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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF METFORMIN HYDROCHLORIDE AND EMPAGLIFLOZIN IN BULK AND IN A SYNTHETIC MIXTURE

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ABSTRACT

The purpose of the investigation was to develop a simple, rapid and accurate RP-HPLC method to determine assay of Metformin Hydrochloride And Empagliflozin in Bulk and synthetic mixture. The chromatographic separation was performed on Kromosil 250 x 4.6 mm, 5µm. Eluents were monitored on PDA detector at a wavelength of 233 nm using a Buffer: Acetonitrile (45:55v/v). The column temperature was maintained at 30°C. Validation parameters such as system suitability, linearity, precision, accuracy, specificity, limit of detection (LOD), limit of quantification (LOQ), Stability of sample and standard stock solutions and robustness were studied as reported in the ICH guidelines. The retention time for Metformin Hydrochloride And Empagliflozin was 2.270 min and 3.413 min respectively. Assay method further evaluated for Metformin Hydrochloride and Empagliflozin analysis at low concentration of analyte and found limit of detection is 0.48 and 0.016 ppm respectively and limit of Quantitation is 1.49 and 0.049 ppm respectively. The percentage recovery of Metformin Hydrochloride And Empagliflozin was 99.64% and 99.47% respectively. The %RSD for Metformin Hydrochloride And Empagliflozin was less than 2. Linearity of Metformin Hydrochloride and Empagliflozin performed from 25% to150% and the R2 is 0.999, intercept and slope found to be y = 26850x + 439840 and y = 47664x + 9394.7 respectively. The method was fast, accurate, precise and sensitive hence it can be employed for routine quality control of Metformin Hydrochloride and Empagliflozin containing drug in quality control laboratories and pharmaceutical industries.

Key words: RP-HPLC, Metformin Hydrochloride and Empagliflozin

INTRODUCTION

Metformin Hydrochloride is a biguanide derivative which is the most widely prescribed drug to treat hyperglycemia in individuals with Type 2 diabetes especially in overweight patients and is recommended, in conjunction with lifestyle modification (diet, weight control and physical activity), as a first line oral therapy in the recent guidelines of the American Diabetes Association and European Association of the Study of Diabetes.Metformin is one of only two oral anti diabetics in the World Health Organisation Model List of Essential Medicines (the other being glibenclamide).Chemically it is known as1,1-dimethylbiguanide hydrochloride. It is marketed under the trade name Glycomet®, Glucophage and Fortamet.Metformin decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization.[1-5]These effects are mediated by the initial avtivation of AMP-activated protein kinase (AMPK), a liver enzyme that plays an

important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats[6-8]. Empagliflozin is a 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. Chemically it is known as (2S,3R,4R,5S,6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl]oxyphenyl]methyl]phenyl]-(hydroxymethyl)oxane-3,4,5-triol. It is marketed under the trade name Jardiance. SGLT2 co-transporters are responsible for reabsorption of glucose from the glomerular filtrate in the kidney.[9-10] The glucuretic effect resulting from SGLT2 inhibition reduces renal absorption and lowers the renal threshold for glucose, therefore resulting in increased glucose excretion. Additionally, it contributes to reduced hyperglycaemia and also assists weight loss and blood pressure reduction[11-12].

The literature survey reveals that there are only two analytical methods available for estimation of Metformin Hydrochloride and Empagliflozin. The reported methods available for the estimation of Metformin Hydrochloride and Empagliflozin individually and in combination are spectrophotometric and HPLC methods.13-28So we have planned to develop a simple, precise, economic and accurate Stability indicating RP-HPLC method development and validation for the estimation of Metformin Hydrochloride And Empagliflozin in synthetic mixture.

EXPERIMENTAL

Materials and methods

Active pharmaceutical ingredients Metformin Hydrochloride and Empagliflozin were obtained as a gift sample from Hetero Drugs Limited, Hyderabad. The pharmaceutical dosage forms (Glycomet®, Jardiance) were purchased from local pharmacy. The solvents used in this work were of HPLC grade and obtained from Rankem.

Instrumentation and chromatographic conditions

The analysis was performed on a high performance liquid chromatography system consists of waters 2695 with 2996 module Photo Diode Array detector equipped with a quaternary solvent delivery pump, automatic sample injector and column thermostat. The data acquisition and analysis was performed by using Empower2 software. The chromatographic separation was performed on Kromosil 250 x 4.6 mm, 5µm. The flow rate was kept at 0.8ml/min. The column temperature was maintained at 30°C. The mobile phase was made of 0.1% Ortho Phosphoric Acid Buffer and Acetonitrile taken in the ratio45:55 ratio had gave acceptable retention time and good resolution between Metformin Hydrochloride and Empagliflozin. The method was optimized at 233nm. Data acquisition and processing was performed by using empower2 system software. The run time was taken as 6min. All the determinations are carried out at an ambient temperature.

