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Research Article

# DEVELOPMENT AND VALIDATION OF STABILITY INDICATING REVERSED PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF METFORMIN AND EMPAGLIFLOZIN IN BULK AND TABLET DOSAGE FORM

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## ABSTRACT

**Objective:** To develop accurate, fast, simple, and precise reversed-phase high-pressure liquid chromatography method for simultaneous determination of the binary mixture of metformin (MET) and empagliflozin (EMPA) in dosage forms.

**Methods:** The method uses a mobile phase consisting of phosphate buffer, acetonitrile, methanol (15:80:5 v/v/v), an octadecyl silica C-18 column (4.6 mm × 250 mm, 5 µ particle size) in isocratic mode, detection wavelength of 227 nm, and a flow rate of 1 mL/minutes.

**Results:** The measured retention times for MET and EMPA and were 2.528 and 4.140 minutes, respectively. The percentage recoveries of MET and EMPA were 101.12% and 100.55%, respectively. The relative standard deviation for assay of tablets was found to be <2%. The correlation coefficient for MET and EMPA was found to be 0.9990. The limit of detection and quantification for MET was 0.10 µg/mL and 0.31 µg/mL and for EMPA 0.01 µg/mL and 0.03 µg/mL.

**Conclusion:** The method was fast, accurate, precise, and sensitive hence it can be employed for routine quality control of tablets containing both drugs in quality control laboratories and pharmaceutical industries.

**Keywords:** Empagliflozin, Forced degradation method, Metformin, Reversed-phase high-performance liquid, Stability-indicating assay.

## INTRODUCTION

Metformin (MET) is chemically named as 4 1-carbamimidamido-N, N-dimethylmethanimidamide and it is as shown in Fig. 1. It is used as a biguanide antihyperglycemic agent used for treating non-insulin-dependent diabetes mellitus (NIDDM) [1,2]. It improves glycemic control by decreasing hepatic glucose production, decreasing glucose absorption, and increasing insulin-mediated glucose uptake. MET may induce weight loss and is the drug of choice for obese NIDDM patients. Use of MET is associated with modest weight loss.

Empagliflozin (EMPA) is chemically named as (2S,3R,4R,5S,6R)-2-[4-chloro-3-({4-[(3S)-oxolan-3-yloxy]phenyl}methyl)phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol and it is as shown in Fig. 2. EMPA sodium glucose co-transporter-2 (SGLT-2) inhibitor indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with Type 2 diabetes. SGLT-2 cotransporters are responsible for reabsorption of glucose from the glomerular filtrate in the kidney. The glucuretic effect resulting from SGLT-2 inhibition reduces renal absorption and lowers the renal threshold for glucose, therefore resulting in increased glucose excretion [3,4]. In addition, it contributes to reduced hyperglycemia and also assists weight loss and blood pressure reduction.

Literature survey revealed that few analytical methods are reported for analysis of both the drugs alone as well as in combination using ultraviolet (UV) spectrophotometry [5], high-pressure liquid chromatography (HPLC) [6-11], and ultra-pressure liquid chromatography [12].

The analytical method employed for the quantitative determination of drugs in formulation plays a significant role in the evaluation and interpretation of combined tablet dosage forms. Hence, an attempt has been made to develop new method for simultaneous estimation and validation of MET and EMPA in tablet formulation in accordance with the International Conference on Harmonization (ICH) guidelines [13].

## METHODS

### Chemicals

The reference samples of MET and EMPA were provided as gift samples from Spectrum Pharma Research Solutions, Hyderabad.

HPLC grade acetonitrile, HPLC grade methanol, and all other chemicals were obtained from Merck chemical division, Mumbai. HPLC grade water obtained from Milli-Q water purification system was used throughout the study. Commercial tablets (Wormazan; Dosage: MET - 850 mg and EMPA - 5 mg) were purchased from the local pharmacy.

### Methodology

#### *Preparation of phosphate buffer pH 4.8*

Accurately weighed 1.36 g of potassium dihydrogen orthophosphate in a 1000 mL of volumetric flask add about 900 mL of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1 mL of orthophosphoric acid then PH adjusted to 4.8 with dil. Orthophosphoric acid solution.

#### *Preparation of mobile phase*

Mixture of phosphate buffer (pH - 4.8), acetonitrile, and methanol (15:80:5 v/v/v).

#### *Preparation of diluent*

A mixture of water and methanol in the ratio 50:50 [%v/v].

#### *Chromatographic parameters*

Mobile phase:Phosphate buffer:Acetonitrile:Methanol (15:80:5% v/v/v) pH adjusted to 4.8

Column: Octadecyl silica C18, 250 mm × 4.6 mm, 5 µm
Wavelength: 227 nm

Flow rate: 1.0 mL/minutes
Injection volume: 10 µL
Run time: 10 minutes
Detector: 2996 photodiode array detector.

### *Blank*

A mixture of water and methanol in the ratio 50:50 [%v/v].

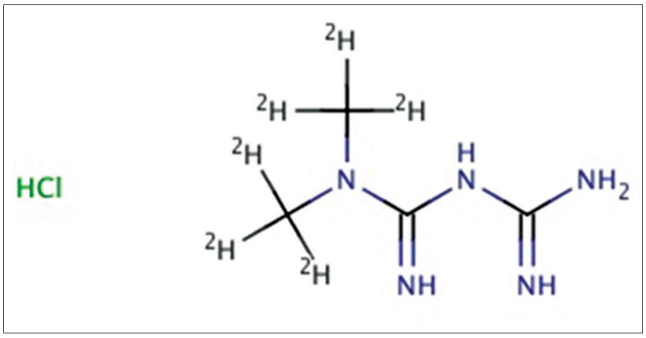

**Fig. 1: Chemical structure of metformin**

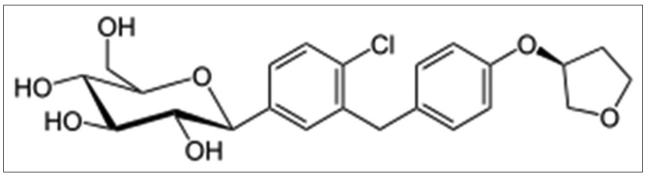

**Fig. 2: Chemical structure of empagliflozin**

### Standard solution

To prepare the standard solution, 1000 ppm of MET and 12.5 ppm of EMPA was taken in to 10 mL of clean dry volumetric flask, add 7 mL of diluent (water:methanol, 50:50), then sonicated for 10 minutes and make up the volume with diluent.

