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ORIGINAL ARTICLE

Estimation of Anagliptin and Metformin HCl in Bulk & Pharmaceutical Dosage Form using Stability Indicating High Performance Thin Layer Chromatography

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ABSTRACT

A simple, accurate and precise stability indicating high performance thin layer chromatography was developed and validated for estimation of Anagliptin and Metformin HCl in bulk and pharmaceutical dosage form. Chromatographic separation was carried out on Merck TLC Aluminium plates precoated with silica gel 60 F254 of size (20 cm x 10 cm) with 250 µm layer thickness with the mobile phase Toluene: 1% Methanolic ammonium acetate (2:4 %V/V) at detection wavelength 243 nm for ANA and MET. The Rf value for ANA and MET were 0.728 ± 0.013 and 0.324 ± 0.009 respectively. The method was linear over the concentration ranges 100-1000 ng/band for ANA and 250-2500 ng/band for MET. The LOD was 8.67 ng/ band for ANA and 67.29 ng/ band for MET. The LOQ was 26.29 ng/ band for ANA and 203.9 ng/ band for MET. During stress conditions, ANA degraded significantly under acidic, alkaline, oxidative and thermal stress conditions; degraded moderately under photolytic stress conditions; and showed negligible degradation under elevated temperature and humidity conditions. MET degraded significantly under acidic, alkaline and oxidative stress conditions; degraded moderately under thermal and humidity stress conditions and showed negligible degradation under photolytic stress condition.

KEYWORDS: *Anagliptin (ANA), Metformin HCl (MET), High performance thin later chromatography (HPTLC), Stability indicating assay method (SIAM), Validation (ICH Q2 R1)*

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INTRODUCTION

Anagliptin is used for type 2 diabetes mellitus and longer period of action for treatment of type 2 non-insulin dependent diabetes mellitus disease as a Dipeptidyl Peptidase 4 inhibitor Anagliptin, chemically N-[2-[[2-[(2S)-2-Cyanopyrrolodin-1-yl]-2-oxoethyl] amino]-2-methylpropyl]-2-methylpyrazolo [1, 5-a] pyrimidine-6-carboxamide (Figure 1) is a Dipeptidyl Peptidase 4 (DPP 4) inhibitor which is used in management of type 2 NIDDM [1]. Dipeptidyl Peptidase 4 enzyme discontinuities the incretins GLP-1 gastrointestinal hormones released in response to a meal. By stopping GLP-1 inactivation, they are able to rise the secretion of insulin and suppress the release of glucagon by the alpha cells of the pancreas. This drives blood glucose levels near normal level [2-3]. This drug is not approved in several of the pharmacopeia. Anagliptin is a very effective pill with minimum risk profile for type 2 diabetes mellitus and longer duration of action for treatment of type 2 non-insulin dependent diabetes mellitus disease [4]. A literature review discovered that few methods are reported for determination of ANA, either alone or in combination, by spectrometric [5-7], HPLC [8-9], LC/MS [10].

Metformin is chemically a 1-Caramimidamido-N, N-Dimethylmethanimidamide [11] (Figure 2) and has based on Biguanides class [12-13]. It suppresses hepatic GNG and glucose output from liver. This is the major action liable for lowering blood glucose in diabetics. It is official in IP [14], BP [15], and USP [16]. Literature analysis studies that many spectroscopic [17-18], HPLC [19-22], HPTLC [23] methods are reported for determination of Metformin hydrochloride (HCl), either alone or in mixture [8].

The aim of the present work was to develop a stability indicating HPTLC method for simultaneous estimation of ANA and MET in bulk and pharmaceutical dosage form. It is pertinent to note that, some of the published methods enabled estimation of drugs in combination products containing two drugs via spectrophotometric and HPLC methods. However, so far, not any HPTLC method was reported for the same. Hence, HPTLC chromatographic conditions were developed with forced degradation studies and the method was validated to establish selectivity with respect to excipients and degradation products.

MATERIAL AND METHODS

Apparatus

Chromatographic separation of drugs was performed on Merck TLC Aluminium plates precoated with silica gel 60 F254 of size (20 cm×10 cm) with 250 µm layer thickness. Samples were applied on the plates using Camag 100 µL sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). Plates were developed in a twin through glasschamber and scanned by Camag TLC scanner 3, operated by winCATS planar chromatography manager software which used Deuterium lamp as a radiation source.

Chemicals and Reagents

The API of Anagliptin and Metfromin procured from Intas Pharmaceuticals LTD, Ahmedabad. Combination test product METOANA Tablets 100/250 mg (manufactured by Sanwa Kagaku Kenkyusho Co. LTD) was procured from the market. All the solvents like Methanol, Toluene and Ammonium acetate were purchased from E. Merck Mumbai. All the chemical reagents were of analytical grade.

Preparation of Standard Stock Solution

The standard solution was prepared by weighing accurately about 10 mg of ANA and 25 mg of MET transferred into clean and dry 10 mL volumetric flask. Initially about 5 mL methanol was added to the flask respectively and sonicated. The volume was made up to the mark with the methanol to achieve 1000 µg/mL of ANA and 2500 µg/mL of MET standard stock solutions.

Preparation of working standard solution

From the above prepared Stock solutions, pipette out 1 mL of ANA and 1 mL MET solution and transferred into a clean and dry 10 mL volumetric flask, methanol was added upto the mark to get final concentration 100 µg/mL& 250 µg/mL respectively, in mixture.

Preparation of test solution

Twenty METOANA tablets were accurately weighed, their average weight was calculated. Amount of finely powdered tablet equivalent to 100 mg ANA and 250 mg MET was weighed and transferred into a 100 mL volumetric flask and the volume was adjusted to mark with methanol. The contents of the flask were sonicated for 30 min to dissolve the active ingredients completely. The solution was filtered through a Whatman filter paper no. 41. From this 1 mL aliquot was transferred into a 10 mL volumetric flask and the volume was made with methanol. This corresponds to 100 µg/ mL of ANA & 250 µg/ mL MET, in mixture was analysed for assay determination. Aliquot of 1 µL of this solution was used for assay determination which gave 100 ng/band of ANA and 250 ng/ band of MET.

