EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 2
404-410
Year: 2018

DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF METFORMIN AND EMPAGLIFLOZIN IN DRUG PRODUCT BY RP-HPLC

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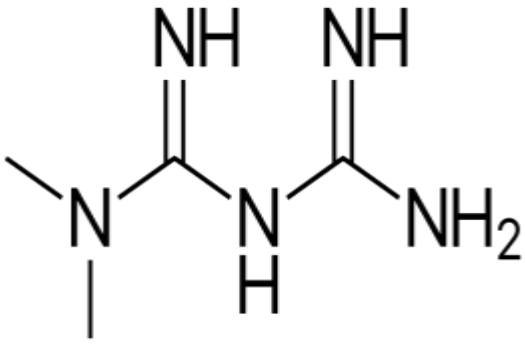
Article Received on 26/11/2017 **Article Revised on 16/12/2017** **Article Accepted on 06/01/2018**

ABSTRACT
New Analytical method was developed for the estimation of Metformin and Empagliflozin in drug product by liquid chromatography. The chromatographic separation was achieved on C18 column (Inertsil ODS 3V 250*4.6mm) at ambient temperature. The separation achieved employing a mobile phase consists of 0.1%v/v Trifluoro acetic acid in water: Acetonitrile: Methanol (200:200:600). The flow rate was 0.8ml/ minute and ultra violet detector at 265nm. The average retention time for Metformin and Empagliflozin found to be 2.626 min and 3.848 min. the proposed method was validated for selectivity, precision, linearity and accuracy. All validation parameters were within the acceptable range. The assay methods were found to be linear from 1700.0 – 5100.0µg/ml for Metformin and 25.0 -75.0µg/ml of Empagliflozin.

KEYWORDS: Metformin and Empagliflozin, Isocratic, HPLC, Eclipse XDB-Phenyl 250*4.6, 5m, Trifluoro acetic acid, Acetonitrile, Methanol and validation.

METFORMIN
Metformin marketed under the trade name **Glucophage** among others, is the first-line medication for the treatment of type 2 diabetes, particularly in people who are overweight.[6] It is also used in the treatment of polycystic ovary syndrome.[4] Limited evidence suggests metformin may prevent the cardiovascular disease and cancer complications of diabetes. It is not associated with weight gain.[8] It is taken by mouth.

Metformin is chemically designated as *N*,*N*-Dimethylimidodicarbonimidic diamide. Its molecular formula is $C_4H_{11}N_5$ and its molecular weight is 129.16364 g/mol.

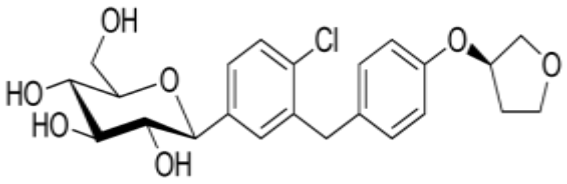


Structure of Metformin

EMPAGLIFLOZIN
Empagliflozin is an inhibitor of the sodium glucose co-transporter-2 (SGLT-2) and causes sugar in the blood to be excreted by the kidneys and eliminated in urine.

Empaglifozin in people with type 2 diabetes reduces the risk of death from cardiovascular disease in those with a previous history of cardiovascular disease.

Empagliflozin is chemically designated as (2*S*,3*R*,4*R*,5*S*,6*R*)-2-[4-Chloro-3-[[4-[(3*S*)-oxolan-3-yl]oxyphenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol. Its molecular formula is $C_{23}H_{27}ClO_7$ and its molecular weight is 450.91 g/mol.



Structure of Empagliflozin

EXPERIMENTAL
Equipments: The chromatographic technique performed on a waters 2695 with 2487 detector and Empower2 software, reversed phase C18 column (Inertsil ODS 3V 250*4.6,5µ) as stationary phase, Ultrasonic cleaner,

Scaletech analytical balance, Vaccum micro filtration unit with 0.45µ membrane filter was used in the study.

Materials: Pharmaceutically pure sample of Metformin/Empagliflozin were obtained as gift samples from Fortune pharma training institute, sri sai nagar colony, KPHB, Hyderabad, India.

HPLC-grade Methanol was from qualigens reagents pvt ltd. Trifluoro acetic acid (AR grade) was from sd fine chem.