Preparation of Standard stock solutions

Accurately Weighed and transferred 125mg of Metformin Hydrochloride and 12.5mg of Empagliflozin working Standards into a 25ml and 100 ml clean dry volumetric flasks respectively, add 3/4th volume of diluents (Water:Acetonitrile in the ratio 50:50), sonicated for 10minutes and make up to the final volume with diluents. 1ml from the above two stock solutions was taken into a 10ml volumetric flask and made up to 10ml with diluent.

Preparation of Sample stock solutions

Accurately Weighed and transferred equivalent to 125mg of Metformin Hydrochloride and 12.5mg of Empagliflozin tablets powder into a 25ml and 100 ml clean dry volumetric flasks respectively, add 3/4th volume of diluent (Water:Acetonitrile in the ratio 50:50), sonicated for 15minutes and make up to the final volume with diluent and filtered. 1ml from the above two filtered stock solutions was taken into a 10ml volumetric flask and made up to 10ml with diluent.

Preparation of buffer

Buffer: (0.1%OPA)

1ML of Ortho Phosphoric Acid solution in a 1000ml of volumetric flask add about 100ml of HPLC grade water and final volume make up to 1000 ml with HPLC grade water.

Method validation

The method was validated according to ICH guidelines. The different validation characteristics which were performed are following: Linearity, accuracy, Precision, limit of detection, limit of quantification, robustness and the stability indicating capability.

System suitability parameters

The system suitability parameters were determined by preparing standard solutions of Metformin Hydrochloride and Empagliflozin and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

Linearity

The linearity of the method is determined by

preparing three individual series of solutions in the range of Metformin Hydrochloride (125-750µg/ml) and Empagliflozin (3.125-18.75µg/ml). The obtained peak areas are plotted against concentration.

Preparation of linearity solutions

Preparation of Standard stock solutions

Accurately weighed and transferred 125 mg of Metformin Hydrochloride and 12.5 mg of Empagliflozin and transferred into a 25ml and 100ml clean dry volumetric flasks seperately. Add3/4thvolume of diluent, sonicated for 10minutes and make up to the final volume with diluent. From two stock solutions pipette out 0.25ml, 0.5ml, 0.75ml, 1.0ml, 1.25ml, 1.50ml into 10ml volumetric flak to get 25%,50%, 75%, 100%, 125%, 150% of standard solutions.

Precision

a) Method precision (repeatability)

The method precision/ repeatability can be determined by injecting six working standard solutions. The areas of all the injections were taken and standard deviation, %Relative standard deviation, %assay were calculated.

b) Intermediate precision

The intermediate precision can be determined by injecting six working standard solutions on different days by different operators or by different instruments. The areas of all the injections were taken and standard deviation, %Relative standard deviation, %assay were calculated. The results obtained were within the acceptance criteria.

Accuracy

Accuracy is tested by the standard addition method at three different levels 50, 100 and 150%. The percentage recoveries of Metformin Hydrochloride and Empagliflozin were calculated.

Preparation of Standard stock solutions

Accurately weighed 125mg of Metformin, 12.5mg of Empagliflozin and transferred to 25ml and 100ml volumetric flasks separately. 3/4 th of diluents was added to both of these flasks and sonicated for 10 minutes. Flasks were made up with diluents and labeled as Standard stock solution 1and 2. (5000µg/ml of MET and 125µg/ml of EMPA)

Preparation of 50% Spiked Solution

0.5ml of each sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution

1.0ml of each sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution

1.5ml of each sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) of Metformin Hydrochloride and Empagliflozin were determined by calibration curve method.

Method robustness

The robustness can be determined by varying the following parameters:

Robustness of the developed method was determined by making small deliberate changes in flow rate (±0.2ml/min), column temperature (±5%), organic mobile phase ratio (±5%), along with the optimized method.

