



Development and validation of stability-indicating HPTLC method for simultaneous estimation of Metformin, Saxagliptin, and Dapagliflozin in their combined matrix using AQB

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ABSTRACT

The present research work developed a simple, precise, and stability-indicating HPTLC method for the simultaneous determination of Metformin, Dapagliflozin, and Saxagliptin in bulk and tablet dosage forms. The conditions were optimized using the Box-Behnken design (BBD) to examine the effect of 3 variables: the Saturation time, the solvent front, and the volume of Methanol at 3 levels. The optimized chromatographic conditions used pre-coated silica gel 60 F254 HPTLC aluminum plates, mobile phase containing Methanol: 0.5 % aqueous ammonium sulphate (8:2, %v/v), pH 5.5, the saturation time of 20 min, and the solvent front of 95 mm. The method was developed in linear ascending order using a Twin through glass chamber. The detection was carried out at 222 nm using a UV detector. The proposed method was validated according to International Council for Harmonization (ICH) guidelines for linearity, precision, accuracy, LOD, LOQ, specificity, and robustness. The optimized method was utilized to evaluate the stability of MET, DAPA, and SAXA in different stress conditions by performing forced degradation studies. The observed regression coefficients for MET, DAPA, and SAXA were > 0.990. The proposed method is precise, and the percentage relative standard deviation was between 0.5 and 2.0. The observed percentage recoveries were between 96.7 and 99.54 for all three compounds. The results from the degradation study stipulated that on exposure to various stressors, namely acid, alkali, oxidative, and UV light, the MET, DAPA, and SAXA.

Keywords: Box-Behnken Design, Dapagliflozin, Metformin, Saxagliptin, Validation

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INTRODUCTION

Metformin hydrochloride (MET) chemically, 1, 1-dimethyl biguanide hydrochloride, is an oral antidiabetic drug (1). Because of its better efficiency, safety profile, and cardiovascular and metabolic benefits, MET is advised as first-line therapy to improve glucose tolerance in patients with type 2 diabetes mellitus by lowering basal and postprandial plasma glucose (2). Unlike sulfonylureas, metformin does not produce hypoglycemia in diabetic patients' diabetes or normal subjects and does not cause hyperinsulinemia (3). Saxagliptin (SAXA) is another oral antidiabetic agent belonging to the dipeptidyl peptidase-4 (DPP-4) inhibitor class of drugs used for treating type 2 diabetes in combination with MET. Chemically it is known as (1S,3S,5S)-2-[(2S)-2-amino-2-(3-hydroxy-1-adamantyl)acetyl]-2-azabicyclo [3.1.0] hexane-3-carbonitrile (4). Saxagliptin and its active metabolite 5-hydroxy Saxagliptin are DPP-4 inhibitors that enhance glycemic control by inhibiting the inactivation of the incretin hormones GLP-1 and glucose-dependent insulinotropic polypeptide. This raises GLP-1 levels, promotes insulin secretion, and lowers postprandial glucagon and glucose levels (5).

Dapagliflozin (DAPA) is a novel Sodium-Glucose Co-Transporter 2 (SGLT2) inhibitor, the primary transporter involved in glucose reabsorption in the kidneys that reduce renal glucose reabsorption to improve glycemic control in type 2 diabetes mellitus patients. It is chemically known as (1s)-1, 5-anhydro-1-C-[4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl] -D-glucitol (6).

Recently, MET, DAPA, and SAXA-containing FDC Qternmet XR extended-release tablets and Qtrilmet modified-release tablets manufactured by AstraZeneca, received US Food and Drug Administration (USFDA) and the European Medicines Agency (EMA) approval respectively for treating type 2 diabetes

mellitus (7) (8). It is indicated to improve glyce mic control in adults aged 18 years and older with type 2 diabetes mellitus when metformin with or without sulphonylurea and either Saxagliptin or dapagliflozin does not provide adequate glyce mic control (9). A literature review revealed that most analytical techniques, including High-Performance Liquid Chromatography (HPLC), Liquid chromatography-mass spectrometry (LC-MS), and Gas Chromatography (GC) have been described for estimating MET, DAPA, and SAXA alone or in combination with other drugs (10–15). However, no High-Performance Thin-Layer Chromatographic (HPTLC) technique is available for stability testing of MET, DAPA, and SAXA.

HPTLC separation procedures based on a “trial and error” approach are time-consuming and can only offer information on the responsiveness of a few key analytical parameters. Furthermore, these methods provide limited information on how the various parameters interact and impact one another. Developing the analytical technique requires thoroughly investigating multiple variables that may impact the sensitivity and specificity (16).

