in **Tables 6 and 7**.

Conditions	RP-TLC Densitometric Method Selectivity (α)		
RP-TLC	RP-TLC	Acid Deg	Oxid Deg
Mobile phase composition (acetonitrile:water:conc ammonia) (4:5:1)(v/v).	4.5:4.5:1	2.5	2.45
	3.5:5.5:1	2.2	2.6
	5:0.5:0.1	2.1	2.52
Saturation time	25	2.05	2.41
	30	2.2	2.6
	35	2.35	2.35

*Selectivity(α)=Rf / Rf.

Table 8: Robustness results for the proposed densitometric-RP-TLC method.

Parameters	Conditions	HPLC Resolution (Rs)
HPLC	HPLC	
	Mobile phase composition (acetonitrile:Water) 40: 60 (v/v).	33:67
		35:65
Temperature (°C)	32	2.19
	27	2
	1	1.98
Flow rate	0.8	2.06
	1.2	2
		2.01

Table 9: Robustness results for the proposed HPLC method.

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Lumigen® (0.03% of Bimatoprost)	Proposed RP-TLC densitometric method Recovery %* of claimed amount % ± RSD	Proposed HPLC method Recovery %* of claimed amount % ± RSD	Manufacture method Recovery%* of claimed amount ± RSD
	93.145 ± 0.89	95.35 ± 0.65	91.58 ± 0.98

Average of five different determinations.

Table 10: Application of the proposed methods for determination of bimatoprost in drug product.

The percent recovery was obtained by the standard addition technique, where different levels of drug substance were added to previously analyzed sample. The mean percent recovery of the drug was calculated. The results are presented in **Table 11**.

Conclusion

The proposed RP-TLC and RP-HPLC methods were suggested to fulfill the aim of this work for determination of BMT in presence of its degradation products without either pretreatment or preliminary separation. In addition, the proposed methods were found to be selective which showed good resolution between the drug and its degradation products.

Preparation	Amount taken Pure		Added Found		Recovery*% ± RSD	
	RP-TLC µg/band	HPLC µg/mL	RP-TLC	HPLC	RP-TLC	HPLC
Lumigen® (0.03% of Bimatoprost)	1.5	30	0.5	10	98.40 ± 0.48	98.00 ± 1.27
			1	20	99.12 ± 0.63	100.37 ± 0.446
			2	30	99.80 ± 0.53	98.50 ± 0.892
			3	40	99.78 ± 0.92	102.00 ± 1.095
			4	50	98.66 ± 1.218	98.60 ± 0.624

*Mean of three different determinations.

Table 11: Results of application of standard addition technique for determination of bimatoprost by the proposed methods.

The advantages of densitometric - RP TLC method is that several samples can be run simultaneously using a small quantity of mobile phase, thus lowering analysis time and cost per analysis and provides high sensitivity and selectivity. Also it can be considered as a stability indicating method as it is able to quantify the drug and resolve it from its degradation products.

From the data obtained it is provide that the proposed HPLC method is sensitive, specific, accurate, and precise over the specified range and could be used for purity testing, stability studies, quality control and routine analysis of BMT in drug substance and drug products. the proposed method provides more sensitivity, simplicity and efficacy than other reported methods.

From the results obtained, we concluded that the suggested RP-TLC Densitometric method showed for the first time determination of BMT in presence its degradations in rang 2-20% ,where neither any literature mention that application nor get that sensitivity for the determination of BMT in drug substance , or in drug product in presence its acid/oxidative degradation products .these methods provide high sensitivity, accuracy and specificity. Moreover, the RP-HPLC method is simple and inexpensive, permitting its application in quality control laboratories for quantitative determination of the studied drug in drug substance, in drug product as well as in presence of its mentioned degradation products.

Conflict of Interest

Walash M, Safaa. T, Nahla S and Maha M, have reviewed the research article. They are the supervisors of that presented work, who help me to finish my idea as it.

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