Forced degradation studies

Oxidation:

To 1 ml of stock solution of Metformin Hydrochloride and Empagliflozin, 1ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept undisturbed for 30 min . For HPLC study, the resultant solution was diluted to obtain 500µg/ml and 12.5µg/ml of all components and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies

To 1ml of sample stock solution of Metformin Hydrochloride and Empagliflozin, 1 ml of 2N Hydrochloric acid was added and the samples were left undisturbed for 30 minutes on a bench top and the solutions were neutralized by adding 1ml of 2N NaoH. The resultant solutions were diluted to obtain 500µg/ml and 12.5µg/ml of Metformin Hydrochloride and Empagliflozin and 10 µl solution were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies

To 1ml of sample stock solution of Metformin Hydrochloride and Empagliflozin, 1 ml of 2N Sodium Hydroxide was adsded and the samples were left undisturbed for 30 minutes on a bench top and

the solutions were neutralized by adding 1ml of 2NH.CL. The resultant solutions were diluted to obtain 500µg/ml and 12.5µg/ml of Metformin Hydrochloride and Empagliflozin and 10 µl solution were injected into the system and the chromatograms were recorded to assess the stability of sample.

Photolytic Degradation Studies:
1 ml of sample stock solution of Metformin Hydrochloride and Empagliflozin was subjected to UV Light upto 200 Watt hours/m2 upto 30 minutes. For HPLC study, the resultant solution was diluted to obtain 500µg/ml and 12.5µg/ml of Metformin Hydrochloride and Empagliflozin and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:
1 ml of sample stock solution of Metformin Hydrochloride and Empagliflozin was treated with 1 ml of HPLC grade water and the samples were left undisturbed for 30 minutes on a bench top and then diluted to obtain 500µg/ml and 12.5µg/ml of Metformin Hydrochloride and Empagliflozin respectively and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

RESULTS AND DISCUSSIONS

Development and optimization of HPLC method
The present work was focused to develop stability indicating RP-HPLC method for the simultaneous estimation of Metformin Hydrochloride and Empagliflozin in synthetic mixture. The solubility of the active pharmaceutical ingredients were checked in different solvents like methanol, water, Acetonitrile and in different ratios but finally the standards were soluble in water: acetonitrile (50:50) so it was chosen as a diluent. The different mobile phases like Acetonitrile and potassium dihydrogen phosphate buffer and water:metanol were used in compositions with a flow rate of 1ml/min but the peak resolution, retention time and tailing factor were not satisfactory so at last 0.1% orthophosphoric acid and Acetonitrile was selected as a mobile phase at flow rate of 0.8ml/min. Initially "Hypersil BDS" (250mm x 4.6mm x 5µ) columns with different temperatures like 30, 35, 40, 45°C were used but the retention time, run time and peak resolution were not exact and the problem was get rid by using "kromosil18"(250mm x 4.6mm x 5µ) kept at 30°c with a run time of 6 minutes. Finally the method was optimized by altering the mobile phase composition / ratio and the optimized wavelength of two drugs Metformin Hydrochloride and Empagliflozin was found to be at 233nm.

Forced degradation studies:
The stability studies were conducted by exposing the dosage forms to different stress conditions like acid, base, peroxide, light and water. It was found that the dosage forms were slightly degraded in acid, base and peroxide but stable in photolytic and hydrolytic conditions.

System suitability parameters
The system suitability tests were conducted before performing the validation and the parameters were within the acceptance criteria like retention times were 2.270min and 3.413min for Metformin Hydrochloride And Empagliflozin, plate count was >2000, peak tailing was <2 and the %RSD of peak areas of six injections were ≤ 2% (Table 1). Hence the proposed method was successfully applied to routine analysis without any problems.

Linearity range
The linearity range was in the interval of Metformin Hydrochloride, (125-750µg/ml) and Empagliflozin (3.125-18.75µg/ml) respectively. These `were represented by a linear regression equation as follows: y (Metformin Hydrochloride) = 26850x+439840(r2=0.999) and y (Empagliflozin) = 47664x + 9394.7. Regression line was established by least squares method and correlation coefficient (r2) for Metformin Hydrochlorideand Empagliflozin were found to be greater than 0.999. Hence the curves established were linear. (Table 2).

Precision
Six replicates injections at the same concentration were analyzed on same day and two different analysts for verifying the variation in the precision and the % RSD for Metformin Hydrochloride and Empagliflozin were within acceptable limit of ≤2. Hence the method is reproducible on different days with different analysts. This indicates that the method is precise (Table 3).

Accuracy
The percentage recoveries for Metformin Hydrochloride and Empagliflozin were shown in (Table4,5). The results of the recovery studies undoubtedly demonstrate accuracy of the proposed method.

