Original Article

A Novel Validated Stability Indicating Analytical Method for Simultaneous Quantification of Metformin Hydrochloride and Empagliflozin in Bulk and Marketed Formulation by HPTLC using Box-Wilson Experimental Design Approach

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ABSTRACT

Background: A novel stability indicating analytical method was developed and validated by High Performance Thin Layer Chromatography (HPTLC) using Design of experiment approach. The proposed method is useful for quantification of Metformin hydrochloride and Empagliflozin in bulk and its dosage forms simultaneously. Design of experiment approach was applied for optimization of chromatographic conditions. **Materials and Methods**: For optimization process independent variables were used as Isopropyl alcohol proportion in mobile phase, saturation time of chamber and distance travelled by mobile phase. Experiments were carried out on silica gel pre-coated plate using mobile phase as 2 % Ammonium acetate: Isopropyl alcohol: Triethylamine (4:6:0.1 v/v/v). Direct evaluation of chromatograms were done by TLC scanner with reflectance/absorbance mode set at 242 nm. Method was validated as per ICH Q2 (R1) requirements. **Results:** Correlation coefficients for calibration curves were found to be 0.985 and 0.988, the calibration curve is in concentration range of 5000-30000 ng band^{-1} and 125–750 ng band^{-1} for Metformin hydrochloride and Empagliflozin respectively. The method showed % recovery between 99.05 to 102.54 % for Metformin hydrochloride and 99.20 to 101.50 % for Empagliflozin. The method has a prospective to determine Metformin hydrochloride and Empagliflozin simultaneously. The Metformin hydrochloride and Empagliflozin were subjected to forced degradation studies like hydrolysis, oxidation, thermolysis and photo-degradation. **Conclusion:** Proposed method has capacity to separate the Metformin hydrochloride and Empagliflozin in its degradation products. Hence one can apply this method effectively for routine analysis and during stability study as per regulatory requirements.

Key words: Method development, Validation, HPTLC, Stability studies, DoE.

INTRODUCTION

Chemically Empagliflozin, 1-chloro-4-(glucopyranos-1-yl)-2-(4-(tetrahydrofuran-3-yloxybenzyl) benzene, [Figure 1 (a)] is an orally available competitive inhibitor of Sodium-glucose Co-transporter-2 (SGLT2) with anti-hyperglycemic activity. Empagliflozin function by inhibiting SGLT-2 present in proximal tubules in the kidneys. Empagliflozin reduces renal reabsorption of glucose leads to increase in urinary excretion of glucose and act as a antidiabetic agent for treatment of type-2 diabetes.[1] Metformin [Figure 1 (b)] is anti-hyperglycemic agent acts by inhibition of hepatic glucose output and therefore, the liver is most likely the principle site of Metformin function.[2] Chemically Metformin is 1-carbamimidamido-N,N-

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dimethylmethanimidamide. For the purpose of increasing solubility, Metformin formulations are present in its salt form as Metformin Hydrochloride. Literature review of Empagliflozin for its analytical method should contain all the following methods in pharmaceutical dosage form, also either alone or in combination with Metformin hydrochloride/ Linagliptin. Extensive literature study showed that High Performance Liquid Chromatography (HPLC),[3-18] High Performance Thin Layer Chromatography (HPTLC)[19] and Spectrophotometry[20-22] methods for analysis of Empagliflozin in bulk or in its marketed dosage form as alone or in combination with some other active chemical entity. To the best of our knowledge, developed method is novel, simple, economical, rapid, selective and more specific for simultaneous estimation of Metformin hydrochloride (MET) and Empagliflozin (EN) in formulations by HPTLC using Design of Experiment (DoE). The proposed method will be validated as per ICH Q2 (R1) requirments.[23] The proposed work aimed to develop a stability indicating analytical method by High Performance Thin Layer Chromatography (HPTLC) using DoE approach. The proposed method will be useful for quantification of MET and EN in bulk and its dosage forms.

MATERIALS AND METHODS

Materials

The drug samples of MET and EN was obtained as gift samples from Lupin Ltd. Pune, Maharashtra, India. The marketed dosage form used in the study was Jardiance Met™ (Boehringer Ingelheim India Private Limited Bandra (East) Mumbai, India) purchased from the local market and each tablet contains 500 mg of MET and 12.5 mg of EN. The required chemicals and solvents were of analytical grade.

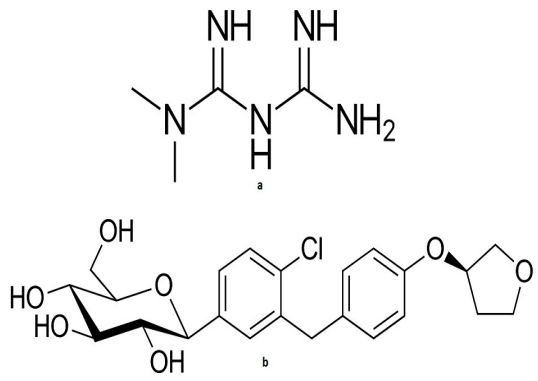


Figure 1: Chemical structures of Metformin hydrochloride (a) and Empagliflozin (b).

Instrumentation

Microsyringe (linomat syringe, Camag, Switzerland), Linomat-5 applicator, Twin trough chamber, UV chamber, Saturation pads, TLC scanner, winCATS software (Camag, Switzerland), Pre-coated silica gel aluminium plates (Merck, Germany) and Microsoft excel sheet were used.

Preparation of standard solutions for MET and EN

Standard stock solutions of MET and EN were obtained by dissolving 500 mg of MET and 12.5 mg of EN in 10 ml of methanol respectively. The concentration of resulted solution was 50000 µg/ml for MET and 1250 µg/ml for EN respectively.

Preparation of sample solutions

Twenty tablets (label claim 500 mg of MET and 12.5 mg of EN), Jardiance Met™ (Boehringer Ingelheim India Private Limited Bandra (East) Mumbai, India) were accurately weighed and then crushed. The powder equivalent to 500 mg of MET and 12.5 mg of EN was accurately weighed and transferred to a ten ml volumetric flask containing methanol (07 ml). Resultant solution was sonicated for 30 min. After 30 min the volume was made up to 10 ml using methanol. The resultant solution was filtered through Whatman quantitative filter paper. Working sample solution and standard solution were freshly prepared by adopting the same method using methanol as solvent.