Standardized Chromatographic conditions

It was developed on pre coated silica gel Aluminium plate 60F254 (10×20 cm with 0.2 mm thickness) pre washed with methanol then dried at room temperature. Samples were applied as 6 mm wide bands with application rate of 7 sec / µL. The plates were conditioned for 20 min in a pre-saturated twin-trough glass chamber (20 cm × 10 cm) with the mobile phase Toluene: 1% Methanolic ammonium acetate (2:4 % V/V) and ascending development was performed till a distance of 90 mm from the point of application. Then the plates were dried with the help of air dryer at 50^0 C for 5 min and densitometry scanning was performed at 243 nm for ANA and MET.

METHOD VALIDATION

The proposed method was validated as per ICH guidelines Q2 R1[22].

Linearity

The working standard solution containing 100 µg/mL ANA and 250 µg/mL MET were spotted on TLC plate with injection volume 1-10 µL to get concentration in the range of 100-1000 ng/band and 250-2500 ng/band, respectively. Six replicates of each concentration were performed and then calibration plots were determined by linear least – squares regression. The plate was developed on previously described mobile phase. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

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Precision
Repeatability was determined by applying six replicates of test solution (100 µg/mL ANA and 250 µg/mL MET). The intraday and interday precisions were determined by responses of six replicates on same and different days for the test concentration. The results were reported in terms of % RSD.

Accuracy
Recovery study was carried out by standard addition method where known amount of standard concentration at 50%, 100% and 150% of the test solution were spiked in the test solution in triplicate. The amount of drugs was estimated by substituting values in regression equation. The % RSDof the recovery was calculated.

LOD and LOQ
The LOD and LOQ of the developed method were calculated from the calibration curve using equations, LOD = $3.3 \times σ/S$ and LOQ = $10 \times σ/S$ where σ is the standard deviation of y- intercept and S is the slope of the curve.

Robustness
By introducing small changes in the detection wavelength (±2 nm) and saturation time for the chamber before plate development (±2 min), the effects on the results were determined. One factor at a time was changed and the effect on peak area of the drug was studied. Robustness of the method was done on single level for six replicates and %RSD was calculated.

Specificity
The specificity of the method was checked by the peak purity of analytes' peak.

FORCED DEGRADATION STUDIES
Force degradation study was intended to ensure the effective separation of ANA and MET and their potential degradation products which are generated under different stress conditions like acid, alkaline and neutral hydrolysis, oxidative degradation, thermal and photolytic degradation.

Acid Hydrolysis
Accurately weighed 10 mg ANA and 25 mg MET were transferred in 10 mL volumetric flask individually and in combination. To this were added 5 mL methanol and 5 mL of 0.1 N HCl and it was kept at room temperature for 5 h. From that solution 1 mL was transferred into 10 mL volumetric flask, neutralized with 0.1 N NaOH and diluted to mark with methanol. Aliquot of 1 µL of resultant solutions corresponding to 100 ng/band ANA and 250 ng/ band MET were applied on TLC plate and developed.

Alkaline Hydrolysis
Accurately weighed 10 mg ANA and 25 mg MET were transferred in 10 mL volumetric flask individually and in combination. To this were added 5 mL methanol and 5 mL of 0.1 N NaOH and it was kept at room temperature for 1 h. From that solution 1 mL was transferred into 10 mL volumetric flask, neutralized with 0.1 N HCl and diluted to mark with methanol. Aliquot of 1 µL of resultant solutions corresponding to 100 ng/band ANA and 250 ng/ band MET were applied on TLC plate and developed.

Oxidative Hydrolysis
Accurately weighed 10 mg ANA and 25 mg MET were transferred in 10 mL volumetric flask individually and in combination. To this were added 5 mL methanol and 5 mL of 3% H2O2 and it was kept at RT for 3 h. From that solution 1 mL was transferred into 10 mL volumetric flask, neutralized with 0.1 N HCl and diluted to mark with methanol. Aliquot of 1 µL of resultant solutions corresponding to 100 ng/band ANA and 250 ng/ band MET were applied on TLC plate and developed.

Thermal Degradation
Accurately weighed 10 mg ANA and 25 mg MET were transferred in 10 mL volumetric flask individually and in combination in petridish. Those were kept at 70^0 C, 4 hfor 4 h. After that those were dissolved in 10 mL methanol. From that solution 1 mL was transferred into 10 mL volumetric flask and diluted to mark with mobile phase. Aliquot of 1 µL of resultant solutions corresponding to 100 ng/band ANA and 250 ng/ band MET were applied on TLC plate and developed. Same condition was applied to formulation and solutions were prepared with the above mention concentrations according to the dilution scheme.

Photolytic degradation
Accurately weighed 10 mg ANA and 25 mg MET were transferred in 10 mL volumetric flask individually and in combination in petridish. It was exposed in photo stability chamber (TH-90S, Thermo lab, Mumbai, India) in UV light at 254 nm for 36 h to get 200 watt hours / m2 intensity. After that those were dissolved in 10 mL methanol. From that solution 1 mL was transferred into 10 mL volumetric flask and diluted to mark with mobile phase. Aliquot of 1 µL of resultant solutions corresponding to 100 ng/band ANA and 250 ng/ band MET were applied on TLC plate and developed. Same condition was applied to formulation and solutions were prepared with the above mention concentrations according to the dilution scheme.