### Sample preparation

The chromatogram was shown in (Fig.3). One tablet was weighed, powdered and then was transferred into a 100 mL volumetric flask, 10 mL of diluent added and sonicated for 25 minutes, further the volume made up with diluent and filtered. From the filtered solution, 1 mL was pipeted out into a 10 mL volumetric flask and made up to 10 mL with diluent.
Label claim: 850 mg MET, 5 mg EMPA.

### Placebo preparation

The placebo was prepared by weighing accurately 85 mg of MET and (5 mg of EMPA) was taken into a 100 mL volumetric flask and add 50 mL of diluent and sonicated for 15 minutes and the diluted to the mark. The chromatogram was shown in (Fig.4).

### Method development

To establish and validate an efficient method for analysis of these drugs in pharmaceutical formulations, preliminary tests were performed. Different chromatographic conditions were employed for the analysis of the MET and EMPA in both bulk and pharmaceutical dosage form. The pure drugs of MET and EMPA were injected into the HPLC system and run using standard organic solvents commonly used for HPLC studies. Water, acetonitrile, and methanol were tested separately and in combination to find the best conditions for the separation of MET and EMPA. In focus to develop good symmetrical peak, water was replaced by phosphate buffer and it was observed that phosphate

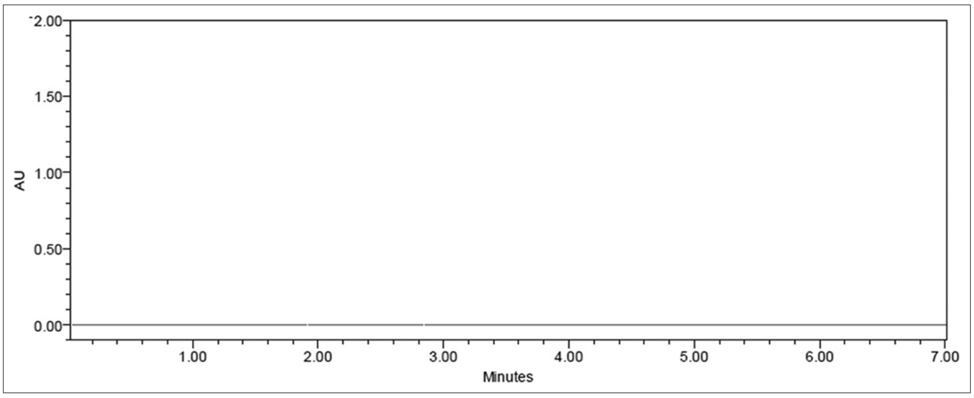

**Fig. 3: Chromatogram of blank**

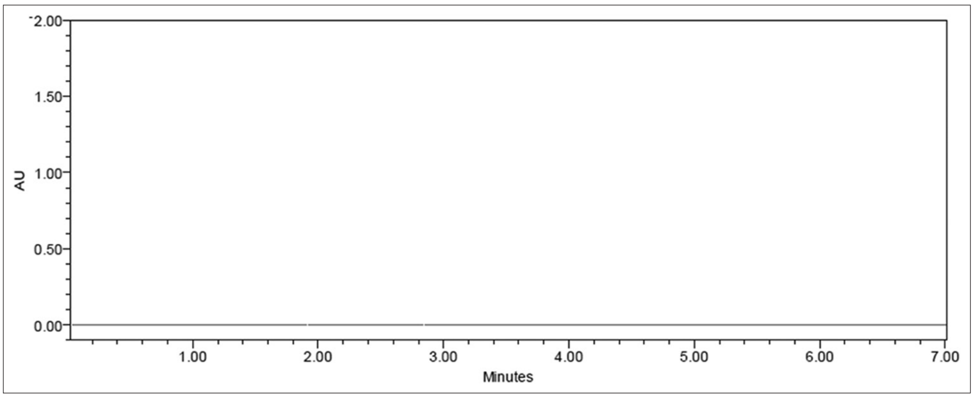

**Fig. 4: Chromatogram of placebo**

buffer, acetonitrile and methanol gave satisfactory results. This mobile phase system was tried with different proportions and with different flow rates. Finally, the optimal condition of the mobile phase was chosen as phosphate buffer: Acetonitrile: Methanol in the ration of 15:80:5% v/v/v. This composition of the mobile phase resolved the two drugs very well. All measurements were carried out at ambient temperature of the column. To optimize the flow rate, various flow rates were used. The optimal flow rate was 1 mL/minute for the presented work. The chromatogram was shown in (Fig.5).

### Validation of quantitative HPLC method

The validation of the method was carried out as per ICH Guidelines [13]. The parameters assessed were specificity, linearity, precision, accuracy, stability, limit of detection (LOD), and limit of quantification (LOQ).

#### *Specificity*

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences including degradation products and related substances.

#### *Accuracy*

The accuracy was determined by calculating percentage recoveries of MET and EMPA. It was carried out by adding known amounts of each analyte corresponding to three concentration levels (50, 100, and 150%) of the labelled claim to the excipients. At each level, six determinations were performed, and the accuracy results were expressed as percent analyte recovered by the proposed method.

#### *Precision*

The precision of an analytical method is usually expressed as the standard deviation (SD). The repeatability studies were carried out by estimating response of MET and EMPA six times. The intra- and inter-day precision studies (intermediate precision) were carried out by estimating the corresponding responses three times on the same day and on three different days for three different concentrations, and the results are reported in terms of relative SD (RSD).

#### *Linearity*

The purpose of the test for linearity is to demonstrate that the entire analytical system (including detector and data acquisition) exhibits a linear response and is directly proportional over the relevant concentration range for the target concentration of the analyte. The linear regression data for the calibration plot is indicative of a good linear relationship between peak area and concentration over a wide range. The correlation coefficient was indicative of high significance.

#### *Robustness*

Robustness of the method was investigated under a variety of conditions including changes of composition of buffer in the mobile phase, flow rate, and temperature. This deliberate change in the method has no affect on the peak tailing, peak area and theoretical plates and finally the method was found to be robust.