Using the design of experiments (DOE), a systematic approach may generate scientifically valid results by systematically identifying and analyzing essential components during the method development with fewer runs required than a conventional technique (17).

Box–Behnken Design (BBD) was employed in the present research work to develop a simple, precise, and stability-indicating HPTLC technique for the simultaneous determination of MET, DAPA, and SAXA in bulk and tablet dosage forms. The method was validated according to International Conference on Harmonization (ICH) guidelines. The drugs’ stability was analyzed by subjecting the drugs to various stress conditions of acid, basic, oxidative, and photolytic degradation.

MATERIAL AND METHODS

Chemicals and reagents

Acetonitrile, methanol, ammonium acetate, ethyl acetate, triethyl amine, chloroform, toluene, benzene, orthophosphoric acid, and triethylamine (all were AR grade) were obtained from Merck Chemicals, Mumbai. Potassium dihydrogen orthophosphate, ammonium sulphate, ammonium Acetate (AR grade) were obtained from SD Fine Chemicals Ltd, Mumbai. Metformin Hydrochloride was received as a gift sample from West Coast Pharmaceutical Works Ltd., Gujarat. Dapagliflozin and Saxagliptin were received as a gift sample from Torrent Pharmaceutical Ltd., Ahmedabad-Gujarat.

Instrumentation

The analysis was carried out on pre-coated silica gel 60 F254 HPTLC aluminum plates (Merck, Germany) using UV chamber and TLC scanner 4 (Camag, Switzerland) with Linomat 5 applicator using Linomat syringe 100 µl, Hamilton-Bonaduz Schweiz (Camag, Switzerland). The instrument was controlled by win CATS software Version 1.4.2 (Camag, Switzerland) and Design-Expert trial Version 13.0.7.0 (Stat-Ease Inc., Minneapolis).

Preparation of Stock Solution

The stock solution containing 1 mg/ ml of each drug was prepared by accurately weighing 25 mg of each MET, SAXA, and DAPA, dissolving in methanol, and making the volume 25 ml with AR grade methanol. The stock solution was further diluted to obtain standard solutions of META, SAXA, and DAPA (1000 µg/band).

Method Optimization

The standard solutions were scanned in a 200 to 400 nm UV region. The overlay of the scan was observed for the selection of the analytical wavelength. Based on the literature review, several mobile phase compositions and their different ratios were tested using different solvents with different pH and combinations. The mobile phase using which good separation of all the 3 drugs was observed was selected. In the present study, BBD design optimized the chromatographic conditions where 3 factors were studied at 3 levels. From the stock solution of each drug (100 µL/ml), a mixed solution of MET, SAXA, and DAPA (10 µL/ml of each drug) was prepared and analyzed using different chromatographic conditions.

Analysis of Synthetic Mixture

A synthetic mixture equivalent to 1000 mg MET, 5 mg SAXA, and 5 mg of DAPA that mimics marketed FDC tablets were prepared and chromatograms were recorded using the optimized chromatographic conditions at wavelength 222 nm.

METHOD VALIDATION

The method was validated following ICH guidelines Q2 (R1) for evaluating various parameters; linearity, precision, accuracy, Limit of Detection (LOD), Limit of Quantification (LOQ), specificity, and robustness.

Linearity

To establish the linearity of the proposed HPTLC method, the relationship between peak area and concentration of MET, SAXA, and DAPA was evaluated over the concentration range of 50,000–150,000 ng/band for MET and 250–750 ng/band for SAXA and DAPA (n=5). Calibration graphs were constructed

by plotting the area under the peak versus the concentration of MET, SAXA, and DAPA using the ordinary least squares regression analysis method.

Precision

The precision of the developed method was evaluated by performing repeatability and interday precision studies. Repeatability was performed with three replicates at three different concentrations 50,000, 100,000, and 150,000 ng/band MET and 250, 500, and 750 ng/band, each of SAXA and DAPA. The peak area measured was expressed as a percentage relative standard deviation (% RSD) for all the developed chromatograms.

Accuracy

The accuracy of this method was ascertained by performing recovery at three levels (50%, 100%, and 150%). Recovery studies were carried out by spiking three different amounts of MET standard (25000, 50000, and 75000 ng/band) into the synthetic mixture, where the MET test concentration was 50000 ng/ml. While recovery study of SAXA and DAPA was performed by spiking three different amounts of SAXA and DAPA standard (120, 250, and 375 ng/band) to the synthetic mixture, where SAXA and DAPA test concentration was 250 ng/band. Recovery studies were performed in triplicate.