Optimized chromatographic conditions

The solvent system was optimized by using thin layer chromatography (Pre-coated silica gel aluminium plates) using mobile phase as Ammonium Acetate: Isopropyl Alcohol: Triethylamine. This mobile phase was further developed for HPTLC run. On the HPTLC plates a small volume in µL of standard and sample solution were applied above 10 mm from bottom and 10 mm from side edges as bands with length 6 mm. For each chromatographic run mobile phase/solvent system comprising of 2 % Ammonium Acetate: Isopropyl Alcohol: Triethylamine (4:6:0.1 v/v/v) was used. The study was performed using ascending development method in chamber (twin trough). The optimized time of saturation for chamber with mobile phase was 15 min at ambient temperature. The system was assisted with saturation pad. The solvent travelled a distance of 8 cm within 18 min. Direct evaluation of chromatograms was done by TLC scanner with reflectance/absorbance mode set at 242 nm by using win CATS software. For evaluation criteria intensity of light reflected and

peak area indicates the concentrations of separated compounds.

Method optimization using DoE

A box-wilson method was applied for optimization of compositional parameters (Isopropyl alcohol proportion in solvent system or mobile phase, saturation time of chamber and distance travelled by mobile phase) and its effects were studied like main, interaction and quadratic on retardation factor (R_f) of MET and EN. A Box-wilson method is an experimental design, helpful in response surface methodology, for construction of a second order polynomial (quadratic response surfaces) model for the response variable. Vital factors and ranges were observed for method parameter optimization using DoE. The mobile phase composition indicates, amount of isopropyl alcohol added in the total volume of ten ml of mobile phase. Three factors were selected with five center points and total of twenty experiments were performed. Responses of MET and EN were R_f of MET and EN which will be proportional with three factors; the isopropyl alcohol volume in mobile phase-A, saturation time of chamber-B and distance travelled-C depicted in Table 1. The nominal values for all three factors like A, B and C were 6 ml, 15 min and 8 cm respectively. Isopropyl alcohol volume range was maintained between 3.5 and 8.5. The values of minimum and maximum for the saturation time of chamber were kept between 8 min and 22 min.

Table 1: Central composite rotatable design arrangement and responses.

Run	Type	Factors			Responses	
		A	B	C	R_f of MET	R_f of EN
1	Center	6	15	8	0.51	0.79
2	Factorial	7.5	11	7.9	0.61	0.95
3	Axial	3.4	15	8	0.44	0.75
4	Center	6	15	8	0.53	0.88
5	Factorial	4.5	11	7.9	0.5	0.96
6	Factorial	7.5	11	8.1	0.61	0.77
7	Axial	6	15	8.1	0.42	0.74
8	Factorial	7.5	19	7.9	0.58	0.85
9	Factorial	7.5	19	8.1	0.55	0.82
10	Axial	6	15	7.8	0.52	0.88
11	Center	6	15	8	0.53	0.79
12	Center	6	15	8	0.51	0.89
13	Axial	6	21.7	8	0.4	0.55
14	Axial	8.5	15	8	0.6	0.91
15	Factorial	4.5	11	8.1	0.49	0.81
16	Axial	6	8.2	8	0.53	0.75
17	Center	6	15	8	0.51	0.87
18	Factorial	4.5	19	8.1	0.41	0.68
19	Center	6	15	8	0.47	0.82
20	Factorial	4.5	19	7.9	0.39	0.6

A= Isopropyl alcohol (ml), B= Chamber saturation time (min), C= Distance travel (cm), MET=Metformin hydrochloride, EN= Empagliflozin.

Analysis of marketed formulation

Twenty tablets were accurately weighed (label claim 500 mg of MET and 12.5 mg of EN), Jardiance Met™ (Boehringer Ingelheim India Private Limited Bandra (East) Mumbai, India) and crushed using mortar and pestle. Accurately weighed powder equivalent to 500 mg of MET and 12.5 mg of EN was transferred to a ten ml of volumetric flask containing methanol (07 ml). The resultant solution was sonicated for 30 min. After 30 min of sonication the volume was made up to 10 ml with methanol. The resultant solution was filtered through whatman quantitative filter paper wetted with methanol. Sample stock solutions were obtained after dilution containing 50000 µg/ml of Metformin hydrochloride and 1250 µg/ml of Empagliflozin. From filtered solution 01 µL of 5000 ng/band of MET and 125 ng/band of EN was applied to TLC plate, followed by development and scanning. Experiments were performed in triplicate.

Method validation

Method was developed and validated in fulfillment with ICH requirements and official parameters were applied for validation of developed method.

Linearity

Linearity between peak area and concentration of the MET and EN were studied over the concentration range of 5000-30000 ng band^{-1} for MET and 125-750 ng band^{-1} for EN by using six replicates.

Precision

Validation of developed method for Precision was carried out by performing intermediate precision and repeatability. Analysis of peak area was determined by using six replicate studies of same band with the help of a sample solution containing 20000 ng band^{-1} of MET and 500 ng band^{-1} of EN.

Recovery studies

Recoveries of MET and EN content were performed by using standard addition method with addition of three known amounts of different concentration from standard API to the marketed drug product. Therefore after sample dilution, 40000, 50000 and 60000 ng band^{-1} of MET and 100, 125 and 150 ng band^{-1} of EN were

added in solution made from the marketed dosage form that containing 20000 and 500 ng band^{-1} of MET and EN respectively.

LOD and LOQ

Determination of detection limit and quantification limit of developed method were studied by using standard formulae. Values of LOD and LOQ for MET and EN were calculated from slop (S) and standard deviation (α) of the calibration curves with y-intercepts respectively. Formulae for calculating the limit of detection and limit of quantification are 3α /S and 10α /S, respectively.

Robustness

Robustness study was performed by evaluating intentional variant in method parameters like change in composition of mobile phase, change in time of saturation and total mobile phase changes. R_f values and peak areas of MET and EN were evaluated with change in each parameter by calculating the relative standard deviations (RSD) for above mentioned parameter. Values and peak areas were evaluated by calculating the relative standard deviations (RSD) for each parameter.