#### *LOD and LOQ*

The LOD can be defined as the smallest level of analyte that gives a measurable response and LOQ was determined as the lowest amount of analyte that was reproducibly quantified. These two parameters were calculated using the formula based on the SD of the response and the slope. LOD and LOQ were calculated by using equations,

$LOD = 3.3 \times s/S$ and

$LOQ = 10 \times s/S$,

Where s = Standard deviation, S = Slope of the calibration curve.

#### *Assay of MET and EMPA in tablet*

Assay of marketed product was carried out using the developed method. Sample solutions were prepared and injected into reversed-phase (RP)-HPLC system. The sample solution was scanned at 227 nm. The percentage drug estimated was found to be 101.12 for MET and 100.10 for EMPA. The chromatogram showed two single peaks of MET and EMPA was observed with retention times of 2.528 and 4.140 minutes Fig. 6.

### Forced degradation studies

Stress studies are performed according to ICH guidelines under conditions of peroxide, acid, alkali, dry heat and UV studies.

#### *Peroxide degradation studies*

To 1 mL of stock solution of MET and EMPA, 1 mL of 20% hydrogen peroxide ($H_2O_2$) was added separately. The solutions were kept for 30 minutes at 60°C. For HPLC study, the resultant solution was diluted to obtain 850 μg/mL and 5 μg/mL solution and 10 μl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

#### *Acid degradation studies*

To 1 mL of s tock s solution MET and EMPA, 1 mL of 2 N hydrochloric acid was added and refluxed for 30 minutes at 60°C. The resultant solution was diluted to obtain 850 μg/mL and 5 μg/mL solution and 10 μl solutions were injected into the system and the chromatograms were recorded to assess the stability of the sample.

#### *Alkali degradation studies*

To 1 mL of stock solution MET and EMPA, 1 mL of 2N sodium hydroxide was added and refluxed for 30 minutes at 60°C. The resultant solution was diluted to obtain 850 μg/mL and 5 μg/mL solution and 10 μl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

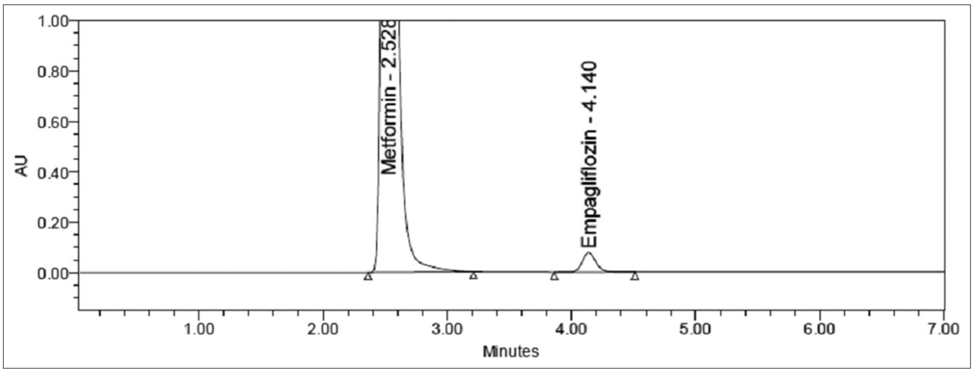

**Fig. 5: Standard chromatogram of metformin and empagliflozin**

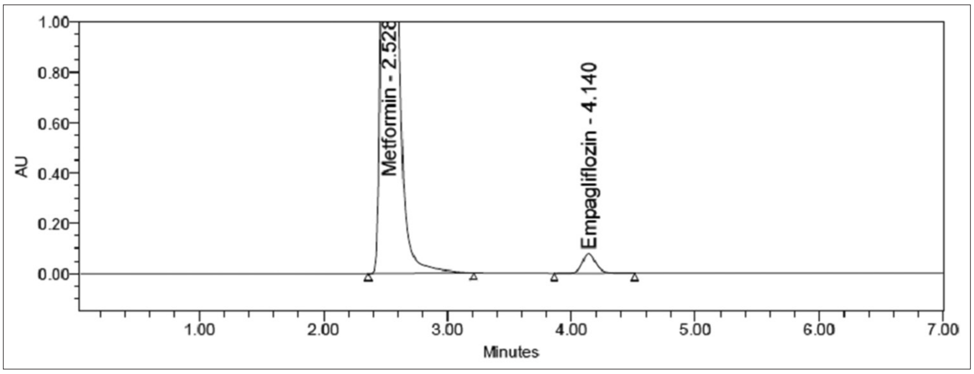

Fig. 6: A typical chromatogram of metformin and empagliflozin in tablet dosage form

*Dry heat degradation studies*

The standard drug solution was placed in oven at 105°C for 6 hrs to study dry heat degradation. For HPLC study, the resultant solution was diluted to 850 µg/mL and 5 µg/mL solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

*UV degradation studies*

The photochemical stability of the drug was also studied by exposing the 850 µg/mL and 5 µg/mL solution to UV light by keeping the beaker in UV chamber for 7 days or 200 Watt hrs/m$^2$ in photostability chamber. For HPLC study, the resultant solution was diluted to obtain 850 µg/mL and 5 µg/mL solutions and 10 µl were injected into the system, and the chromatograms were recorded to assess the stability of sample.

## RESULTS AND DISCUSSION

From this study, it was found that a simple, precise, accurate, sensitive, and efficient stability indicating RP-HPLC method has been developed and validated for simultaneous estimation of MET and EMPA in bulk and pharmaceutical dosage form. To establish and validate an efficient method for analysis of these drugs in pharmaceutical formulations, optimized chromatographic conditions used for the estimation of MET and EMPA are given as in Table 1.

Linearity was established by analyzing different concentrations of MET and EMPA. The calibration curve was plotted with the area obtained versus concentration of both MET and EMPA Figs. 7 and 8. In the present study, six concentrations were chosen ranging between 10 and 60 µg/mL of MET and 30-50 µg/mL of EMPA.

The regression equation and correlation coefficient for MET and EMPA was found to be:

$y=9338x + 641.8$ and $R^2=0.9990$ and

$y=48884x + 1054$ and $R^2=0.9990$, respectively, and results were given in Table 2.

The accuracy of the method was proved by performing recovery studies on the commercial formulation at 50, 100 and 150% level. Percentage recoveries of MET and EMPA ranges from 100.01% to 100.10% in simultaneous equation method, and the results were shown in Table 3.

Precision was evaluated by a known concentration of MET and EMPA was injected six times and corresponding peaks were recorded and percentage RSD was calculated and found within the limits. The low percentage RSD value was indicated that the method was precise and reproducible and the results were shown in Table 4.