Limit of detection (LOD) and limit of quantitation (LOQ)
The determined values of LOD and LOQ were calculated by using slope and Y-intercept. The LOD

and LOQ values for Metformin Hydrochloride and Empagliflozin were found to be 0.48, 1.49 µg/ml and 0.016,0.048µg/ml respectively (Table 6).

Robustness

Robustness of the proposed method demonstrated a non-significant alteration through analysis of thesample and standard Metformin Hydrochloride and Empagliflozin solution (Table 7). After this the results obtained were compared with that of optimized method. It was confirmed that by the deliberate changes in the parameters there were no significant changes in standard deviation, relative standard deviation, theoretical plates, retention time and USP tailing factor.

Assay

The Content of and Metformin Hydrochloride and Empagliflozin in the pharmaceutical dosage forms was found by using the developed method. The percentage purity of and Empagliflozin were found to be 100.31 and 99.0% and %RSD values for Metformin Hydrochloride,and Empagliflozin were within limit of ≤2.

Forced degradation studies

The forced degradation studies were conducted and all the parameters for Metformin Hydrochloride and Empagliflozin were within the limits. Metformin Hydrochloride and Empagliflozin have shown significant sensitivity towards the treatment of HCl, NaOH and peroxide solutions. The drugs gradually undergone degradation with time and prominent degradation was observed. Metformin Hydrochloride and Empagliflozin were stable under forced photolytic and neutral degradations. From the degradation studies, Peak purity test results derived from PDA detector, confirmed that the Metformin Hydrochloride and Empagliflozin peaks were homogeneous and pure in all the analyzed stress samples. The mass balance of stressed samples was close to 97.61%.

CONCLUSION

A new, simple, rapid and precise stability indicating high performance liquid chromatographic method was developed for the simultaneous estimation of Metformin Hydrochloride and Empagliflozin in synthetic mixture. Hence this method can be applied for the estimation of Metformin Hydrochloride and Empagliflozin in drug testing laboratories and pharmaceutical industries.

ACKNOWLEDGEMENTS

The authors were thankful to Hetero Drugs Limited, Hyderabad for providing Metformin Hydrochloride and Empagliflozin reference standards as a gift sample and Spectrum Pharma Research Solutions for providing the facilities to carry out the research work.

DISCLOSURE OF INTEREST

The authors declare that they have no conflicts of interest concerning this article.

Table 1: System suitability parameters for Metformin Hydrochloride and Empagliflozin

S no	Metformin Hydrochloride			Empagliflozin		
	RT(min)	TP	Tailing	RT(min)	TP	Tailing
1	2.270	4643	1.14	3.407	11363	1.40
2	2.273	4737	1.13	3.410	11424	1.42
3	2.277	5172	1.12	3.413	10913	1.41
4	2.280	4602	1.16	3.417	11237	1.38
5	2.282	4589	1.14	3.435	11233	1.39
6	2.285	4797	1.14	3.449	11544	1.38

Table 2: Linearity table for Metformin Hydrochloride and Empagliflozin.

Metformin Hydrochloride		Empagliflozin	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
125	3882347	3.125	162811
250	7052509	6.25	304378
375	10384230	9.375	447658
500	14218085	12.5	606118
625	17107156	15.625	766196
750	20526823	18.75	896555

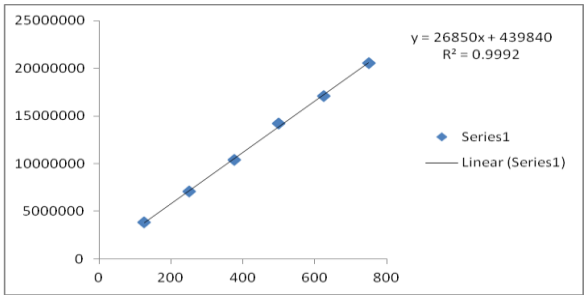

Fig 1Calibration curve of Mefformin Hydrochloride

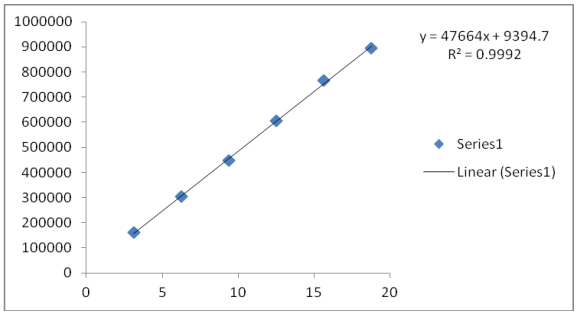

Fig 2 Calibration curve of Empagliflozin

Table 3 Determination of repeatability and intermediate precision

Drug Name	Repeatability			Intermediate		
	Peak Area	Std Dev	%RSD	Peak Area	Std Dev	%RSD
Metformin Hydrochloride	14578085	121476	0.8	14715250	181135	1.2
Empagliflozin	608194	5734	0.9	594038	7680.9	1.3