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RESULT AND DISCUSSION

Optimization of chromatographic Conditions

For separation of ANA and MET and their degradation peaks different mobile phases with different solvents in different ratio were tried like (1) Chloroform: Methanol: GAA (4:3:1 %V/V/V) (2) Chloroform: Methanol: GAA (3:5:0.5 %V/V/V) (3) Toluene: Chloroform: Methanol: GAA (3:5:1:1%V/V/V) (4) Acetone: Chloroform : Methanol: Formic acid (3:5:1:1 %V/V/V) (5) Toluene: Chloroform : Methanol 1%ammonium acetate (1:4:3 %V/V/V). Finally, the mixture of Toluene: 1% Methanolic ammonium acetate (2:4% V/V) showed well resolved peaks with better peak shape. The drugs were resolved with Rf values 0.728 ± 0.013and 0.324 ± 0.009 for ANA and MET at, respectively. Determination of ANA and MET was done at wavelength 243 nm to minimize the noise and improve baseline conditions. Both the drugs and potential degradation products could be detected at 243 nm. The spot appeared more compact and peak shape more symmetrical when the HPTLC plate were pre-treated with methanol and activated at 50^0 C for 5 min. (Figure 3).

Linearity

Aliquots of standard solution were applied in the concentration range of 100 -1000 ng/ band and 250 - 2500 ng/ band for ANA and MET respectively and densitogram was developed under above optimized condition. The calibration curve obtained by the least square regression analysis between average peak area and concentration showed linear relationship with a correlation coefficient of 0.9992 and 0.9993 for ANA and MET respectively. The linear regression equation were y = 18.311x + 273.1and y = 6.9405x + 2862.6 for ANA and MET respectively. (Figure 4, Figure 5, Figure 6), (Table 1)

Precision

The % RSD for repeatability was found to be 1.47 for ANA and 1.28 for MET. The % RSD of Intraday Precision was found to be 1.64 for ANAand 1.74 for MET. The % RSD of Interday precision was found to be 1.72 for ANA and 1.74 for MET. Hence, confirming precision of the developed method. (Table 2)

Accuracy

The accuracy of the developed method was established by standard addition method by adding known standard concentration solutions to the pre-analysed samples. Recoveries were in between 99.99-101.64 % for ANA and 99.68-101.38 % for MET which is in accordance with ICH guidelines which proves method to be accurate. (Table 3)

LOD and LOQ

The LOD calculated by formulae was found to be 8.67 ng/ band for ANA and 67.29 ng/ band for MET. The LOQ calculated by formulae was found to be 26.29 ng/ band for ANA and 203.9 ng/ band for MET.

Robustness

Slight change in the chromatographic condition of the developed method like saturation time of chamber, detection wavelength did not affect the result significantly. The % RSD values were found below 2 indicated the method to be robust. (Table 4)

Analysis of marketed formulation

The developed method was applied to marketed tablet preparation. The assay results of ANA and MET were 100.57 ± 1.07 % and 100.69 ± 1.21 %, respectively of the labelled amount. **(**Figure 7), (Table 5)

Forced degradation studies

The result of forced degradation studies are summarized in (Table 6 and Table 7). Under the optimized chromatographic conditions degradation products of analytes were well resolved and the percent degradation was calculated by comparing peak area with standard preparation.

During stress degradation experiments, it was observed that ANA degraded significantly under acidic, alkaline, oxidative and thermal stress conditions; degraded moderately under photolytic stress conditions; and showed negligible degradation under neutral hydrolysis condition. MET degraded significantly under alkaline and oxidative stress conditions; degraded moderately under acidic, neutral and thermal stress condition and showed negligible degradation under photolytic stress condition.

The purity of the drug (analyte peak) was ascertained by analysing spectrum at peak start, peak end and peak apex position which showed no interference of any excipients or process impurities in analyte peak. The method is also deemed to be specific from potential degradation products as most of the degradation products are adequately resolved from both analyte peaks. The sample tablet was exposed to thermal and photolytic degradation conditions as per ICH Q1A R2 guidelines [23]. The method was therefore being considered to be stability indicating for tablet solid dosage form. (Figure 8, Figure 9, Figure 10, Figure 11, Figure 12 and Figure 13),.

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Table 1: Linearity range of ANA and MET

Sr No.	ANA			MET		
	Conc (ng/band)	Peak Area* ± SD	%RSD	Conc (ng/band)	Peak Area * ± SD	%RSD
1	100	1947.883 ± 28.79	1.47	250	4568.033 ± 66.68	1.45
2	200	3842.567 ± 39.59	1.03	500	6133.883 ± 66.23	1.07
3	300	5682.133 ± 58.07	1.02	750	8291.55 ± 102.54	1.23
4	400	7819.367 ± 108.02	1.38	1000	9762.95 ± 123.89	1.26
5	500	9655.65 ± 93.54	0.96	1250	11724.57 ± 185.48	1.58
6	600	11354.6 ± 144.03	1.26	1500	13241.2 ± 163.26	1.23
7	800	14868.6 ± 184.98	1.24	2000	16610.2 ± 276.28	1.66
8	1000	18427.15 ± 222.16	1.20	2500	20238.7 ± 222.02	1.09

*Average of six determinations

Table 2: Repeatability, Intraday and Interday Precision of ANA and MET

Parameter	ANA			MET		
	Conc (ng/band)	Peak Area* ± SD	%RSD	Conc (ng/band)	Peak Area * ± SD	%RSD
Repeatability	100	2128.0± 31.42	1.47	250	4653.08± 73.94	1.58
Intraday Precision	100	2142.66± 35.15	1.64	250	4640.38 ± 80.78	1.74
Interday Precision	100	2154.33 ± 37.08	1.72	250	4645.38± 81.22	1.74