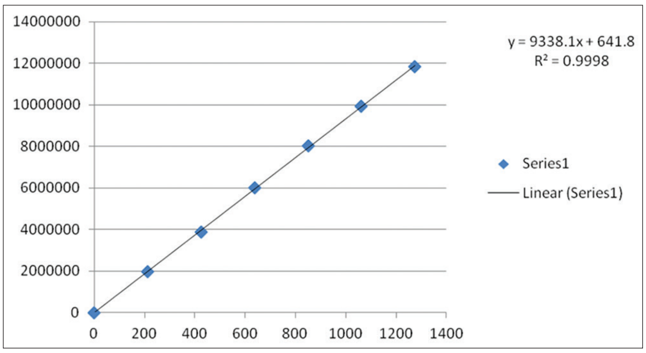

Fig. 7: Linearity curve of metformin

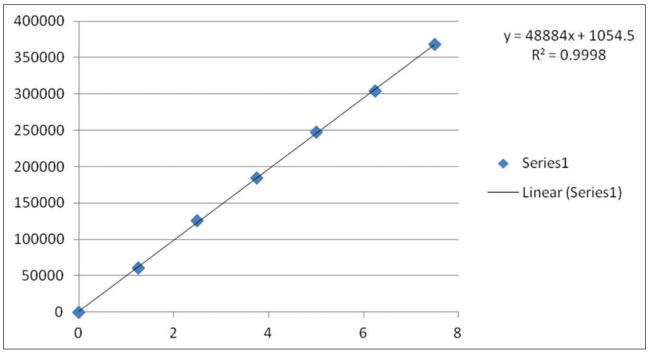

Fig. 8: Linearity curve of empagliflozin

Table 1: Optimized chromatographic conditions

| Parameter | Condition |
|---|---|
| Mobile phase | Phosphate buffer: Acetonitrile: Methanol (15:80:5%V/V/V) pH adjusted to 4.8 |
| Column | ODS C18, 250×4.6 mm, 5µ |
| Wave length | 227 nm |
| Flow rate | 1.0 mL/min |
| Injection volume | 10 µL |
| Run time | 10 min |
| Diluent | Water: Methanol (50:50) |

ODS: Octadecyl silica

Robustness of the method is the ability of the method to remain unaffected by small deliberate changes in parameters like flow rate, mobile phase composition and column temperature. To study the effect of flow rate of the mobile phase, it was changed to 0.1 units from 1.0 mL to 0.9 mL and 1.1 mL. The effect of column temperature also checked by changing temperature to ±5°C. This deliberate change in the above parameters has no significant effect on the chromatographic behavior

**Table 2: Linearity data for MET and EMPA**

| Serial number | MET | | EMPA | |
|---|---|---|---|---|
| | Concentration (μg/ml) | Peak area | Concentration (μg/ml) | Peak area |
| 1 | 212.5 | 1,978,270 | 1.25 | 60,899 |
| 2 | 425 | 3,888,258 | 2.5 | 125,922 |
| 3 | 637.5 | 6,025,370 | 3.75 | 184,711 |
| 4 | 850 | 6,025,370 | 5 | 247,276 |
| 5 | 1062.5 | 8,010,015 | 6.25 | 303,966 |
| 6 | 1275 | 11,848,754 | 7.5 | 367,820 |

MET: Metformin, EMPA: Empagliflozin

**Table 3: Accuracy data for MET and EMPA**

| Drug | Spiked level (%) | Percentage recovery | Percentage RSD |
|---|---|---|---|
| MET | 50 | 100.64 | 1.41 |
| | 100 | 100.89 | 0.41 |
| | 150 | 100.83 | 0.96 |
| EMPA | 50 | 101.34 | 0.26 |
| | 100 | 99.95 | 0.41 |
| | 150 | 100.34 | 0.96 |

RSD: Relative standard deviation, MET: Metformin, EMPA: Empagliflozin

**Table 4: Precision method of proposed RP-HPLC method**

| Drug | Mean area | Percentage RSD |
|---|---|---|
| MET | 7,993,232 | 0.59 |
| EMPA | 245,611 | 1.3 |

RSD: Relative standard deviation, RP-HPLC: Reversed phase high-pressure liquid chromatography, MET: Metformin, EMPA: Empagliflozin

of the samples and results were given in Table 5 and the chromatograms are shown in the Figs. 9-14.

LOD and LOQ of MET and EMPA were evaluated based on RSD of the response and slope of the calibration curve. The detection limits were found to be 0.10 μg/mL and 0.01 μg/mL for MET and EMPA, respectively. The quantitation limits were found to be 0.31 μg/mL and 0.03 μg/mL for MET and EMPA, respectively. The results were given in Table 6. Moreover, the chromatograms are shown in Figs. 15 and 16.

Forced degradation studies were performed to establish the stability indicating property and specificity of the proposed method. Degradation studies were carried out under conditions of hydrolysis, dry heat, oxidation, UV light and photolysis and the drug substances were degraded in all conditions.

Acid and base hydrolysis was performed by exposing the drug substances with 2 N HCl and 2 N NaOH at 60°C for 30 minutes and it was showed degradation of MET and EMPA with degraded products peak at retention time 2.61 minutes and 3.02 minutes, respectively.

Degradation studies under oxidative conditions were performed by heating the drug sample with 20% $H_2O_2$ at 60°C and degraded product peaks were observed. Both MET and EMPA are sensitive to acid and alkali and there was no degradation occurs under UV light and thermal conditions. The results of forced degradation studies were given in Table 7 ant chromatograms are shown in Figs. 17-21.