LOD and LOQ

As per ICH guidelines, the LOD and LOQ were calculated from the standard deviation of the response and mean slope of the calibration curve using the formula,

$$\text{Limit of Detection} = 3.3 \times \sigma / S$$

$$\text{Limit of Quantitation} = 10 \times \sigma / S$$

Where “ σ ” is the Standard deviation of the Response

“S” is the Mean of the slope of the calibration curve

Here, known analytes in low concentrations were compared with the signals of the blank samples, and the resulting chromatograms were analyzed.

Specificity

The specificity of the method was ascertained by analyzing the peak purity of the standard drugs and samples. The spots obtained for MET, SAXA, and DAPA in the sample were confirmed by comparing the Rf values and spectra of all the three drugs with that of the standard. The peak purity of each drug was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M), and peak-end (E) position of the spot.

Robustness

In the present research work, a deliberate change in saturation time (20 min \pm 2.0 min) was made and its effect on retention time and peak area was observed (n=6). The effect was checked by observing % RSD.

FORCED DEGRADATION STUDY

The standard drugs were subjected to stress degradation conditions like acid and base hydrolysis, oxidation, and photodegradation to evaluate the stability-indicating property of the developed HPTLC method. 1000 mg MET, 5 mg SAXA, and 5 mg DAPA was accurately weighed and the drug was transferred individually to 10 mL volumetric flask and diluted with methanol to obtain a final concentration of 1000 μ g/band MET, 5 μ g/band SAXA and DAPA. This solution was further subjected to following forced degradation study.

Acid-induced Degradation Study

To 5 mL of the above standard drug solution, 5 mL of (0.1 N, 0.5 N, and 1 N separately) hydrochloric acid was added and refluxed at 40 and 60°C for 6 h. Neutralized 20 μ l solution was directly applied to the HPTLC plate followed by the resultant solutions, on regular intervals, and chromatograms were run.

Base-induced Degradation Study

To 5 mL of the above standard drug solution, 5 mL of (0.1 N, 0.5 N, and 1 N separately) sodium hydroxide was added and refluxed at 40 and 60°C for 6 h. Neutralized 20 μ l solution was directly applied to the HPTLC plate followed by the resultant solutions, on regular intervals and chromatograms were run.

Oxidative Degradation Study

To 5 mL of the above standard drug solution, 5 mL of 1% hydrogen peroxide was added and refluxed at 40 and 60°C for 6 h. Then was applied on an HPTLC plate followed by the resultant solutions, on regular intervals and chromatograms were run.

Photodegradation Study

For the photodegradation study, mixed standard powder samples of MET, SAXA, and DAPA were exposed to UV light (in a UV chamber, 254 nm) for 5 days. From the resultant solutions, 20 μ l of solution were directly injected into HPTLC, and chromatograms were run under optimized chromatographic conditions.

RESULTS AND DISCUSSION

Selection of detection wavelength

The common detection wavelength selected for simultaneous analysis of MET, DAPA, and SAXA was 222 nm, as all three drugs showed good absorption at this wavelength.

Optimization of Chromatographic Condition

Selection of mobile phase

Of various combinations screened, the composition of Methanol: 0.5 % aqueous ammonium sulphate (8:2, v/v) was selected as the mobile phase as it resulted in well-defined peaks, different retention times, and good resolution for each drug as shown in **Figure 2**.

Selection of chromatographic conditions using AQbD approach

Responses of 17 experimental runs following the 3 factors 3 variable BBD are presented in **Table 1**.

A three-factorial, Box-Behnken statistical experimental design was performed using 17 experiments trial run. The independent variables and the responses for all 17 optimized trials and experimental runs are given in Table 2. It was observed that the best-fitted model was the quadratic model and the comparative values of r^2 , SD, and % c.v. for the different proposed models is given in Table 3 A positive value represents an effect that favors the optimization, while a negative value indicates an inverse relationship between the factor and the response (Myers and Montgomery, 2002). Comparative values of RSD were found to be 0.94 and % cv was found to be 9.92 for the proposed model along with the regression equation generated for the finally selected response found to be $R1=8.660+0.0538A+0.0362 B+0.0625C+0.0025AB-0.075AC+0.005BC-0.342A2-0.0092B2-0.0118C2$.

Using the response surface curves, the chromatographic conditions were optimized to 20 min saturation time, using 8 ml Methanol, and a solvent front of 95 mm (as shown in **Table 2**). The chromatogram with the optimized chromatographic condition is shown in **Figure 4**.