Chromatographic conditions: The sample separation was achieved on a (5 µ, 250 cm X 4.6 mm i.d.) Inertsil ODS 3V C18 column, aided by mobile phase mixture of 0.1%v/v Trifluoro acetic acid in water: Acetonitrile: Methanol (20:20:60). The flow rate was 0.8 ml/ minute and ultra violet detector at 265nm that was filtered and degassed prior to use, Injection volume is 10 µl and ambient temperatures.

Preparation of mobile phase

Buffer Preparation: Taken accurately 1ml of Trifluoro acetic acid in 1000mL of water.

Mobile phase: Then added 20 volumes of buffer, 20 volumes of Acetonitrile and 60 volumes of Methanol mixed well and sonicated for 5 min.

Diluents: Water: Methanol 50:50 v\v

Preparation of standard stock solution: A 150 mg of pure Metformin and 25 mg of Empagliflozin were weighed and transferred to 25 ml of volumetric flask and dissolved in diluent. The flask was shaken and volume was made up to mark with diluent to give a primary stock solution. From the above solution 0.4ml of solution is pipette out into a 10 ml volumetric flask and volume was made up to mark with water to give a solution containing 3400µg/ml of Metformin and 50 µg/ml Empagliflozin.

Preparation of sample solution: Accurately weighed twenty tablets were ground to obtain fine powder equivalent to 150mg of Metformin and 25mg of Empagliflozin sample were weighed and transferred to 25 ml of volumetric flask and dissolved in diluent. The flask was shaken and volume was made up to mark with diluent to give a primary stock solution. From the above solution 0.2 ml of solution is pipette out into a 10 ml volumetric flask and volume was made up to mark with diluents to give a solution containing 3400 µg/ml of Metformin and 50 µg/ml Empagliflozin.

RESULTS AND DISCUSSIONS

Determination Of Working Wavelength (λ max): 10 mg of the Metformin and Empagliflozin standard drug is taken in a 10 ml volumetric flask and dissolved in Diluent and volume made up to the mark, from this solution 0.1ml is pipette into 10 ml volumetric flask and made upto the mark with the Water to give a concentration of 10 µg/ml. The above prepared solution is scanned in uv between 200-400 nm using Water as blank. The λmax was found to be 265nm After several initial trails with mixtures of methanol, water, ACN and buffer in various combinations and proportions, a trail with a mobile phase mixture of 0.1%v/v TFA in water: Acetonitrile: Methanol (20:20:60). The flow rate was 0.8 ml/ minute brought sharp peaks. The chromatogram was shown in Figure-1**.**

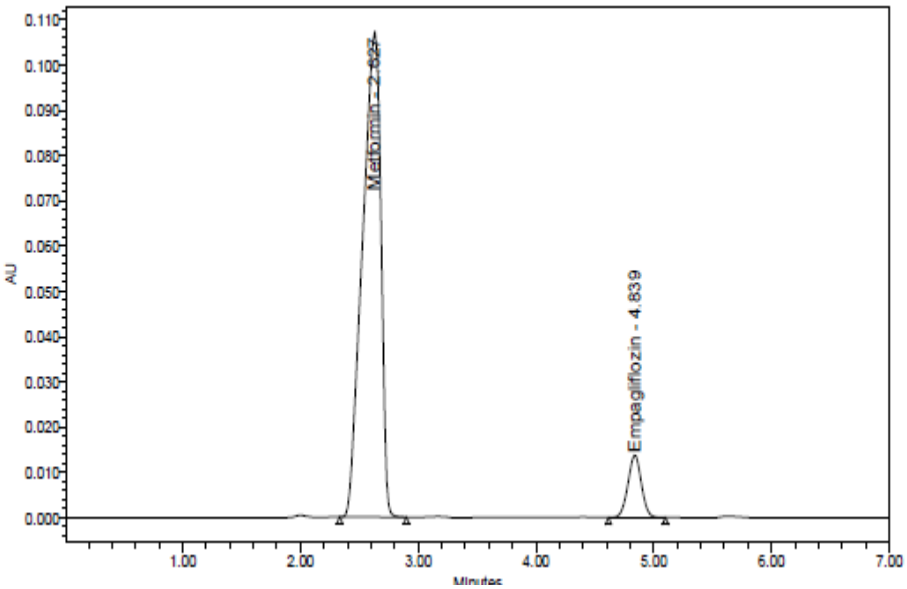


Figure 1: Chromatogram of Metformin/Empagliflozin.