*Average of six determinations

Table 3: Recovery study data of ANA and MET

Drug	Amount of Test Solution (ng/band)	Amount of Stand. added (ng/band)	Peak Area* ± SD	Total Amount Found (ng/band)	Recovered amount (ng/band)	% Recovery	% RSD
ANA	100	0	2111.83 ± 21.99	100.41	---	100.41	1.19
	100	50	3019.53 ± 28.20	149.98	49.98	99.99	1.02
	100	100	3944.06 ± 48.45	200.47	100.47	100.23	1.31
	100	150	4926.06 ± 58.78	254.10	154.10	101.64	1.26
MET	250	0	4612.3 ± 21.87	252.09	---	100.84	1.25
	250	125	5501.26 ± 26.66	380.18	130.18	101.38	1.01
	250	250	6352.2 ± 52.14	502.78	252.78	100.55	1.49
	250	375	7186.7 ± 58.63	624.71	374.71	99.68	1.35

*Average of three determinations

Table 4: Robustness study of ANA and MET

Condition	Variation	% Assay* ± SD		% RSD#	
		ANA	MET	ANA	MET
Normal Test Method	N/A	100.57 ± 1.07	100.69 ± 1.20	1.06	1.20
Detection Wavelength (243±2 nm)	241 nm	101.10 ± 1.23	99.98 ± 1.24		
	245 nm	101.04 ± 0.87	100.96 ± 1.35		
Chamber Saturation time (20 ± 2 min)	22 min	100.91 ± 0.91	100.91 ± 0.91		
	18 min	100.63 ± 1.65	100.63 ± 1.65		

* Average of six determinations

#RSD of original and modified conditions

Table 5: Analysis of marketed tablet formulation

Drug	Amount of drug		% Label claimed (% Assay* ± SD)	% RSD
	Labeled	Estimated		
ANA	100	100.57	100.57 ± 1.07	1.06
MET	250	251.73	100.69 ± 1.21	1.20

*Average of six determinations

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Table 6: Summary of Forced Degradation study of individual API in mixture of ANA and MET

Degradation condition	Rf Value of Analyte		No. of degradation peaks		%Degradation	
	ANA	MET	ANA	MET	ANA	MET
0.1 N HCl at RT, 5 h	0.71	0.36	1	2	3.79	18.97
0.1 N NaOH at RT, 1 h	0.74	0.36	2	2	28.63	21.75
Water, RT, 3 h	0.69	0.36	--	--	5.42	1.37
3% H_2O_2, RT, 3 h	0.67	0.35	1	1	11.69	17.64
Thermal at 70^0 C, 4 h	0.71	0.36	1	2	1.83	8.09
UV light, 254 nm, 36 h	0.72	0.41	1	1	2.15	4.77

Table 7: Summary of Forced degradation study in Pharmaceutical Dosage form

Degradation condition	Rf Value of Analyte		No. of degradation peaks		%Degradation	
	ANA	MET	ANA	MET	ANA	MET
Thermal at 70^0 C, 4 h	0.69	0.35	1	2	1.65	7.99
UV light, 254 nm, 36 h	0.71	0.39	1	1	3.08	4.58

Table 8: Summary of Validation Parameters

Sr. No.	Parameter	ANA	MET
1	Specificity	Specific	Specific
2	Rf value	0.728 ± 0.013	0.324 ± 0.009
3	Linearity Range	100-1000 ng/band	250-2500 ng/band
4	Regression Line equation	y = 18.311x + 273.1	y = 6.9405x + 2862.6
5	Correlation Coefficient	$R^2 = 0.9992$	$R^2 = 0.9993$
6	Precision	% RSD	
	Repeatability	1.47	1.58
	Intraday Precision	1.64	1.74
	Interday Precision	1.72	1.74
7	Accuracy (% Recovery)	99.99-101.64	99.68-101.38
8	LOD (ng/band)	8.67ng/band	67.29 ng/band
9	LOQ (ng/band)	26.29 ng/band	203.9 ng/band
10	Robustness	Robust	Robust