Analysis of Synthetic Mixture

The synthetic mixture, when analyzed using the developed HPLC method, showed good recovery, demonstrating that the method can be applicable in routine quality control testing of the tablet dosage form. The % recovery for MET was 98.37-99.68%, SAXA 98.90-99.49% for and for DAPA 98.27-99.29% as shown in **Table 2**

Validation

Linearity

If there is a linear relationship, test results should be evaluated by appropriate statistical methods like least squares regression analysis. All three drugs showed a good correlation over a concentration range of 50000-150000 ng/band for MET, 250-750 FOR SAXA and DAPA with respect to peak area. Data from the regression provide mathematical estimates of the degree of linearity(18)(19). The regression coefficient (R²) values for MET, SAXA and DAPA were noted to be 0.985, 0.996 and 0.999, respectively. The data from the linearity study is shown in **Table 3** and **Table 4**.

LOD and LOQ

LOD is the smallest quantity of analyte in a sample that can be identified, although not necessarily quantitatively, under given specified experimental circumstances. LOQ is the smallest quantity of analyte in a sample that can be quantified with acceptable precision and accuracy under the specified experimental circumstances (19). LOD of MET, SAXA, and DAPA was found to be 2962.22, 11.08, and 17.90 ng/band, respectively, while LOQ of MET, SAXA, and DAPA were found to be 8976.44, 33.59, and 54.29 ng/band for the proposed method. These values indicated that the method is sensitive.

Precision

The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation of a series of measurements. MET, SAXA and DAPA showed %RSD of less than 2% (as shown in **Table 5**), indicating acceptable precision in terms of repeatability of peak area measurement performed in the same laboratory, indicating the ruggedness of the method. The low value of %RSD showed that the method is precise within the acceptance limit of 2% as dictated by the ICH guidelines.

Accuracy

By spiking previously analyzed test solution 50000 ng/band, 250 ng/band SAXA and DAPA to three different concentration level of each analyte described in Table 6. The proposed method when used for evaluation of recovery at three concentrations levels, 50%, 100% and 150% after spiking with standard, showed percentage recovery between 99.39 to 99.72% for MET, 98.15 to 99.05% for SAXA and 96.71 to 99.54% for DAPA. As shown in **Table 6** the results are in good agreement with acceptable values for the validation of an analytical procedure proving that the method is accurate. The low value (<1.48) of the %RSD is indicative of the repeatability of the method.

Specificity

The chromatogram of the synthetic mixture obtained using the developed method showed a peak at Rf value of 0.21, 0.46 and 0.88 min for MET, SAXA and DAPA, respectively that was found to be at the same Rf value for all three standard drugs by comparison of chromatograms. It was observed from the results that

purity was more than 0.99 for all peaks, indicating the specificity of analytes in the presence of excipients. Results of Specificity study is shown in **Table 7**.

Robustness

As defined by the ICH, the robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters. There were no significant changes in retention time, area of peaks obtained when deliberate change of saturation time was made in the analysis demonstrating the developed method's resistance to variances in chromatographic conditions. The developed method was robust as % RSD of less than 2 % was observed. Results of robustness study are shown in **Table 8**.

There were very slight changes in the peak area, Rf and resolution. The lower value of SD and %RSD indicate the robustness of method.

Forced Degradation Study

Acid-Induced Degradation Study

The results of acid-induced degradation of a mixed solution of MET, SAXA, and DAPA using various concentrations of acid (0.1, 0.5, 1 N HCl) at 40°C and 60°C analysed at different time duration like 60, 120, 180, 240, 300, and 360 min, using optimized chromatographic conditions are presented in **Table 9**. At 1 N HCl concentration and 60°C %drug degradation was found to be 60.23, 74.17 and 42.17 for MET, SAXA and DAPA, respectively. Half-life was found to be 0.5-2.0 h for MET, 0.16-0.38 h for DAPA and 0.38-0.6 h for SAXA at 60 °C 1 N HCl. Shelf-life was found to be 0.1 h, 0.05 h and 0.08 h for MET, DAPA and SAXA, respectively.

Base Induced Degradation Study

The results of MET, SAXA and DAPA using various concentration of base (0.1, 0.5, 1 N NaOH) at 40 °C and 60 °C at different time duration like 60, 120, 180, 240, 300 and 360 min, analysed using optimized chromatographic conditions are presented in **Table 10**. At 1 N NaOH concentration and 60°C %drug degradation was found to be 43.52, 95.68 and 66.5 for MET, SAXA and DAPA, respectively. Half-life was found to be 0.38-0.57 h for MET, 0.16-0.21 h for DAPA and 0.08-0.21 h for SAXA at 60 °C 1 N NaOH. Shelf-life was found to be 0.07 h, 0.03 h and 0.03 h for MET, DAPA and SAXA, respectively.