METHOD VALIDATION

Linearity

Linearity was studied by analyzing five standard solutions covering the range of 1700.0 -5100.0µg/ml for Metformin and 25.0 -75.0µg/ml Empagliflozin. From the primary stock solution 1.0ml, 1.5ml, 2.0ml, 2.5ml, 3.0 ml of aliquots are pipette into 10 ml volumetric flasks and made up to the mark with the water to give a concentrations of 1700.0 µg /mL, 2550.0µg/mL, 3400.0µg/mL, 4250.0µg/mL and 5100.0 µg/mL of Metformin and 25.0g/mL, 37.0µg/mL, 50.0µg/mL, 62.0µg/mL and 75.0 µg/mL Empagliflozin.

Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions and the obtained data were subjected to regression analysis using the least squares method.

Table No. 1: Linearity data of Metformin.

Level	Concentration (mg/mL)	Peak area
50%	1.700	526587
75%	2.550	836711
100%	3.400	1143186
125%	4.250	1414751
150%	5.100	1675434

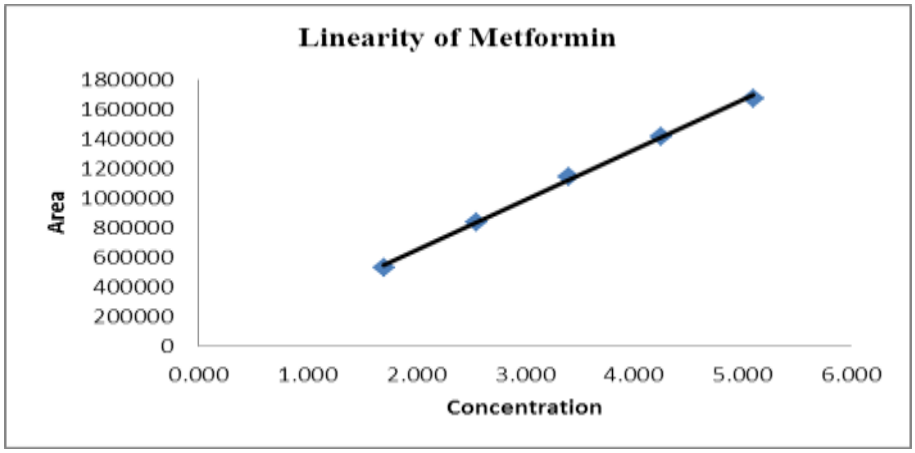


Figure No. 2: Linearity (calibration) curve of Metformin.

Table No. 2: Linearity data of Empagliflozin.

Level	Concentration (mg/mL)	Peak area
50%	0.025	48670
75%	0.038	78665
100%	0.050	108201
125%	0.063	134439
150%	0.075	160729

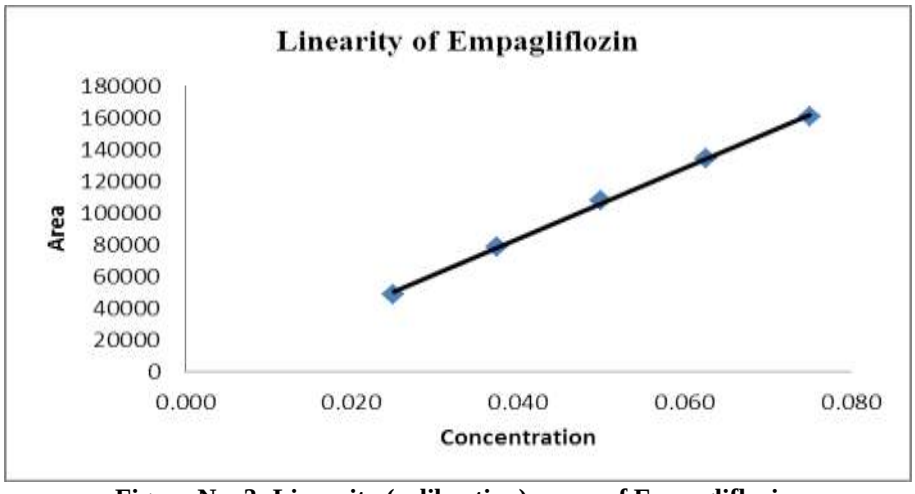


Figure No. 3: Linearity (calibration) curve of Empagliflozin.