Table 4 Determination of Accuracy of Metformin Hydrochloride

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	250	247.68	99.07	99.6
	250	250.96	100.38	
	250	248.41	99.36	
100%	500	492.84	98.57	99.41
	500	501.67	100.34	
	500	496.62	99.33	
150%	750	749.01	99.87	99.91
	750	751.14	100.15	
	750	747.74	99.70	

Table 5 Determination of Accuracy of Empagliflozin

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	6.25	6.23	99.72	99.76
	6.25	6.21	99.4	
	6.25	6.26	100.1	
100%	12.5	12.34	98..7	99.16
	12.5	12.39	99.13	
	12.5	12.45	99.6	
150%	18.75	18.68	99.67	99.48
	18.75	18.60	99.24	
	18.75	18.66	99.53	

Table 6 Sensitivity table of Metformin Hydrochloride and Empagliflozin

Molecule	LOD(µg/ml)	LOQ(µg/ml)
Metformin Hydrochloride	0.49 µg/ml	1.48µg/ml
Empagliflozin	0.016 µg/ml	0.048 µg/ml

Table 7 Robustness data for Metformin Hydrochloride and Empagliflozin.

S.no	Condition	%RSD of Metformin	%RSD of Empagliflozin
1	Flow rate (-) 0.6ml/min	0.9	0.6
2	Flow rate (+) 1.0ml/min		0.4
3	Mobile phase (-) 40B:60A	0.8	0.2
4	Mobile phase (+) 50B:50A	0.5	0.6
5	Temperature (-) 25°C	0.7	0.2
6	Temperature (+) 35°C	0.3	0.2

Table 8 Degradation data for Metformin Hydrochloride and Empagliflozin.

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	3.69	1.122	1.384
2	Alkali	2.84	0.149	1.600
3	Oxidation	2.09	2.555	4.766
5	UV	1.03	6.244	6.932
6	Water	0.83	6.629	8.341

Table 9 Degradation Data of Empagliflozin

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	3.13	0.063	0.793
2	Alkali	2.85	1.050	1.132
3	Oxidation	2.01	0.692	0.777
5	UV	0.67	0.630	0.831
6	Water	0.27	0.578	0.806