Oxidative Degradation Study

The results of MET, SAXA and DAPA using 1% H₂O₂ at 25 °C, 40 °C and 60 °C at different time duration like 1440, 4320 and 7200 min analysed using optimized chromatographic condition are presented in **Table 11**. At 1% hydrogen peroxide concentration and 60°C %drug degradation was found to be 76.49, 93.99 and 86.97 for MET, SAXA and DAPA, respectively. Half-life was found to be 0.18-0.61 h for MET, 0.13-0.17 h for DAPA and 0.09-0.10 h for SAXA at 60 °C 1% hydrogen peroxide. Shelf-life was found to be 0.03 h, 0.02 h and 0.02 h for MET, DAPA and SAXA, respectively.

Table-1: Box-Behnken Design Domain for Optimization of Mobile Phase with Responses

Experimental runs	A: Saturation time(min)	B: Solvent front (mm)	C: Volume of Methanol (mL)	Rf of MET	Rf of SAXA	Rf of DAPA
1	15	98	8	0.18	0.42	0.99
2	20	95	8	0.21	0.45	0.86
3	15	95	7	0.12	0.34	0.76
4	20	95	8	0.22	0.46	0.85
5	15	92	8	0.45	0.72	0.81
6	20	92	9	0.3	0.5	0.97
7	20	98	7	0.11	0.3	0.71
8	20	92	7	0.51	0.66	0.76
9	15	95	9	0.28	0.51	0.94
10	20	98	9	0.08	0.26	0.94
11	20	95	8	0.23	0.48	0.88
12	25	92	8	0.26	0.5	0.65
13	25	95	9	0.09	0.28	0.73
14	20	95	8	0.22	0.48	0.86
15	25	95	7	0.19	0.43	0.85
16	20	95	8	0.21	0.46	0.88
17	25	98	8	0.08	0.19	0.84

Photolytic Degradation

The results of MET, SAXA and DAPA following Photolytic degradation in UV chamber is shown in **Table 12**. First order rate constant, half-life and shelf life were also calculated from the slope of the straight lines at each temperature for photolytic degradation processes. At 254 nm wavelength of UV-Vis spectroscopy %drug degradation was found to be 55.35, 33.01 and 11.43 for MET, SAXA and DAPA, respectively. Half-life was found to be 5.1 h for MET, 37.6 h for DAPA and 15.08 h for SAXA at 60 °C 1% hydrogen peroxide. Shelf-life was found to be 0.77 h, 5.70 h and 2.29 h for MET, DAPA and SAXA, respectively.

Table-2: Results of Synthetic Mixture Analysis			
Conc. (ng/band)	Mean Conc. Found (n=3)	% CV	% Assay (mean ± SD)
MET			
50000	49185.52	0.7248	98.37±0.71
100000	98826.45	0.8556	98.83±0.85
150000	149515.83	0.2618	99.68±0.26
SAXA			
250	247.25	1.8090	98.90±1.78
500	495.01	0.8231	99.00±0.81
750	746.15	1.7956	99.49±1.79
DAPA			
250	245.68	0.4737	98.27±0.47
500	493.12	0.4747	98.62±0.47
750	744.70	0.3830	99.29±0.38

Table-3: Results of Linearity Study					
MET					
Conc. (ng/band)	50,000	75,000	1,00,000	1,25,000	1,50,000
Avg Peak Area (n=5)	12920.10	17940.15	21670.03	26678.04	30106.01
% RSD	1.224	0.974	0.532	0.590	0.561
SAXA					
Con. (ng/band)	250	375	500	625	750
Avg Peak Area (n=5)	969.71	1468.42	2173.09	2870.79	3480.11
% RSD	1.838	1.435	0.777	0.543	0.427
DAPA					
Con. (ng/band)	250	375	500	625	750
Avg Peak Area (n=5)	4307.53	6112.33	7515.65	8887.46	10808.15
% RSD	1.440	1.230	1.067	0.751	0.536

Table-4: Linear regression parameters for MET, SAXA, and DAPA			
Parameters	MET	SAXA	DAPA
Calibration range (ng/band)	50000-150000	50-150	50-150
Regression equation	$y = 0.1724x + 4619$	$y = 0.0051x - 376.85$	$y = 0.0126x + 1215.7$
Regression coefficient (r ²)	0.9963	0.9971	0.9962
LOD (ng/band)	2962.22	11.08	17.90
LOQ (ng/band)	8976.44	33.59	54.29
a ⁿ =5 replicates, b Confidence interval at 95% confidence level and six degree of freedom			