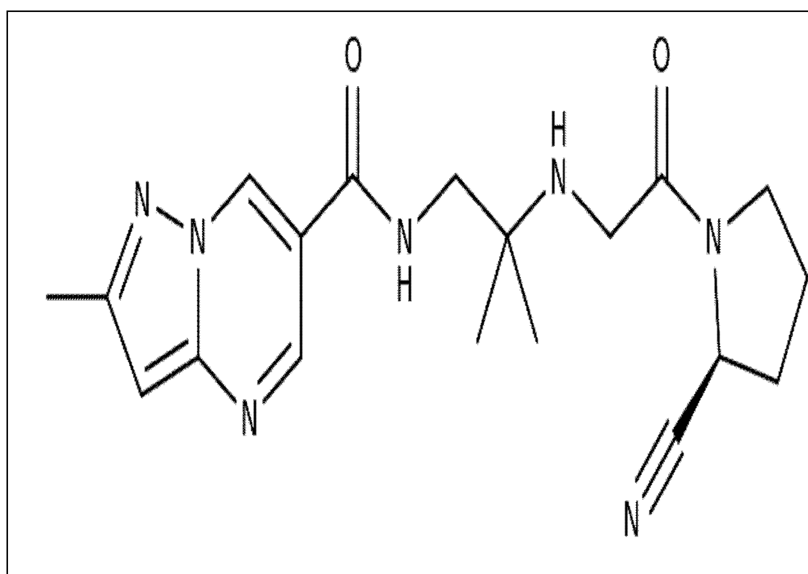

Figure 1: Chemical Structure of Anagliptin

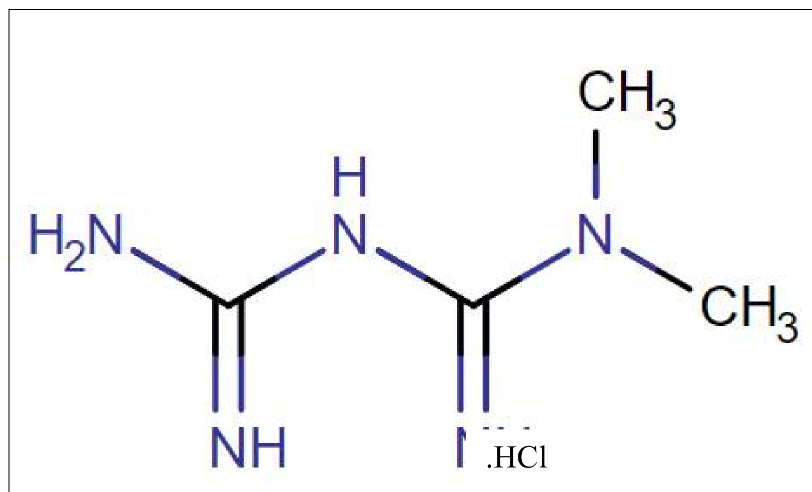

Figure 2: Chemical structure of Metformin HCl

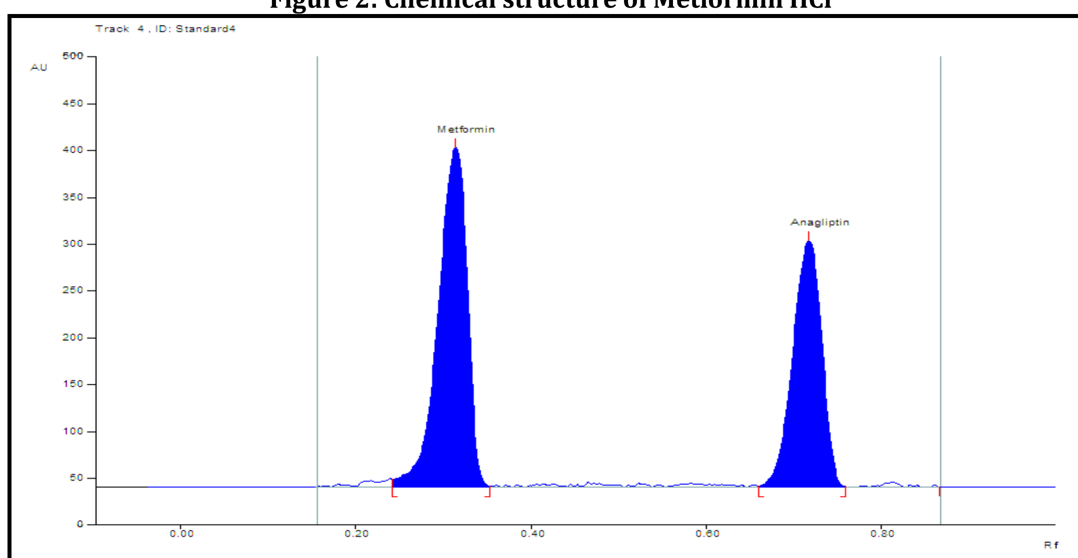

Figure 3: Chromatogram of STD ANA (100 ng/ band) and MET (250 ng/band) at 243 nm

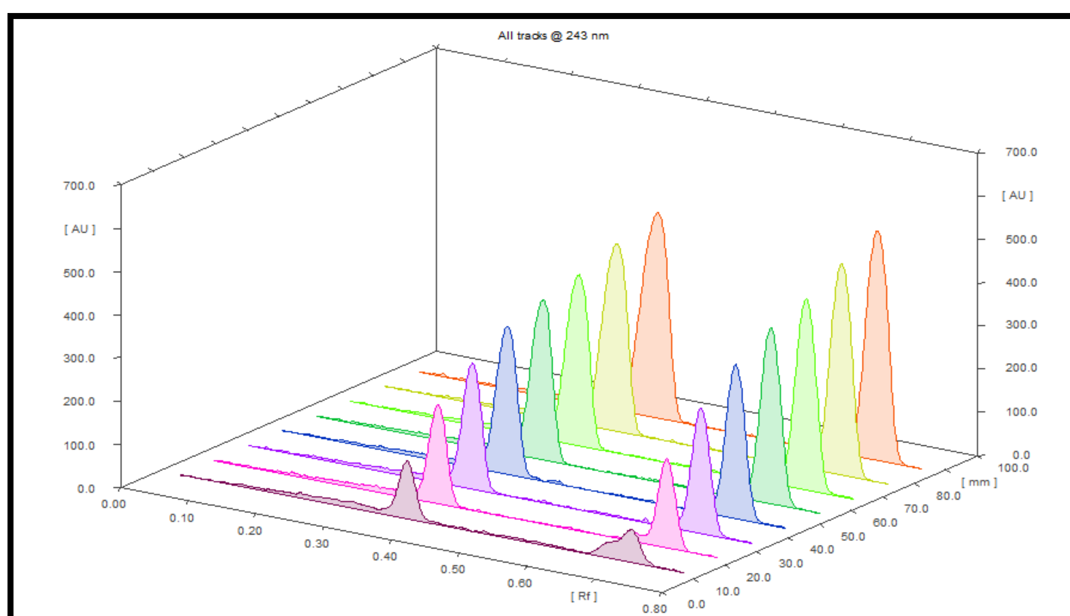

Figure 4: 3D overlain chromatogram of Linearity of ANA (100-1000 ng/band) and MET (250 -2500 ng/band) at 243 nm