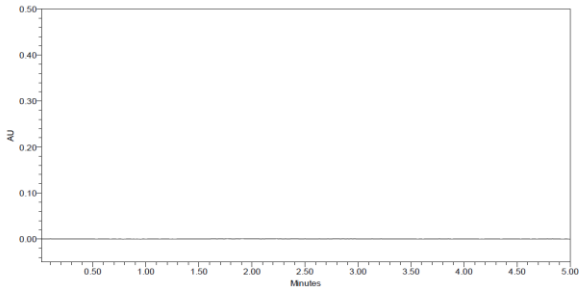

Fig.3 Blank chromatogram of Metformin HCl& Empagliflozin

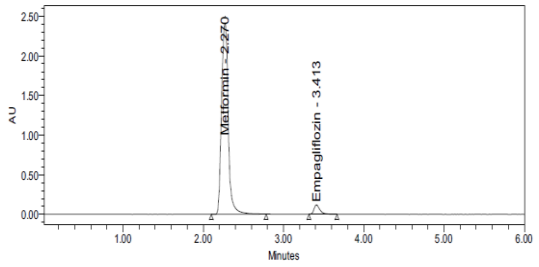

Fig.4 Standard chromatogram of Metformin HCl & Empagliflozin

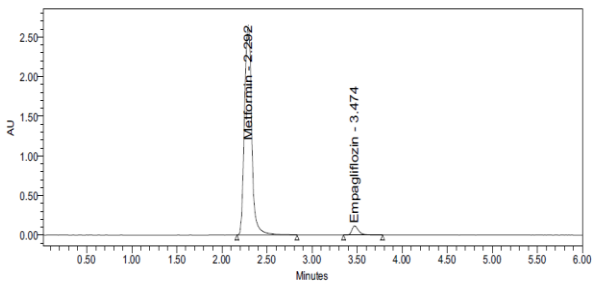

Fig.5 Linearity chromatogram of Metformin HCl & Empagliflozin

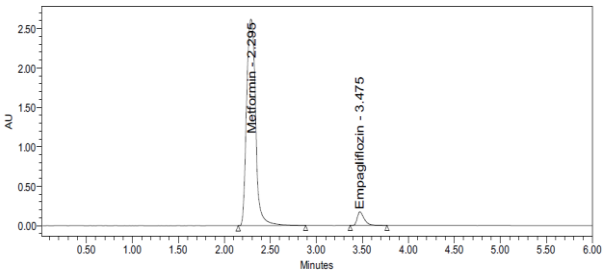

Fig.6 Accuracy chromatogram of Metformin HCl & Empagliflozin

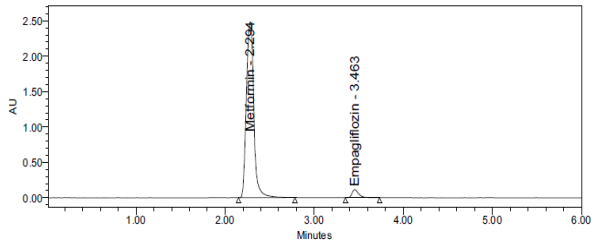

Fig.7 sample chromatogram of Metformin HCl & Empagliflozin

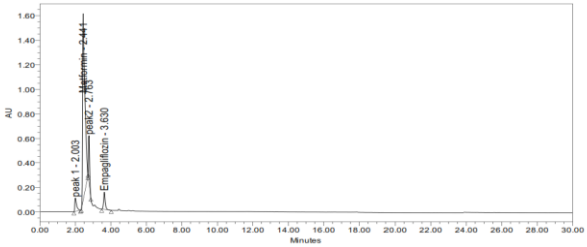

Fig.8 Acid degradation chromatogram of HCl & Empagliflozin

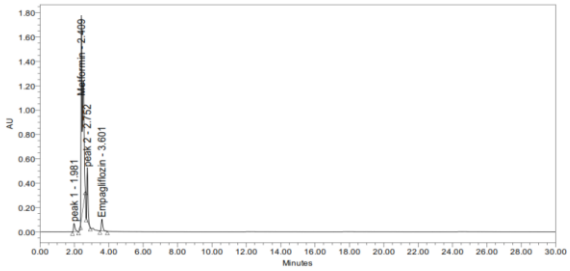

Fig.9 Alkali degradation chromatogram of Metformin HCl & Empagliflozin

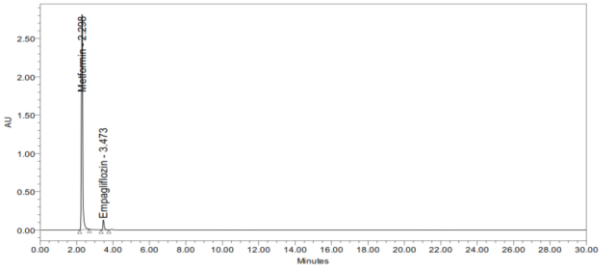

Fig.10 Peroxide degradation chromatogram of Metformin HCl & Empagliflozin

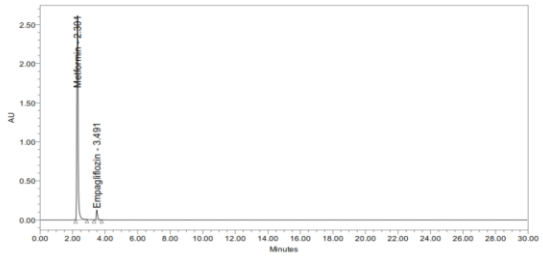

Fig.11 UV degradation chromatogram of Metformin HCl & Empagliflozin

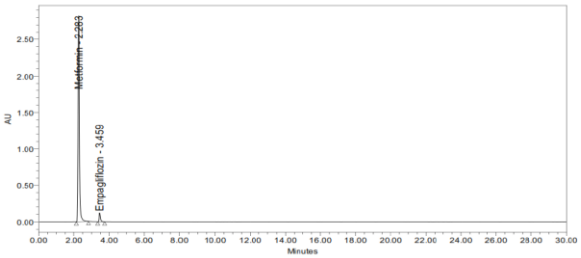

Fig.12 Water degradation chromatogram of Metformin HCl & Empagliflozin

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