## CONCLUSION

A new stability - indicating RP-HPLC method has been developed for estimation of MET and EMPA in bulk and pharmaceutical dosage form. The developed method was validated and it was found to be simple, sensitive, precise, and robust and it can be used for the

**Table 5: Robustness data for MET and EMPA**

| Parameters | Changed condition | Mean peak area | | USP plate count | |
|---|---|---|---|---|---|
| | | MET | EMPA | MET | EMPA |
| Flow rate (mL/minute) | | | | | |
| | 0.9ml | 25,943,381 | 625,201 | 2711.17 | 12,942.5 |
| | 1.0ml | 7,984,114 | 245,123 | 2867.83 | 9433 |
| | 1.1ml | 23,407,466 | 570,391 | 2632.33 | 13,144.5 |
| Temperature (±5) | | | | | |
| | 25°C | 26,407,278 | 624,757 | 2402.17 | 13,202.2 |
| | 30°C | 7,984,114 | 245,123 | 2867.83 | 9433 |
| | 35°C | 23,507,485 | 566,202 | 2628 | 13,219.5 |
| Mobile phase (±5%) | | | | | |
| | 10:85:45%v/v | 26,295,148 | 624,135 | 2405.33 | 13,209.3 |
| | 15:80:5%v/v | 7,984,114 | 245,123 | 2867.83 | 9433 |
| | 20:75:5%v/v | 24,446,476 | 598,229 | 2774.67 | 9406.67 |