Specificity

Peak purity study for both MET and EN were evaluated for Specificity study. At three different levels sample band and standard band were scanned, levels were peak start, peak apex and peak end. Standard stock solutions were prepared and employed from MET and EN (1000 μg ml^{-1}) in this study.

Stability studies

Forced degradation studies were executed in accordance to the ICH guiding principle for the purpose of evaluating stability indicating properties of the developed method by HPTLC. The standard API of MET and EN were subjected to hydrolysis, oxidation, thermolysis and photo-degradation studies.

Acid-induced degradation (Hydrolysis)

The acid degradation study for MET and EN were carried out. The dilution was made from the stock solution to obtain concentration 50000 μg/ml for MET and 1250 μg/ml for EN. The individual solution of concentration 50000 μg/ml for MET and 1250 μg/ml for EN was added to 5 ml stock solution of methanol containing HCl (0.1N, 3 ml) in 10 ml volumetric flasks. The individual mixtures of MET and EN were refluxed at 40°C for 30 min. After reflux volume was made upto 10 ml using methanol. Study was carried out in dark environment to eliminate the probable effect of degradation by light. The resultant 0.4 μL individual solutions of MET and EN (20000 ng band^{-1} for MET and 500 ng band^{-1} for EN) were applied to TLC plates and the chromatograms were allowed to run.

Base-induced degradation

The base degradation study for MET and EN were carried out. The dilution was made from the stock solution to obtain concentration 50000 μg/ml for MET and 1250 μg/ml for EN. The individual solution of concentration 50000 μg/ml for MET and 1250 μg/ml for EN was added to 5 ml stock solution of methanol containing NaOH (0.1N, 3 ml) in 10 ml volumetric flasks. The individual mixtures of MET and EN were refluxed at 40°C for 30 min. After reflux volume was made upto 10 ml using methanol. Study was carried out in dark environment to eliminate the probable effect of degradation by light. The resultant 0.4 μL individual solutions of MET and EN (20000 ng band^{-1} for MET and 500 ng band^{-1} for EN) were applied to TLC plates and the chromatograms were allowed to run.

Hydrogen peroxide-induced degradation (oxidation)

The degradation (oxidation) study for MET and EN were carried out. The dilution was made from the stock solution to obtain concentration 50000 μg/ml for MET and 1250 μg/ml for EN. The individual solution of concentration 50000 μg/ml for MET and 1250 μg/ml for EN was added to 5 ml stock solution of methanol containing H_2O_2 (3%, 3 ml) in 10 ml volumetric flasks. The individual mixtures of MET and EN were refluxed at 40°C for 30 min. After reflux volume was made upto 10 ml using methanol. Study was carried out in dark environment to eliminate the probable effect of degradation by light. The resultant 0.4 μL individual solutions of MET and EN (20000 ng band^{-1} for MET and 500 ng band^{-1} for EN) were applied to TLC plates and the chromatograms were allowed to run.

Wet degradation study

The wet degradation study for MET and EN were carried out. The dilution was made from the stock solution to obtain concentration 50000 μg/ml for MET and 1250 μg/ml for EN. The individual solution of concentration 50000 μg/ml for MET and 1250 μg/ml for EN was added to 03 ml distilled water in ten ml volumetric flasks. The individual mixtures of MET and EN were refluxed at 40°C for 30 min. After reflux volume was made upto 10 ml using methanol. Study was carried out in dark environment to eliminate the probable effect of degradation by light. The resultant 0.4 μL individual solutions of MET and EN (20000 ng band^{-1} for MET and 500 ng band^{-1} for EN) were applied

to TLC plates and the chromatograms were allowed to run.

Heat degradation (Thermolysis)

Dry heat degradation study was carried out for both MET and EN in an oven at 40°C for 24 hr. The dilution was made from the stock solution to obtain concentration 50000 µg/ml for MET and 1250 µg/ml for EN. The individual solution of concentration 50000 µg/ml for MET and 1250 µg/ml for EN was added to 3 ml distilled water in ten ml volumetric flasks then volume was made upto 10 ml using methanol. Chromatograms were allowed to run and analyzed under optimized conditions.

Photo-degradation (Photolysis)

During the photo-degradation, MET and EN were exposed to UV light, kept for 24 hr in a photo-stability chamber. The dilution was made from the stock solution to obtain concentration 50000 µg/ml for MET and 1250 µg/ml for EN. The individual solution of concentration 50000 µg/ml for MET and 1250 µg/ml for EN was added to 3 ml distilled water in ten ml volumetric flask then volume was made upto 10 ml with methanol. Chromatograms were allowed to run and analyzed under optimized conditions.

RESULTS AND DISCUSSION

HPTLC method optimization

Selection of solvent system or mobile phase which effectively separates MET and EN is major objective of the proposed work. To achieve this, variation in composition of solvent system was carried out by changing polarity of system. Number of runs for samples was carried out on Pre-coated silica gel aluminum plates with respect to optimize the composition of solvent system. The TLC plate showed separation of individual drugs (MET and EN) when applied as combination spot for standard and sample respectively [Figure 2 (a) and Figure 2 (b)]. For selection of analytical wavelength and quantification the standard spots were applied on TLC plate and scanned for MET and EN. The overlain spectra were obtained for MET and EN on the HPTLC instrument. The overlain spectra showed intense absorbance at 242 nm for both drugs; MET and EN. The wavelength of 242 nm was selected for further study (Figure 3). The peak purity of MET and EN in marketed

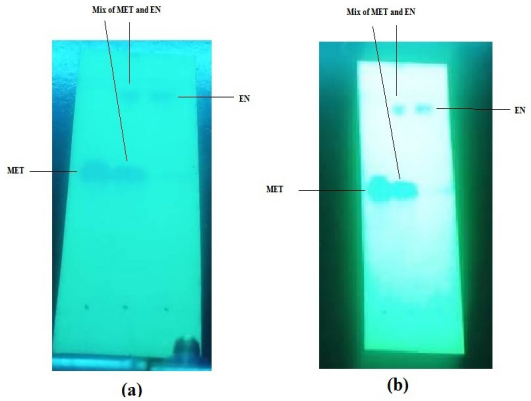


Figure 2: TLC of drugs MET, EN and combination spot for standard (a) and sample (b).