Table-5: Results of Precision Study						
Conc. (ng/ml)	Day 1		Day 2		Day 3	
	Peak Area	%RSD	Peak Area	%RSD	Peak Area	%RSD
MET						
50,000	12952.90	1.2715	12821.57	1.6934	12851.23	1.8750
1,00,000	21552.67	0.9887	21687.50	1.1625	21691.43	1.1360
1,50,000	28036.17	0.5529	27778.60	0.8514	27878.83	0.8660
SAXA						
250	973.87	1.8046	951.98	1.9004	950.03	2.0097
500	2164.95	1.2576	2168.57	1.2511	2155.92	1.3427
750	3434.29	0.9752	3739.01	0.9889	3749.51	1.1383
DAPA						
250	4353.93	1.4455	4343.20	1.7202	4459.03	1.7354
500	7564.90	0.7141	7531.57	1.3351	7144.05	1.6480
750	10789.07	0.5398	10789.07	0.5398	10789.07	0.5398

Table-6: Results of Accuracy Study					
Conc. of test (ng/band)	Conc. of Std added (ng/band)	Total Conc. (ng/band)	Mean Conc. Found	%CV	%Recovery (mean \pm SD)
MET					
50000	25000	75000	74739.60	1.0236	99.65 \pm 1.02
50000	50000	100000	99388.59	1.3913	99.39 \pm 1.39
50000	75000	125000	124652.13	0.8876	99.72 \pm 0.89
SAXA					
250	125	375	368.05	0.6897	98.15 \pm 0.68
250	250	500	493.17	0.5295	98.63 \pm 0.52
250	375	625	619.31	0.4399	99.08 \pm 0.44
DAPA					
250	125	375	362.67	1.5177	96.71 \pm 1.47
250	250	500	490.31	1.1713	98.06 \pm 1.15
250	375	625	622.12	0.3213	99.54 \pm 0.32

Table-7: Results of Specificity study			
Name of the drug	Rf	Area	Height
MET	0.21	13106.1	289.4
SAXA	0.46	1036.5	27.4
DAPA	0.88	1206.5	34.2

Table-8: Results of Robustness study						
Saturation time	MET		SAXA		DAPA	
	Rf	Peak Area	Rf	Peak Area	Rf	Peak Area
18 min	0.19	21708.50	0.44	1169.92	0.75	4356.32
Std. Dev.	0.0036	42.7405	0.0055	13.9010	0.0052	61.9859
%RSD	1.9400	1.1969	1.2600	1.1882	0.6916	1.4223
20 min	0.20	21731.17	0.45	1166.15	0.76	4745.13
Std. Dev.	0.0032	10.8969	0.0052	11.8399	0.0024	72.8433
%RSD:	1.5888	0.0501	1.1561	1.0153	0.3088	1.5351
22 min:	0.2	21708.68	0.46	1173.07	0.78	4750.36
Std. Dev.	0.0046	42.8092	0.0052	10.9973	0.0058	87.1837
%RSD:	2.3238	0.1972	1.1308	0.9375	0.7434	1.8353

Table-9: Acid-induced degradation study results of drugs at various concentrations of acid (0.1, 0.5, 1 N HCl)							
HCl (N)	C0 (Initial Conc.) (µg/band)	C360 (Conc after 6 h) (µg/band)	Log C	Degradation rate constant K x 10⁻³(min⁻¹) from graph	Half Life(h)	Shelf Life(h)	% Drug degraded at 6 h
MET at 40°C							
0.1	1000	824.35	4.91	0.54	1.15	0.17	17.56
0.5	1000	820.85	4.91	0.55	1.11	0.16	17.91
1	1000	807.61	4.90	0.59	1.07	0.16	19.23
DAPA at 40°C							
0.1	5	3.90	2.59	0.68	1.00	0.15	21.80
0.5	5	3.26	2.51	1.18	0.66	0.10	34.60
1	5	2.98	2.47	1.43	0.42	0.06	40.28
SAXA at 40°C							
0.1	5	4.14	2.61	0.52	1.00	0.15	17.05
0.5	5	3.47	2.54	1.01	0.47	0.07	30.50
1	5	3.44	2.53	1.04	0.41	0.06	31.12
MET at 60°C							
0.1	1000	820	4.91	0.55	0.91	0.13	17.99
0.5	1000	646	4.81	1.21	0.51	0.07	35.39
1	1000	395.68	4.59	2.58	0.20	0.03	60.43
DAPA at 60°C							
0.1	5	2.76	2.44	1.65	0.38	0.06	44.70
0.5	5	2.0	2.30	2.54	0.22	0.03	59.86
1	5	1.29	2.11	3.76	0.16	0.02	74.17
SAXA at 60°C							
0.1	5	3.62	2.55	0.90	0.60	0.09	27.57
0.5	5	3.38	2.52	1.09	0.41	0.06	32.36
1	5	2.86	2.45	1.55	0.38	0.06	42.78