MET: Metformin, EMPA: Empagliflozin

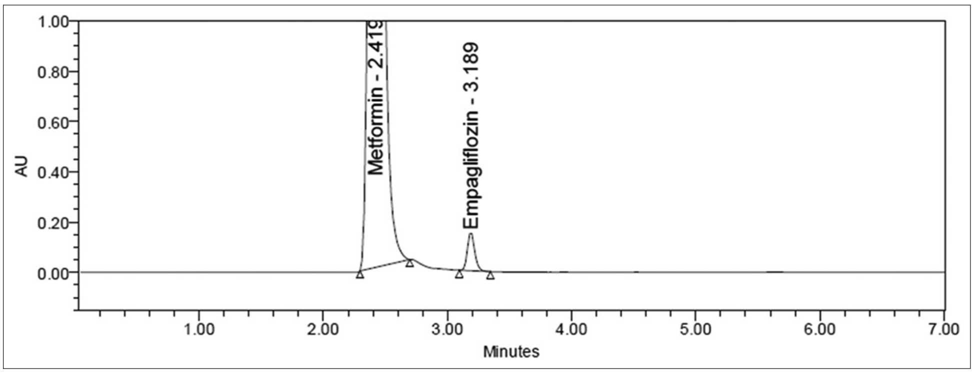

Fig. 9: Chromatogram of flow minus

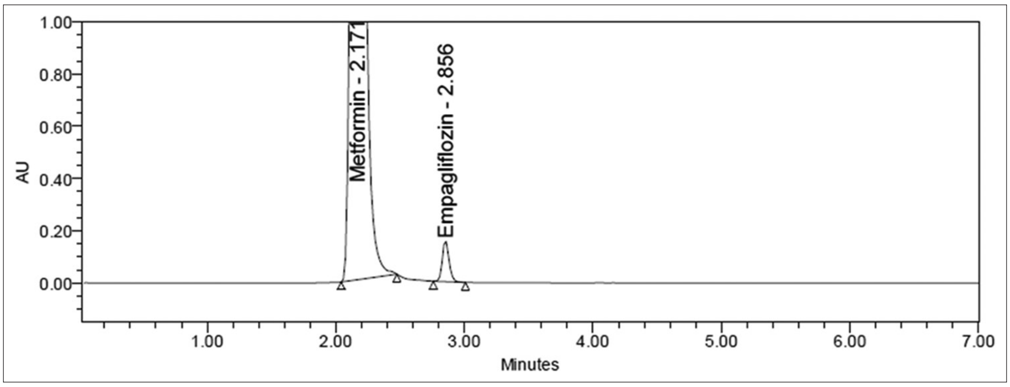

Fig. 10: Chromatogram of flow plus

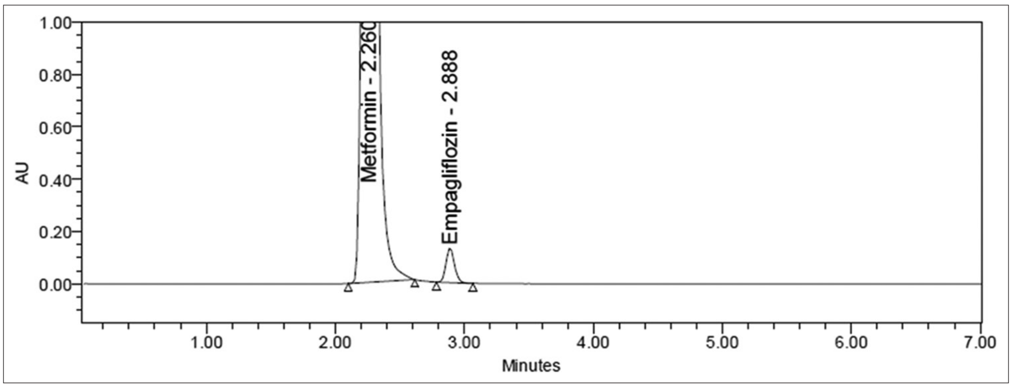

Fig. 11: Chromatogram of mobile-phase minus

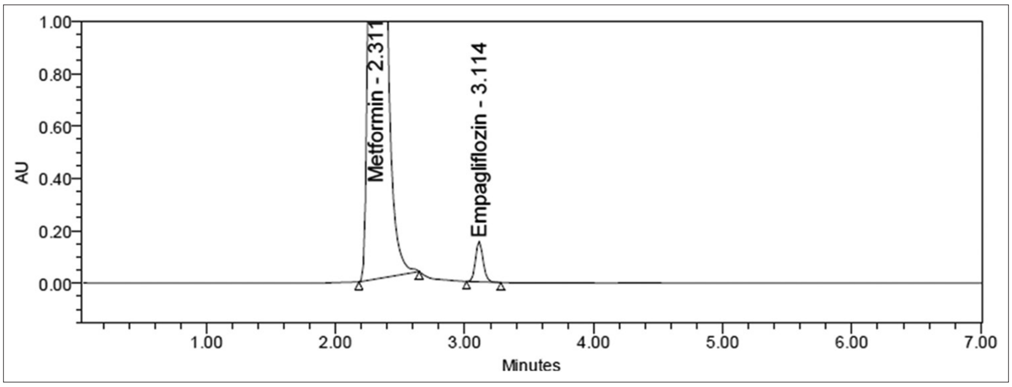

Fig. 12: Chromatogram of mobile-phase plus

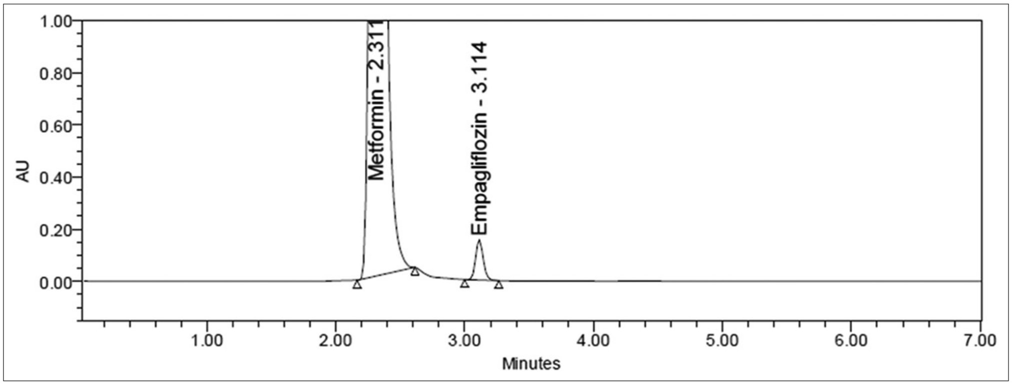

Fig. 13: Chromatogram of temperature minus

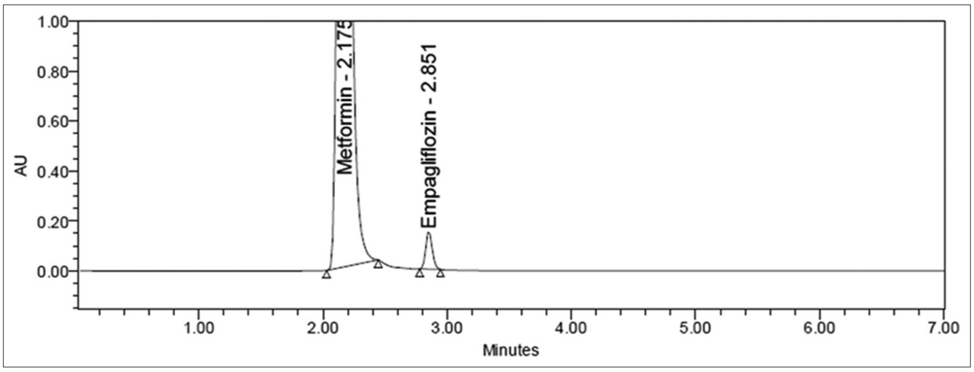

Fig. 14: Chromatogram of temperature plus

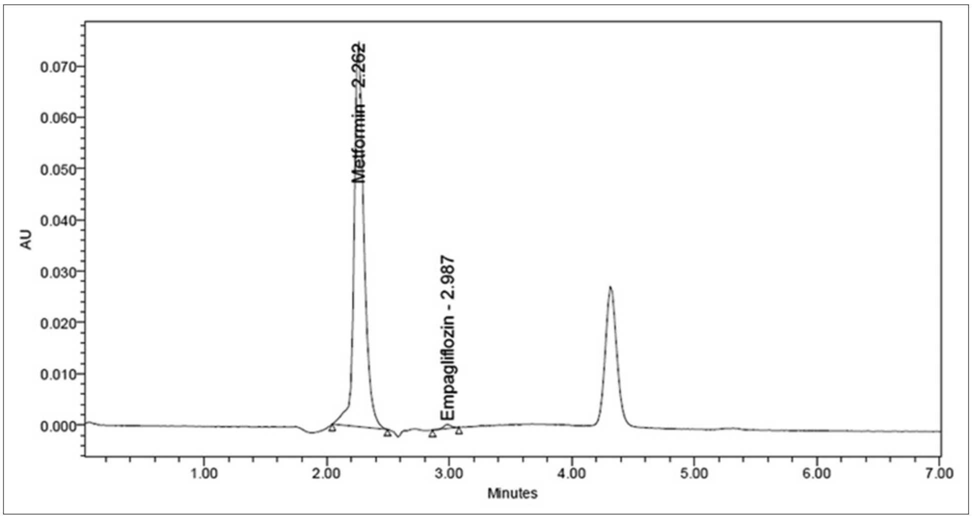

Fig. 15: Chromatogram of limit of detection

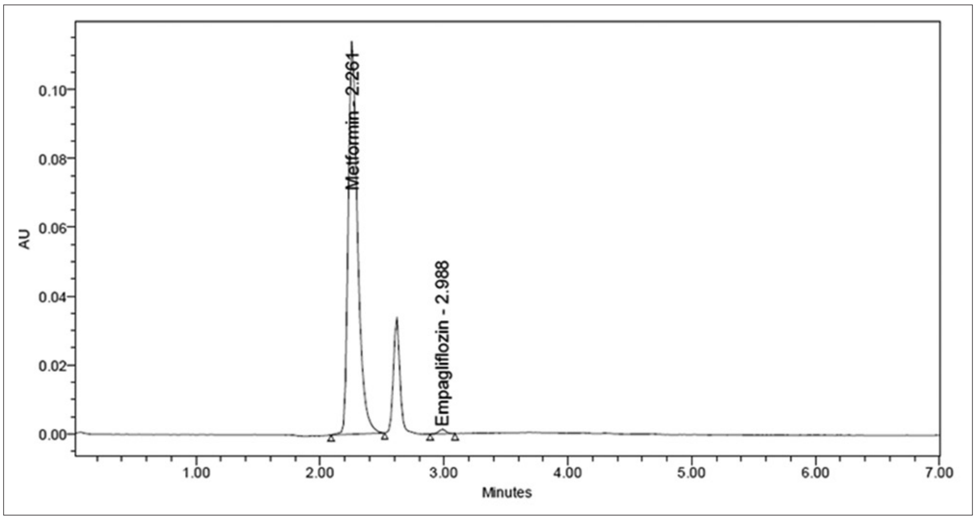

Fig. 16: Chromatogram of limit of quantification

**Table 6: LOD and LOQ data for MET and EMPA**

| Drug | LOD (µg/ml) | LOQ (µg/ml) |
|---|---|---|
| MET | 0.10 | 0.31 |
| EMPA | 0.1 | 0.03 |

LOD: Limit of detection, LOQ: Limit of quantification, MET: Metformin, EMPA: Empagliflozin

routine analysis of MET and EMPA in both bulk and pharmaceutical dosage forms. The forced degradation studies were carried out in accordance with ICH guidelines, and the results revealed suitability of the method to study stability of MET and EMPA under various degradation conditions such as acid, base, oxidative, thermal, UV and photolytic degradations. Finally, it was concluded that the method is simple, sensitive and has the ability to separate the