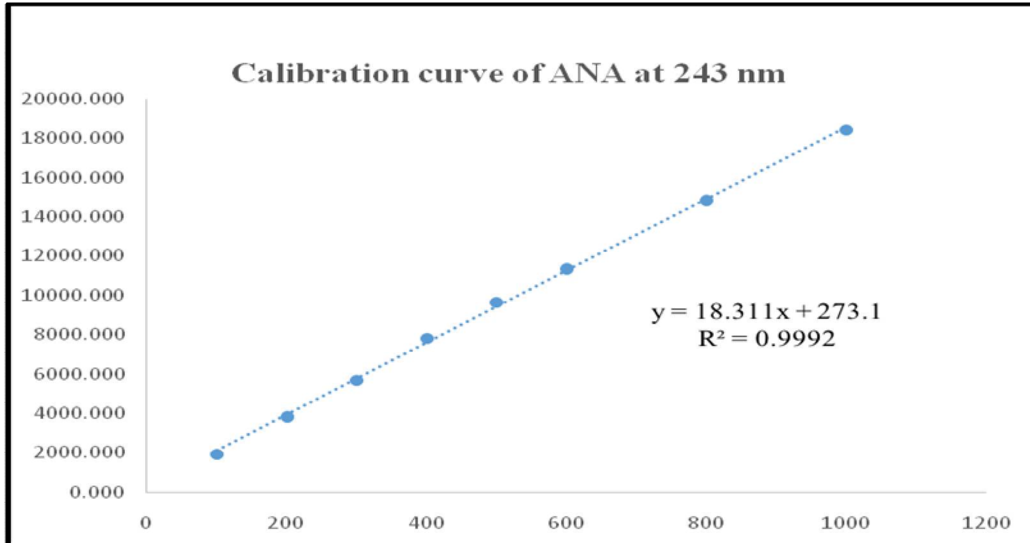

Figure 5: Calibration Curve of Linearity of ANA at 243 nm

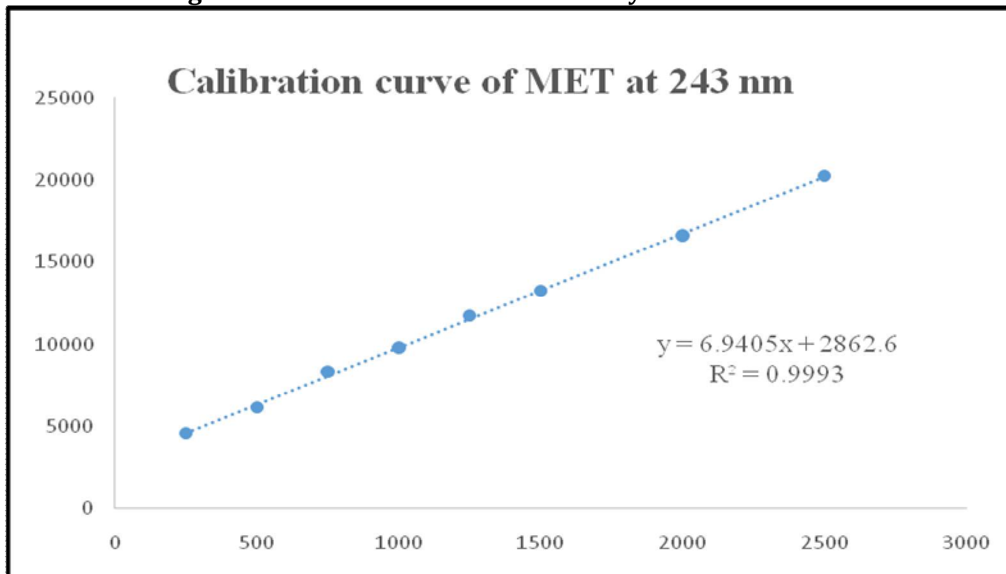

Figure 6: Calibration Curve of Linearity of MET at 243 nm

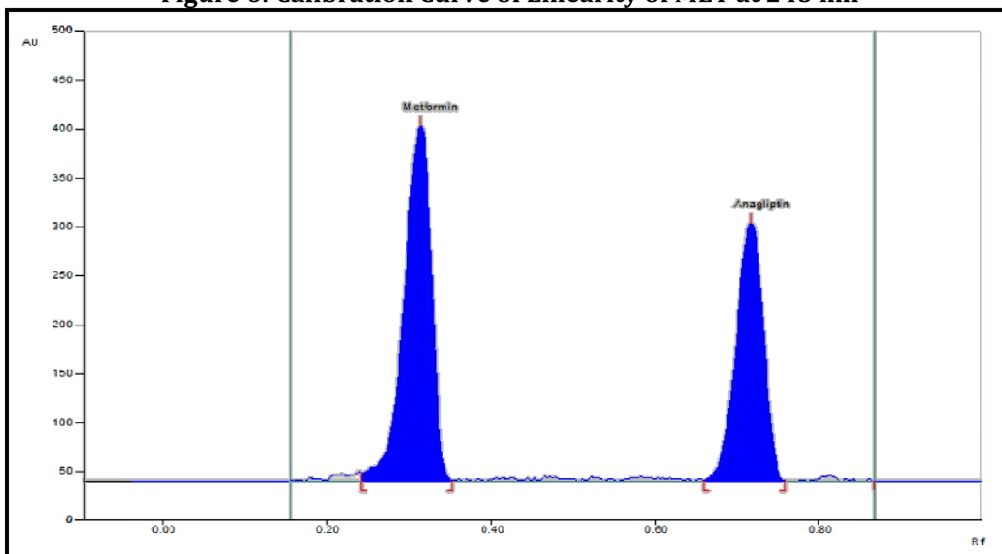

Figure 7: Chromatogram of test solution of ANA (100 ng/ band) and MET (250 ng/ band)