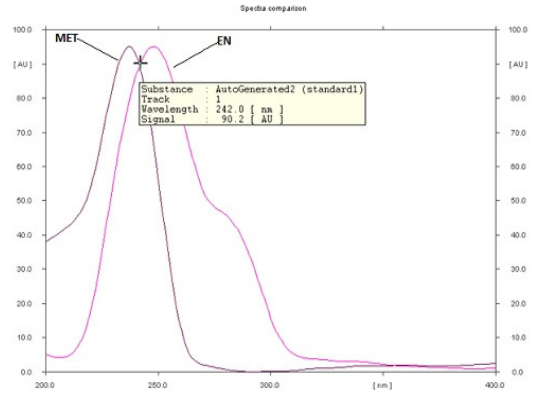


Figure 3: Overlain absorption spectra for (MET) and (EN) at 242 nm.

Table 2: Predicted response models and statistical parameters obtained from the ANOVA for CCD.

Response (Rf)	Type of model	Polynomial equation model for Y	Standard deviation	Model p-value	% CV	Adequate precision
MET	Linear	MET =+0.51+0.061 * A-0.037 * B	0.0315	0.0001	6.248	15.716
EN	Quadratic	EN = +0.84+0.045 * A-0.064 * B-0.038 * C+0.055 * A * B-0.017 * A * C+0.048 * B * C+6.239E-003 * A^2-0.057 * B^2-8.325E-004 * C^2	0.0512	0.0003	5.355	12.324

MET=Metformin hydrochloride, EN= Empagliflozin.

tablet was confirmed by comparison with standard MET and EN (pure drug) of the overlaid spectra band at different peak positions; peak start, peak apex, peak end (Figure 4). The densitogram of the mobile phase without MET and EN (Figure 5) were carried out and result showed no peak (blank) interference at 242 nm, it confirms purity for proposed mobile phase. Different studies were carried out to optimize the mobile phase. Various combinations of mobile phase were tried as two solvent system of toluene with methanol, chloroform with methanol, Three solvent system of methanol: hexane and GAA, chloroform:diethyl ether and ethyl acetate, chloroform:ethyl acetate and acetic acid as well as four solvent system of acetone:methanol:toluene and GAA. From the various combinations of polar solvents the mobile phase comprising of 2% Ammonium Acetate: Isopropyl Alcohol: Triethylamine as 4:6:0.1 v/v/v proportion resulted excellent resolution as sharp and well defined peaks for MET and EN at R_f values of 0.82 ± 0.02 and 0.50 ±0.02 respectively. (Figure 6)

Optimization of chromatographic conditions using DoE

The 20 trial responses for experiments are summarized in Table 1. For the model selection process, based on R_f values of MET and EN the model that best fits were quadratic and linear respectively. The *p*-value obtained was less than 0.0500 specify models are significant. The analysis of variance (ANOVA) data obtained was validated by quadratic and linear model using Design expert software and summarized in Table 2. The precision measures are an adequate response to noise ratio as ratio obtained is greater than 4 as standard v alue.[24] For MET and EN obtained ratio value was greater than 4. A coefficient of variation (% CV) was less than 10% as desirable and predicted R^2 is in reasonable agreement with the adjusted R^2 values. It indicates best correlation between experimental data and fitted models. The final equation shown in Table 2 gives the terms of actual components and factors. Perturbation plots and three-dimensional surface response plot were drawn and evaluated for the effect of the factors on R_f of MET and EN. In Figure 7, perturbation plots showed for the predicted model. Perturbation plots were helpful for the inspected procedures. Figure 7 (a) showed that the isopropyl alcohol content-A and distance travelled-C has showed significant effect on the R_f value of MET as compared to other factors. Saturation time of chamber-B has showed significant effects on R_f of EN, succeeded by the distance travelled-C [Figure 7 (b)]. A variation in the R_f value of MET and EN as a function of saturation time of chamber and isopropyl alcohol concentration by keeping distance travelled as constant is shown in Figure 8. The correlation between the R_f value and distance travelled for MET and EN were inverse. The three-dimensional response surface

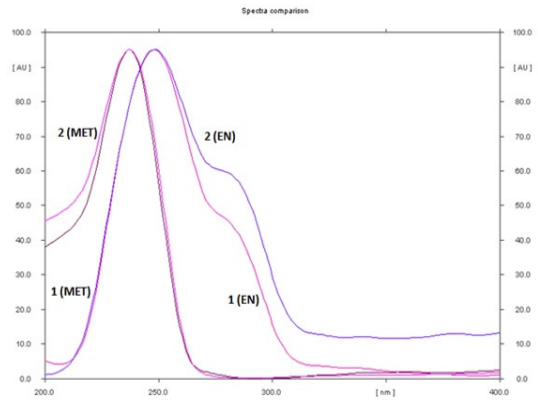


Figure 4: UV spectra comparison (Pick Purity) of the spots of standard (1) and dosage form (2) for (MET) and (EN).

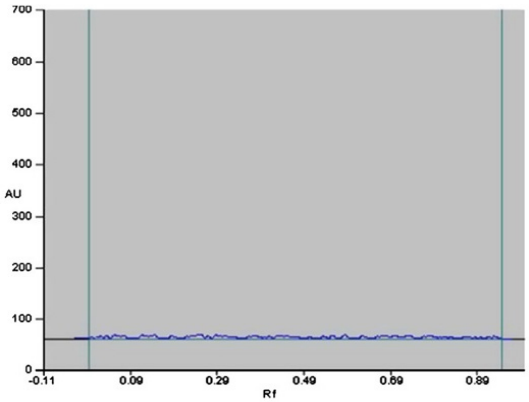


Figure 5: Typical densitogram of blank mobile phase.

Table 3: Comparison of experimental result and predicted values obtained from DoE.

Number	Drug	Factors			Experimental Result (R_f)	Predicted Response (R_f)	Predicted error %
		A	B	C			
1	MET	06	15	08	0.500	0.505	0.990
2	EN				0.820	0.838	1.204

A= Isopropyl alcohol (ml), B= Chamber saturation time (min), C= Distance travel (cm), MET=Metformin hydrochloride, EN= Empagliflozin.

plots and perturbation plots revealed that the isopropyl alcohol content-A and saturation time of chamber-B significantly affects the responses in comparison with distance travelled-C. Comparison of experimental result and predicted values obtained from DoE is shown in Table 3 which indicated closeness of R_f value.