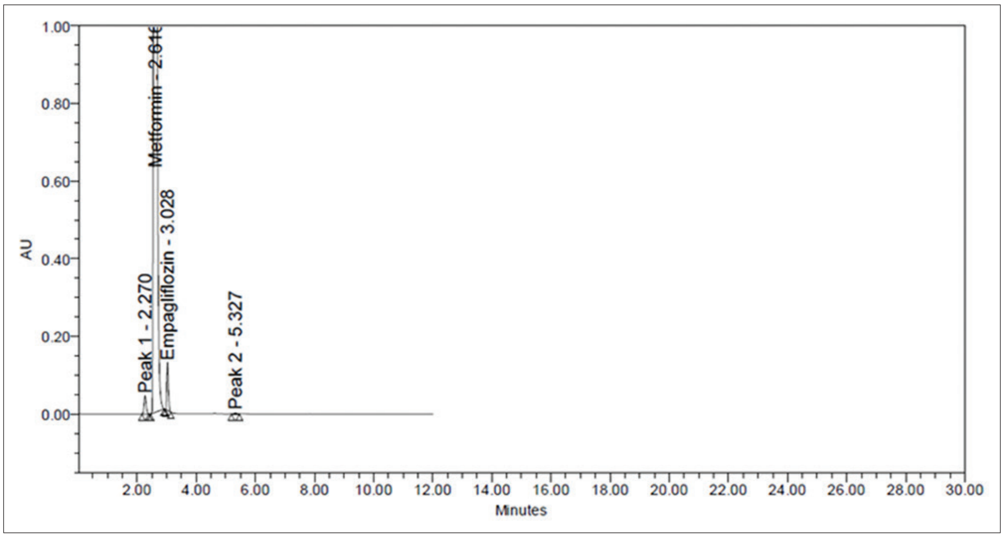

Fig. 17: Chromatogram of acid degradation

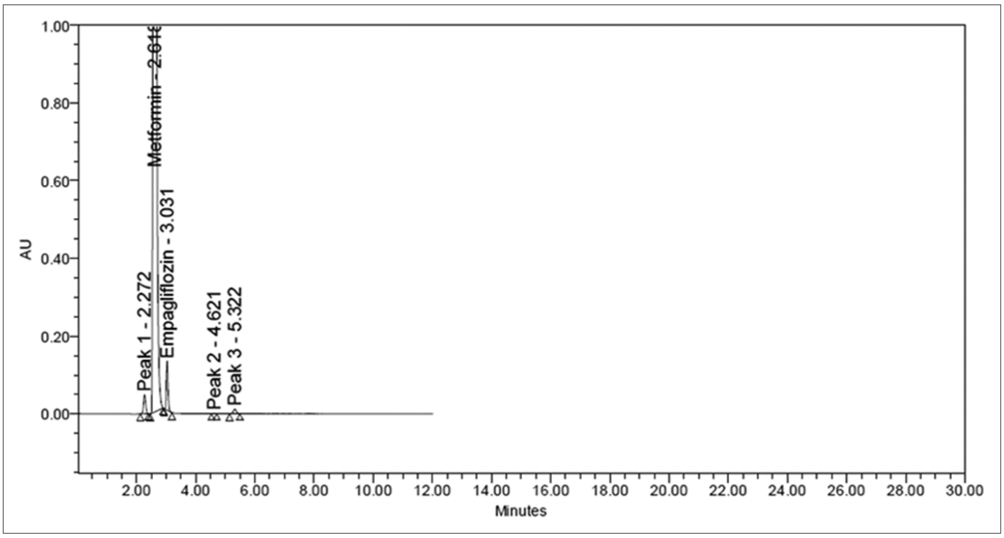

Fig. 18: Chromatogram of alkali degradation

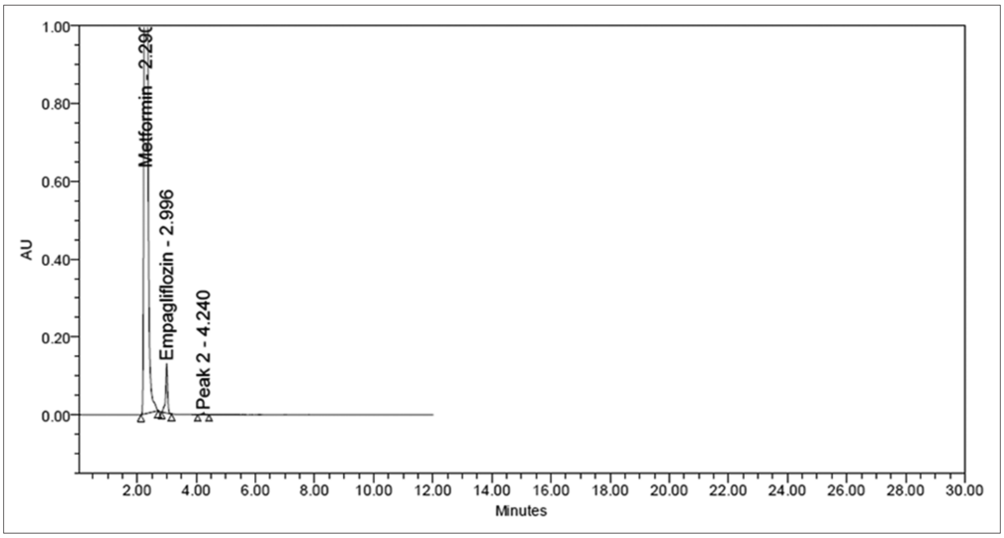

Fig. 19: Chromatogram of peroxide degradation

**Table 7: Results of forced degradation studies**

| Serial number | Injection | MET | | EMPA | |
|---|---|---|---|---|---|
| | | Percentage assay | Percentage degradation | Percentage assay | Percentage degradation |
| 1 | Acid degradation | 95.28 | 2.61 | 95.60 | 3.02 |
| 2 | Base degradation | 96.54 | 2.61 | 96.15 | 3.02 |
| 3 | Peroxide | 97.79 | 2.29 | 97.85 | 2.90 |
| 4 | Thermal degradation | 97.90 | 2.28 | 98.60 | 3.0 |
| 5 | UV degradation | 98.16 | 2.28 | 98.60 | 3.0 |
| 6 | Neutral degradation | 99.01 | 2.29 | 99.82 | 3.0 |