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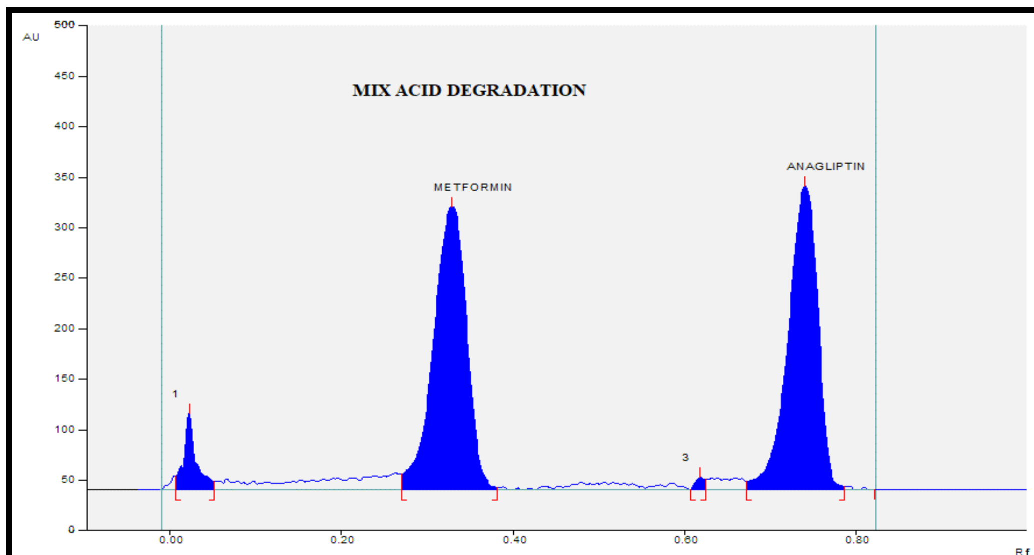

Figure 8: Chromatogram of Acid hydrolysis of mixture of ANA and MET at 243 nm

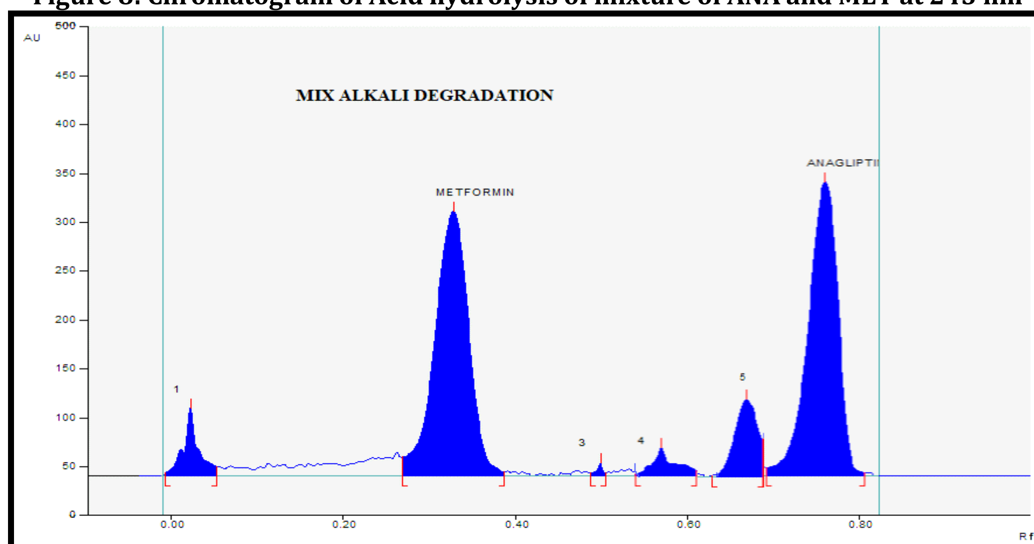

Figure 9: Chromatogram of Alkali hydrolysis of mixture of ANA and MET at 243 nm

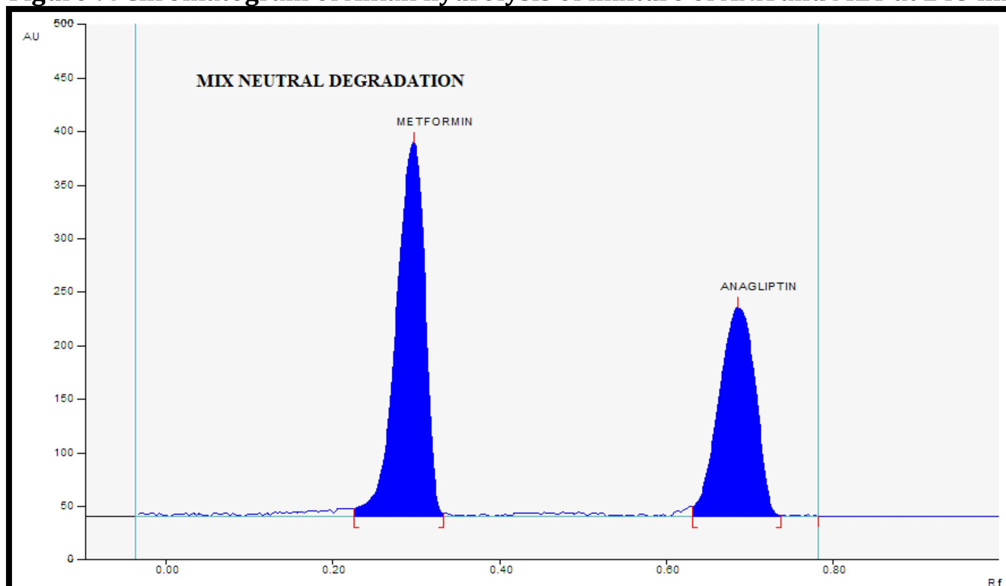

Figure 10: Chromatogram of Neutral hydrolysis of mixture of ANA and MET at 243 nm