Analysis of dosage form in marketed formulation

Marketed tablet formulation (Jardiance Met™) was quantified by using developed HPTLC method. Chromatogram for tablet containing composition of MET and EN showed two peaks at R_f value of 0.51 and 0.81 for MET and EN respectively (Figure 9). It indicates no interference of excipients at 242 nm wavelength. The MET and EN content were determined by comparing peak areas of test (marketed) with standard MET and EN (Table 4).

Method validation

Linearity

The calibration curve for MET and EN were observed as linear in the concentration range of 5000-30000 ng band^{-1} and 125-750 ng band^{-1} respectively with regression coefficient (r^2) values of 0.992 and 0.994 respectively (Figure 10). The statistical data for linearity is summarized in Table 5.

Precision

Repeatability and intermediate precision were carried out for purpose of method validation and expressed as relative standard deviation (RSD) of peak area. Repeatability, intraday and interday variation in results at concentration of 20000 ng band^{-1} and 500 ng band^{-1} for MET and EN were found to be within acceptable range. Results indicates coefficients of variation value less than 1% for MET and EN for intraday and interday studies. (Table 5).

Accuracy/recovery studies

Recovery studies were executed at three levels as 80%, 100% and 120% of the marketed tablet concentrations (Test Sample) as per ICH guidelines. The percentage recovery studies results was found to be satisfactory (Table 6) for recovery of MET and EN at all three levels. The recovery were found to be 99.05 % to 102.54 % and 99.20 % to 101.50 % for MET and EN respectively.

Table 4: Assay results of the pharmaceutical dosage form.

Number	component	Amount percent (mg per tablet)	% Amount found*	SD	% RSD
1.	Empagliflozin	12.5	99.41%	25.423	0.072
2.	Metformin HCl	500	98.67%	3.055	0.138

*Denotes average of three estimations

Table 5: Summary of linear regression and validation data.

Number	Parameters*	Empagliflozin	Metformin HCl
1.	Linearity Range	125-750 ng band^{-1}	5000-30000 ng band^{-1}
2.	Linear regression equation	y = 4.335x - 7.761	y = 0.870x + 16443
3.	Slope ±SD	4.335±0.174	0.870±0.008
4.	Intercept ±SD	59.507±32.386	16443.167±185.971
5.	Correlation coefficient (r^2)	0.988	0.985
6.	Limit of Detection (LOD)	24.65 ng band^{-1}	705.21ng band^{-1}
7.	Limit of Quantitation (LOQ)	74.70 ng band^{-1}	2136.99 ng band^{-1}
8.	Accuracy (% Recovery)	99.20 % - 101.50 %	99.05 % - 102.54 %
9.	**Precision (% RSD)**		
9.1	Intra-day (n=6)	1.451	0.900
9.2	Inter-day (n=3)	0.267	0.066

*Denotes average of six estimations

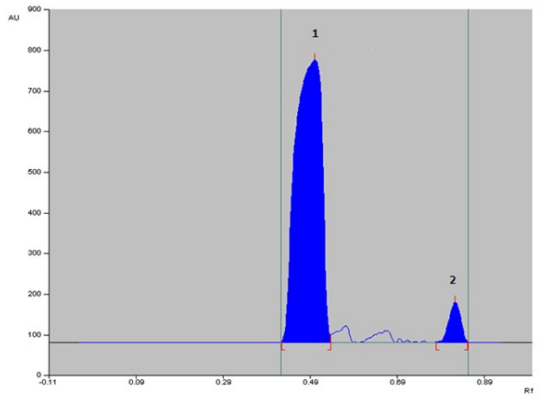


Figure 6: Standard densitogram of Metformin hydrochloride (1) and Empagliflozin (2).
1. Metformin hydrochloride=R_f (0.50) 2. Empagliflozin=R_f(0.82)

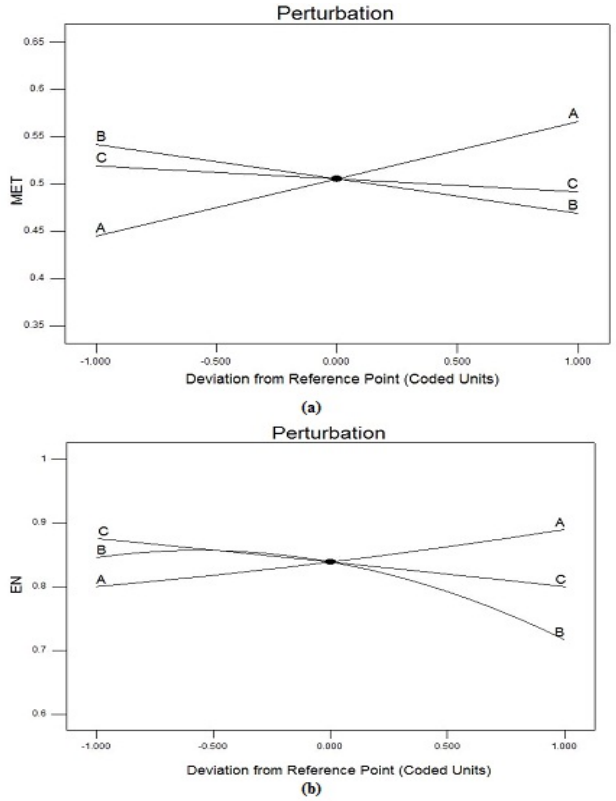


Figure 7: Perturbation graph showing the effect of each factor, A, B and C, on the (a) R_f value of MET and (b) the R_f value of EN.