Table-10: Base Induced Degradation study results of all the three drugs using various concentration of base (0.1, 0.5, 1 N NaOH)							
NaOH (N)	C0 (Initial Conc.) (µg/band)	C360 (Conc after 6 h) (µg/band)	Log C	Degradation rate constant K x 10⁻³(min⁻¹) from graph	Half Life(h)	Shelf Life(h)	% Drug degraded at 6 h
MET at 40°C							
0.10	1000.00	793.73	4.89	0.64	1.00	0.15	20.63
0.50	1000.00	790.23	4.90	0.65	0.86	0.13	20.98
1.00	1000.00	691.03	4.84	1.03	0.63	0.09	30.90
DAPA at 40°C							
0.10	5.00	3.05	2.48	1.36	0.43	0.07	38.82
0.50	5.00	2.76	2.44	1.65	0.26	0.04	44.70
1.00	5.00	1.80	2.25	2.83	0.19	0.03	63.95
SAXA at 40°C							

0.10	5.00	4.06	2.60	0.57	1.43	0.22	18.68
0.50	5.00	2.73	2.43	1.68	0.34	0.05	45.33
1.00	5.00	2.30	2.36	2.15	0.30	0.05	53.82
MET at 60°C							
0.1	1000	737.54	4.86	0.85	0.57	0.09	26.25
0.5	1000	623.12	4.79	1.31	0.44	0.07	37.69
1	1000	564.81	4.75	1.59	0.38	0.06	43.52
DAPA at 60°C							
0.1	5	2.01	2.30	2.53	0.21	0.03	59.71
0.5	5	1.68	2.22	3.04	0.18	0.03	66.53
1	5	1.67	2.22	3.02	0.16	0.02	66.31
SAXA at 60°C							
0.1	5	2.18	2.33	2.30	0.21	0.03	56.34
0.5	5	1.33	2.12	3.66	0.18	0.03	73.27
1	5	0.21	1.33	8.72	0.08	0.01	95.68

Table-11: Oxidative Degradation study results of all the three drugs

Temp (°C)	C0 (Initial Conc.) (µg/ml)	C360 (Conc after 6 h) (µg/ml)	Log C	Degradation rate constant $K \times 10^{-3}$ (min ⁻¹) from graph	Half Life(h)	Shelf Life(h)	% Drug degraded at 6 h
MET							
25	1000	691.03	4.84	1.03	0.61	0.09	30.90
40	1000	318.27	4.50	3.18	0.22	0.03	68.17
60	1000	235.11	4.37	4.02	0.18	0.03	76.49
DAPA							
25	5	1.71	2.23	2.98	0.17	0.03	65.79
40	5	1.64	2.21	3.09	0.16	0.02	67.16
60	5	0.65	1.81	5.66	0.13	0.02	86.97
SAXA							
25	5	0.33	1.52	7.50	0.10	0.02	93.28
40	5	0.36	1.56	7.25	0.10	0.02	92.65
60	5	0.30	1.47	7.81	0.09	0.01	93.99

Table-12: Photolytic Degradation study results of all the three drugs

Drug	C0 (Initial Conc.) (µg/ml)	C7200 (Conc after 6 h) (µg/ml)	Log C	Degradation rate constant $K \times 10^{-3}$ (min ⁻¹) from graph	Half Life(h)	Shelf Life(h)	% Drug degraded at 120 h
MET	1000	446.47	4.65	0.11	5.10	0.77	55.35
DAPA	5	4.42	2.64	0.02	37.61	5.70	11.43
SAXA	5	3.34	2.52	0.06	15.08	2.29	33.01