MET: Metformin, EMPA: Empagliflozin, UV: Ultraviolet

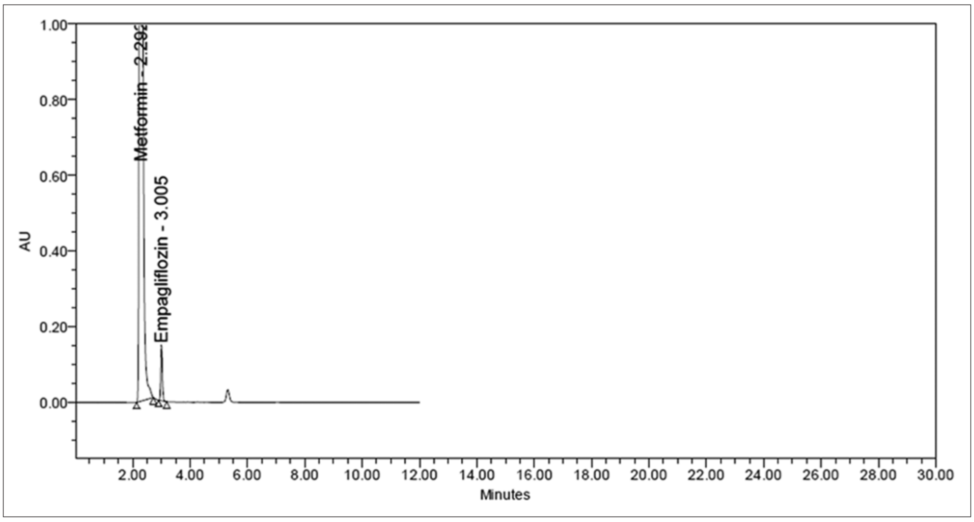

Fig. 20: Chromatogram of thermal degradation

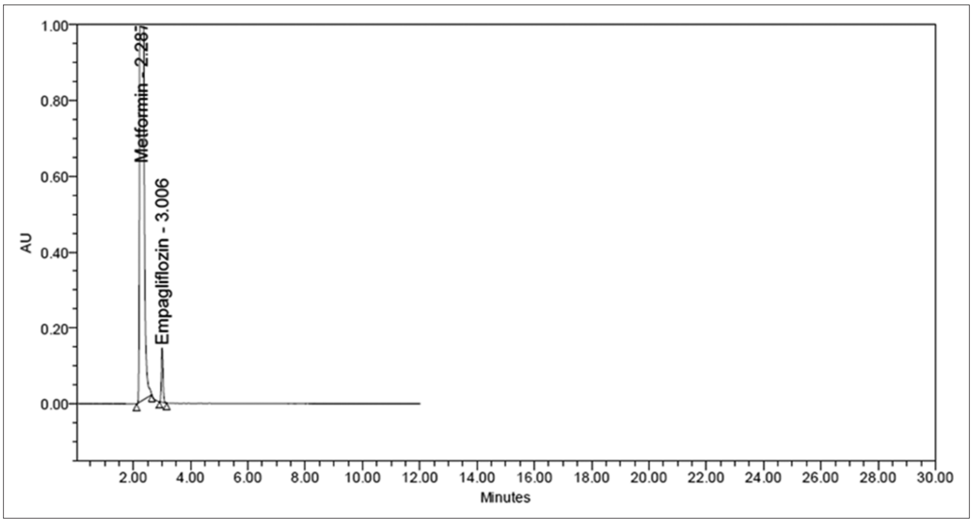

Fig. 21: Chromatogram of ultraviolet degradation

drug from degradation products and excipients found in the dosage form.

## REFERENCES

1. Hermann LS, Kulhmann I, Plus W. Clinical pharmacology of biguanides. In: Hand Book of Experimental Pharmacology. Heidelberg, Germany: Springer; 1995. p. 374-407.
2. Metformin. Available from: http://www.drugbank.ca/drugs/DB00331.
3. Diabetes (Type 2)-Empaglifozin. Appraisal Consultation Document. NICE Technology Appraisal. 28 August, 2014.
4. Nair S, Wilding JP. Sodium glucose cotransporter 2 inhibitors as a new treatment for diabetes mellitus. J Clin Endocrinol Metab 2010;95(1):34-42.
5. Madhukar A, Prince A, Kumar RV, Sanjeeva Y, Jagadeeshwar K, Raghupratap D. A simple and sensitive analytical method development and validation of metformin hydrochloride by RP-HPLC. Int J Pharm Pharm Sci 2011;3(3):117-20.
6. Rajesh T, Lakshmi KS, Sharma S. Simultaneous determination of metformin and pioglitazone by reversed phase HPLC in pharmaceutical dosage forms. Int J Pharm Pharm Sci 2009;1(2):162-6.
7. Akula A, Prajwala N, Sandhya M. Development and validation of RPHPLC method for simultaneous estimation of metformin hydrochloride and Gliclazide in bulk and combined dosage form. Int J Pharm Pharm Sci 2013;5(4):511-7.
8. Bhoomaiah B, Shree AJ. Development and validation of RP-HPLC

method for simultaneous determination of metformin and miglitol in bulk and pharmaceutical formulation. Int J Pharm Pharm Sci 2014;6(6):135-41.
9. Prathap GM, Muthukumaran M, Krishnamoorthy B. Development and validation of simultaneously estimation of vildagliptin and metformin hydrochloride by RP-HPLC in bulk and oral dosage form. Int J Adv Pharm Gen Res 2014;2(1):24-33.
10. Shyamala, Nirmala K, Mounika J, Nandini B. Validated stability-indicating RP-HPLC method for determination of empagliflozin. Der Pharm Lett 2016;8(2):457-64.
11. Padmaja N, Veerabhadram G. Method development and validation of RP-HPLC method for the estimation of empagliflozin in API. Int J Pharm Sci Res 2015;7(2):724-7.
12. Ayoub BM. UPLC simultaneous determination of empagliflozin, linagliptin and metformin. RSC Adv 2015;5(116):95703-9.
13. ICH and Q2B, Harmonized Tripartite Guideline, Validation of Analytical Procedure Methodology. IFPMA. In: Proceedings of the International Conference on Harmonization; 1996.