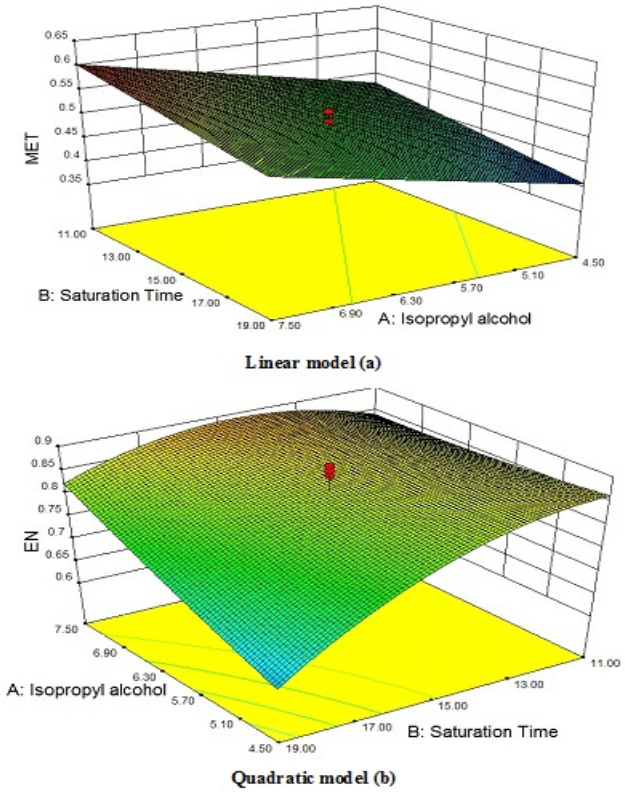


Figure 8: Three-dimensional plots of the RSM (Response surface model) for both responses (a), (b) variation in the R_f of MET and EN as a function of A and B for a fixed value of C.

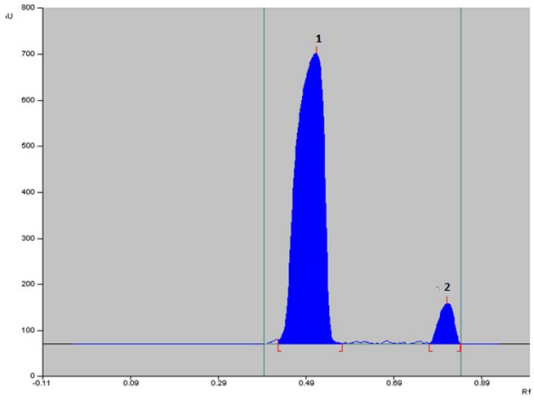


Figure 9: Typical densitogram of Metformin hydrochloride (1) and Empagliflozin (2) in pharmaceutical dosage form.
Metformin hydrochloride=R_f (0.51) 2. Empagliflozin=R_f(0.81)

LOD and LOQ

The values for LOD and LOQ were found to be 705.21 ng band^{-1}, 2136.99 ng band^{-1} for MET and 24.65 ng band^{-1}, 74.70 ng band^{-1} for EN respectively.

Robustness of the method

The results for robustness of the method was found to be low i.e. less than 2.0 % of RSD indicates method is significantly robust (Table 7).

Specificity

Peak purity study for both MET and EN were found to be specific at selected wavelength 242 nm, hence it indicates standard MET and EN are in pure form.

Stability studies

The forced degradation studies results were summarized for MET and EN using 2 % Ammonium acetate:

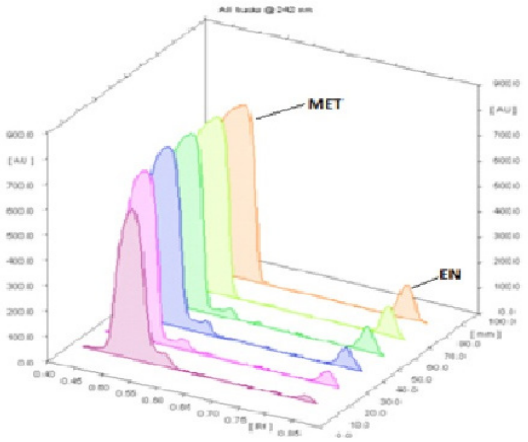


Figure 10: Three-dimensional densitogram for the linearity of MET and EN at 242 nm.

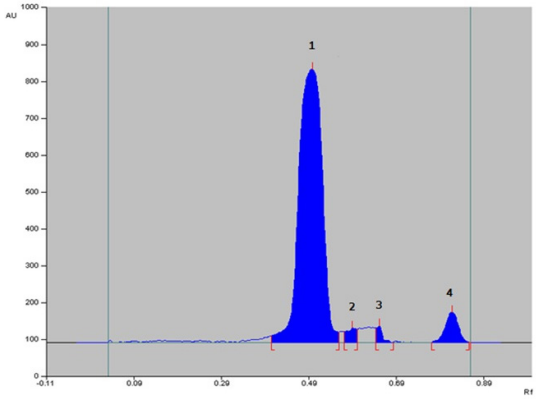


Figure 11: Typical densitogram of MET, EN and degradation products in the acid degradation study. 1. Standard Metformin hydrochloride –R_f (0.50) 2. Degredation product – R_f 0.59 (2.07 %) 3. Degredation product – R_f 0.65 (1.44 %) 4. Standard Empagliflozin- R_f (0.82)

Table 6: Recovery study of the method (using the standard addition method) for Empagliflozin and Metformin hydrochloride.

Number	Drug	Recovery level (%)	Initial amount	Amount added	% Recovery*	% RSD
1.	Empagliflozin	80	12.5 mg	10 mg	99.20 %	1.86 %
		100	12.5 mg	12.5 mg	100.42 %	1.14 %
		120	12.5 mg	15 mg	101.50 %	1.33 %
2.	Metformin HCl	80	500 mg	400 mg	99.05 %	1.66 %
		100	500 mg	500 mg	100.32 %	1.63 %
		120	500 mg	600 mg	102.54 %	1.41 %

*Denotes average of three estimations at each level of recovery

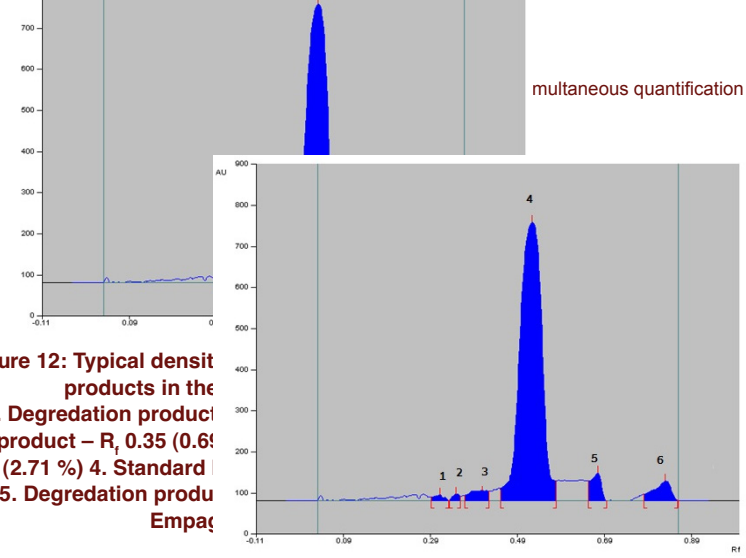
Table 7: Robustness study for the developed method.