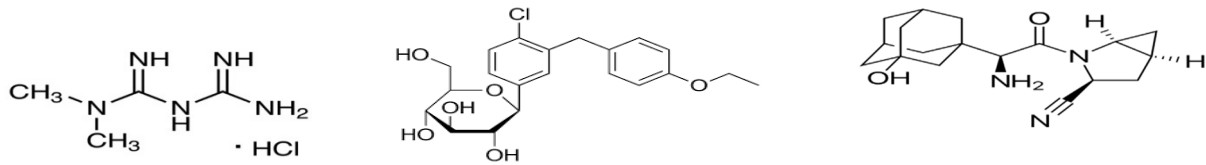


Fig. 1: -Phytochemical Chemical Structures of (a) MET, (b) DAPA, and (c) SAXA

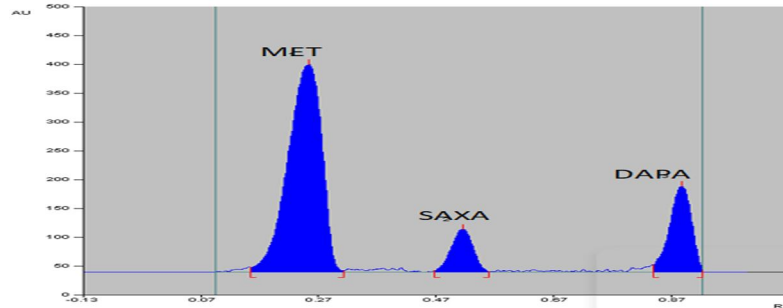


Fig. 2: -Chromatogram of MET, SAXA and DAPA

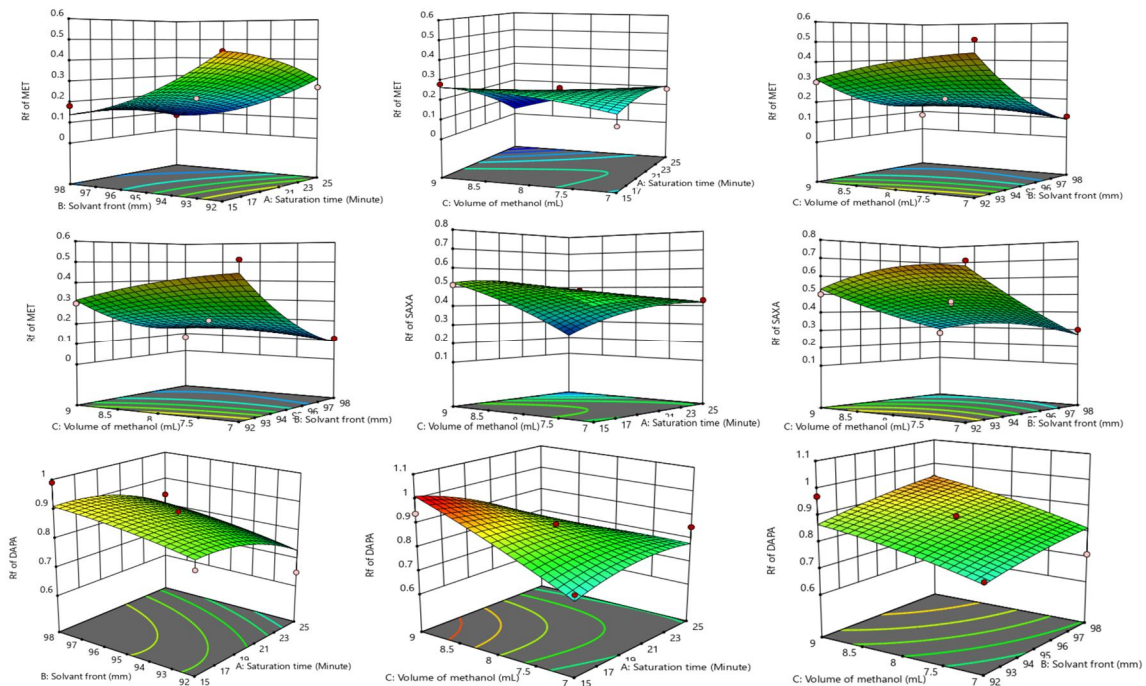


Fig. 3: -Response surface plots for response (a) Rt of MET (b) Rt of SAXA (c) Rt of DAPA

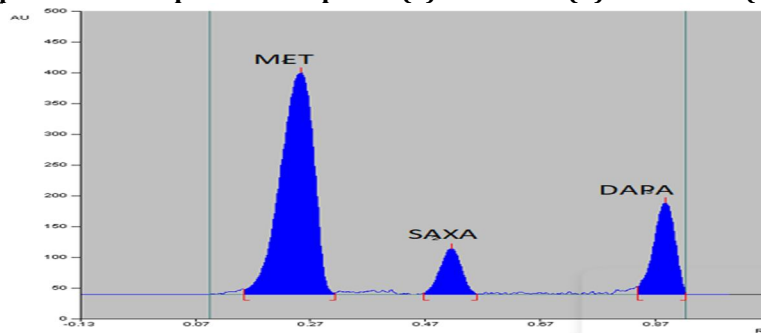


Fig. 4: - Chromatogram of MET, SAXA and DAPA with Optimized Chromatographic Condition

CONCLUSION

A simple and accurate RP-HPTLC method for simultaneous estimation of MET, DAPA, and SAXA was developed and validated as per ICH guidelines. The developed method was validated as per ICH guidelines

for its linearity, precision, accuracy, robustness, LOD and LOQ parameters. The forced degradation studies were carried out to evaluate the changes in the assay values of drugs without any interference from excipients and degradation products. The Box-Behnken design, which was an extremely effective procedure for conducting method optimization, showed that the outcome of the analysis could be altered by tweaking the amount of methanol, saturation time and solvent front. This developed method satisfies the design space concept and is suitable for regulatory submission under regulatory flexibility.

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