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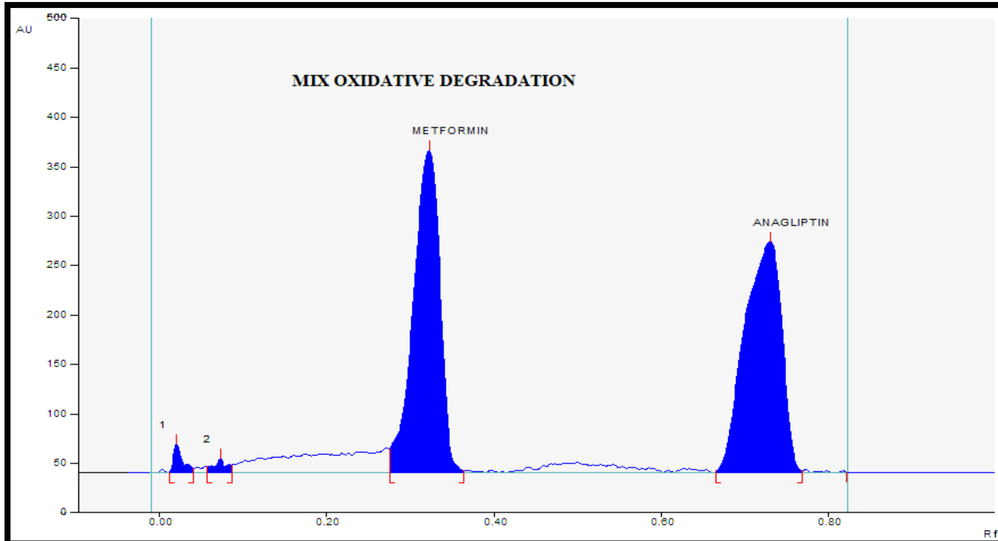

Figure 11: Chromatogram of Oxidative hydrolysis of mixture of ANA and MET at 243 nm

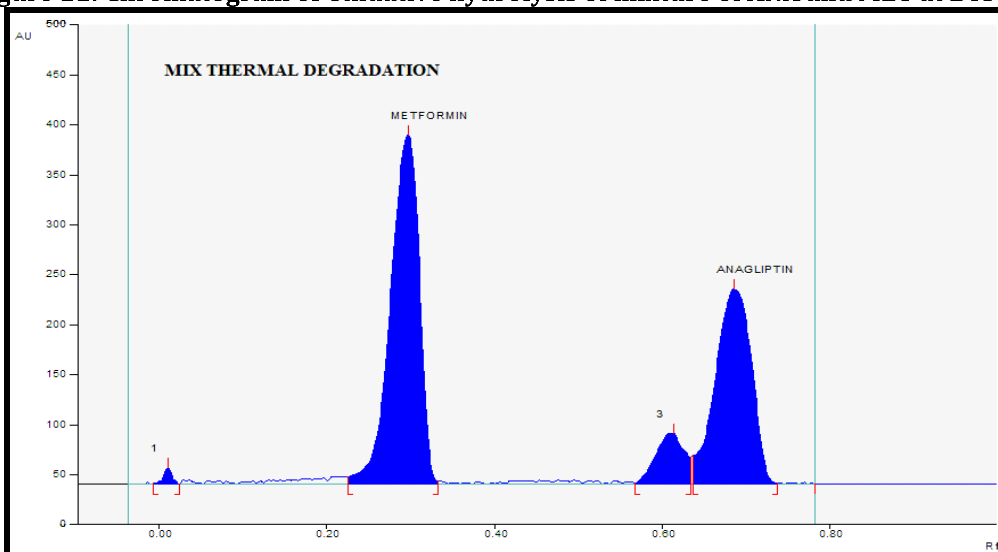

Figure 12: Chromatogram of Thermal degradation of mixture of ANA and MET at 243 nm

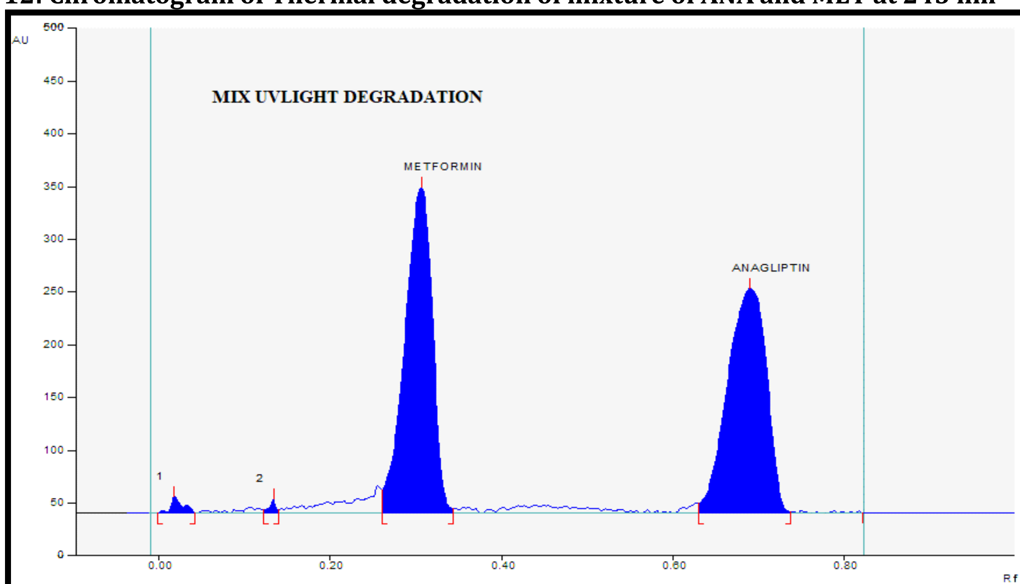

Figure 13: Chromatogram of Photolytic (UV Light)degradation of mixture of ANA and MET at 243 nm

CONCLUSION

The proposed HPTLC method is precise, specific, linear and accurate for the estimation of ANA and MET in pharmaceutical dosage form without interference from the excipients and potential degradation products in various stress conditions like acid hydrolysis, alkaline hydrolysis, neutral hydrolysis, oxidative, thermal and photolytic degradation conditions. The developed method is validated as per ICH guidelines. The results showed the suitability of developed method for stability studies of the pharmaceutical dosage form.

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COMPETING INTERESTS:

The authors have declared that no competing interest exists.

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