Number	Parameters	Drug	SD*	% RSD*
1.	Change in saturation time (±2 min)	Empagliflozin	0.0100	1.234
		Metformin HCL	0.0057	1.139
2.	Change in mobile phase composition (±1 ml)	Empagliflozin	0.0115	1.431
		Metformin HCL	0.0057	1.124
3.	Total mobile phase changes (±1ml)	Empagliflozin	0.0100	1.282
		Metformin HCL	0.0057	1.139

*Denotes average of three estimations at each level

Table 8: Stability studies for the developed method.

Number	Degradation condition	Number of degradation products (R_f values)	Area of degradation products (%)
1.	Acid	2(0.59,0.65)	2.07,1.44
2.	Base	4(0.31,0.35,0.41,0.67)	0.84,0.69,2.71,4.10
3.	Oxidative	2(0.59,0.65)	5.63,2.04
4.	Wet	3(0.58,0.62,0.80)	3.45,2.34,1.23
5.	Heat	2(0.55,0.65)	4.92,3.14
6.	Photo	1(0.65)	1.20



ure 12: Typical densit
products in the
Degredation product
product – R_f 0.35 (0.6
(2.71 %) 4. Standard
5. Degredation produ
Empa

Figure 12: Typical densitogram of MET, EN and degradation products in the base degradation study. 1. Degredation product – R_f 0.31 (0.84 %) 2. Degredation product – R_f 0.35 (0.69 %) 3. Degredation product – R_f 0.41(2.71 %) 4. Standard Metformin hydrochloride – Rf (0.52) 5. Degredation product – R_f 0.67 (4.10 %) 6. Standard Empagliflozin- R_f (0.83)

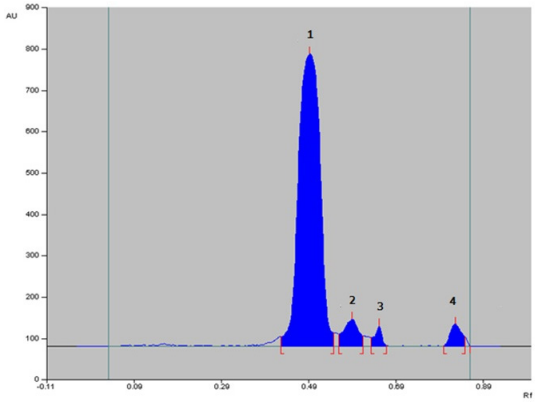


Figure 13: Typical densitogram of MET, EN and degradation products in the oxidative degradation study. 1. Standard Metformin hydrochloride – R_f (0.49) 2. Degredation product – R_f 0.59 (5.63 %) 3. Degredation product – R_f 0.65 (2.04 %) 4. Standard Empagliflozin- R_f (0.82)

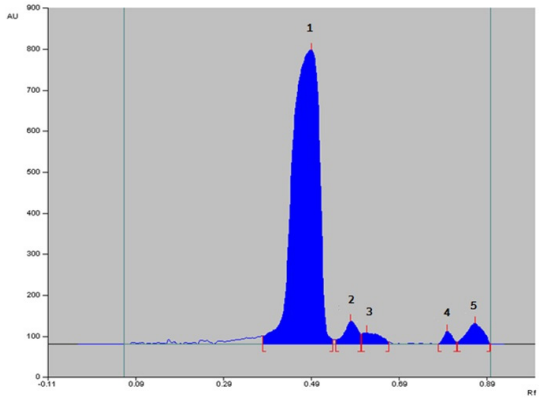


Figure 14: Typical densitogram of MET, EN and degradation products in the wet degradation study. 1. Standard Metformin hydrochloride –R_f (0.49) 2. Degredation product – R_f 0.58 (3.45 %) 3. Degredation product – R_f 0.62 (2.34 %) 4. Degredation product – R_f 0.80 (1.23 %) 5. Standard Empagliflozin- R_f (0.86)

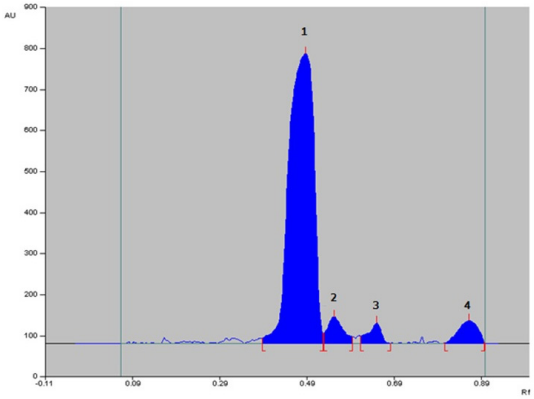


Figure 15: Typical densitogram of MET, EN and degradation products in the heat degradation study. 1. Standard Metformin hydrochloride – R_f (0.49) 2.Degredation product – R_f 0.55 (4.92 %) 3.Degredation product – R_f 0.65 (3.14 %) 4.Standard Empagliflozin- R_f (0.86)

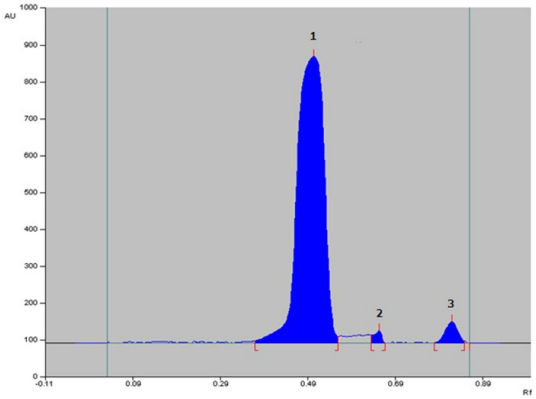


Figure 16: Typical densitogram of MET, EN and degradation products in the photo-degradation study. 1. Standard Metformin hydrochloride – R_f (0.50) 2. Degredation product – R_f 0.65 (1.20 %) 3. Standard Empagliflozin- R_f (0.82)

Isopropyl alcohol: Triethylamine [4:6:0.1 (v/v/v)] as the mobile phase (Table 8)

Acid induced degradation

The chromatogram of possible acid degradation product appeared as additional peak bands at R_f values of 0.59, 0.65 (approximate 2.07 %, 1.44 % degradation) along with the R_f values of 0.50 and 0.82 for MET and EN respectively. (Figure 11)

Base induced degradation

The chromatogram of possible base degradation product appeared as additional peak bands at R_f values 0.31, 0.35, 0.41, 0.67 (approximate 0.84%, 0.69%, 2.71%, 4.10 % degradation) along with the R_f values of 0.50 and 0.82 for MET and EN respectively. (Figure 12)

Oxidative induced degradation

The chromatogram of possible H_2O_2 degradation product appeared as additional peak bands at R_f values 0.59, 0.65 (approximate 5.63%, 2.04 % degradation) along with the R_f values of 0.50 and 0.82 for MET and EN respectively. (Figure 13)

Wet degradation

The chromatogram of possible wet degradation product appeared as additional peak bands at R_f values 0.58, 0.62, 0.80 (approximate 3.45%, 2.34%, 1.23% degradation) along with the R_f values of 0.50 and 0.82 for MET and EN respectively. (Figure 14)

Heat degradation

The chromatogram of possible wet degradation product appeared as additional peak bands at R_f values at 0.55, 0.65 (about 4.92%, 3.14 % degradation) along with the R_f values of 0.50 and 0.82 for MET and EN respectively. (Figure 15)

Photo-degradation

The chromatogram of possible wet degradation product appeared as additional peak bands at R_f values at 0.65 (about 1.20 % degradation) along with the R_f values of 0.50 and 0.82 for MET and EN respectively. (Figure 16)

CONCLUSION

A novel forced degradation using HPTLC method was successfully developed and validated by DoE for quantification of MET and EN in bulk and its dosage forms simultaneously. Experimental study was performed on plates, pre-coated with silica gel using mobile phase as 2 % Ammonium acetate: Isopropyl alcohol: Triethylamine (4:6:0.1 v/v/v). Direct evaluation of chromatograms was done by TLC scanner with reflectance/absorbance mode set at 242 nm. The proposed method was validated as per ICH Q2 (R1) requirements. A box-wilson method was applied for optimization of compositional parameters (Isopropyl alcohol proportion in mobile phase, saturation time of chamber and distance travelled by mobile phase) and its effects were studied like main, interaction and quadratic on the retardation factor (R_f) of MET and EN. The obtained results suggested the use of a Box-wilson methodology and multidimensional decision making approach is a reliable path which may minimizes the number of essential experimental trails for the method development and optimization by HPTLC. The developed method is novel, simple, economical, rapid, selective and more specific for estimation of MET and EN simultaneously in formulations by HPTLC using DoE. The HPTLC method suggest rewards over HPLC methods with respect to the filtration and degassing for solvents are not compulsory required, Visual detection is possible since it is an open method, low maintenance cost as compared to liquid chromatography, low cost and less analysis time is required, learning and operating is easy, sensitivity range of analysis usually occurs at the pictogram and nanogram, we can apply standard and sample at same time which is not possible in liquid chromatography. The method has a prospective to determine MET and EN simultaneously. The MET and EN were subjected to forced degradation studies like hydrolysis, oxidation, thermolysis and photo-degradation. The method has capacity to separate the MET and EN from its degradation products; hence the method can be effectively used for routine analysis and during stability study as per regulatory requirements.

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CONFLICT OF INTEREST

The author declares no conflicts of interest.

ABBREVIATIONS

HPTLC: High performance thin layer chromatography; **MET:** Metformin Hydrochloric acid; **EN:** Empagliflozin; **ICH:** International conference on harmonization; **RSD:** Relative standard deviation; **SD:** Standard deviation; **LOD:** Limit of detection; **LOQ:** Limit of Quantitation; **R_f:** Retention factor; **SGLT-2:** Sodium-glucose co-transporter 2; **API:** Active pharmaceutical ingredient, **NaOH:** Sodium hydroxide; **HCL:** Hydrochloric acid; **H_2O_2:** Hydrogen peroxide; **UV:** Ultraviolet; **GAA:** Glacial Acetic acid.

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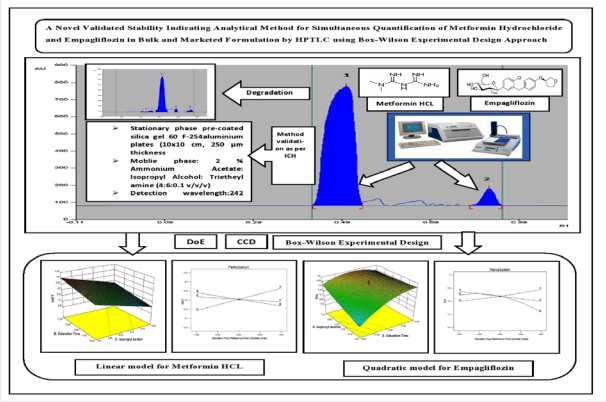
PICTORIAL ABSTRACT



SUMMARY

- A forced degradation by HPTLC method was successfully developed and validated by DoE for quantification of MET and EN in bulk and its dosage forms simultaneously. mobile phase used as 2 % Ammonium acetate: Isopropyl alcohol: Triethylamine (4:6:0.1 v/v/v).
- Box-wilson methodology and multidimensional decision making approach is reliable path which may minimizes the number of essential experimental trails for method development and optimization by HPTLC.
- R_f values for Metformin Hcl and Empagliflozin were found to be 0.50 and 0.82 respectively.

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