RESULT

A linear relationship between peak areas versus concentrations was observed for Metformin and Empagliflozin in the range of 50% to 150% of nominal concentration. Correlation coefficient was 0.9992 and 0.9994 for both Metformin and Empagliflozin which prove that the method is linear in the range of 50% to 150%.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (1) and (2), respectively.

LOD = 3.3 σ /S ……. (1)

LOQ =10 σ /S …….. (2)

Where,

σ = the standard deviation of the response (STEYX)

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Table no. 3: LOD and LOQ values Calculated from calibration curve.

	Metformin mg	Empagliflozin mg
LOD	0.205	0.003
LOQ	0.622	0.008

Method precision (repeatability)

The precision of the method was checked by repeated preparation(n=6) of 3400.0µg/ml of Metformin and 50.0µg/ml Empagliflozin without changing the parameter of the proposed chromatographic method. And measure the peak areas and retention times.

Table 4: Summary of peak areas for method precision of Metformin.

Sample No	Retention time	Peak area	% Assay
1	2.627	1150968	99.2
2	2.627	1154050	99.5
3	2.627	1151170	98.8
4	2.626	1142037	98.2
5	2.625	1132001	99.7
6	2.627	1151694	99.1
Mean	2.627	1146987	99.1
%RSD	**0.03**	**0.73**	**0.53**

Table 5: Summary of peak areas for method precision of Empagliflozin.

Sample No	Retention time	Peak area	% Assay
1	4.849	109238	100.1
2	4.85	109239	99.7
3	4.85	109184	99.4
4	4.849	108222	98.8
5	4.848	107248	99.7
6	4.847	109089	99.6
Mean	4.849	108703	99.5
%RSD	0.02	0.75	0.44

RESULT

Results of variability were summarized in the above table. Percentage relative standard deviation (%RSD) was found to be less than 2.0% which proves that method is precise.

Accuracy (recovery study)

The accuracy of the method was determined by calculating the recoveries of Metformin and Empagliflozin by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of Metformin and Empagliflozin. The percentage recovery results obtained are listed in Table 6 &7.

Table No. 6: Recovery data of Metformin.

LEVEL	S.NO	%Recovery of Metformin	Average
50	1	99.9	99.4%
	2	99.4	
	3	98.7	
100	1	99.2	99.2%
	2	99.5	
	3	98.8	
150	1	98.9	99.3%
	2	99.3	
	3	99.8	

Table No. 7: Recovery data of Empagliflozin.

LEVEL	S.NO	%Recovery of Empagliflozin	Average
50	1	99.1	99.3%
	2	99.5	
	3	99.4	
100	1	100.1	99.7%
	2	99.7	
	3	99.4	
150	1	99.5	99.5%
	2	99.3	
	3	99.8	

RESULT

Results of accuracy study are presented in the above table. All the results indicate that the method is highly accurate.

Robustness: Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2nm and flow rate was varied ±0.2 ml/min. The results were shown in (Table no. 8&9).

Table No. 8: Results of Metformin.

parameter	Rt of Metformin	Theoretical plates	Asymmetry
Decreased flow rate (0.7ml/min)	2.996	2522	0.95
Increased flow rate (0.9ml/min)	2.340	2612	0.99
Wave Length 263nm	2.627	2645	0.96
267nm	2.625	2656	0.96

Table No. 9: Results of Empagliflozin.

parameter	Rt of Empagliflozin	Theoretical plates	Asymmetry
Decreased flow rate (0.7ml/min)	5.530	10222	1.02
Increased flow rate (0.9ml/min)	4.312	7832	0.99
Wave Length 263nm	4.839	8553	1.00
267nm	4.840	8525	0.99

RESULT

The results of Robustness of the present method had shown that changes are not significant we can say that the method is Robust.

Ruggedness: The ruggedness of the method was studied by analyzing the sample and standard preparations by two analysts. The results were shown in Table no.10&11.

Table No. 10: Results of Metformin.

		%Assay	%RSD
Analyst-1	**METFORMIN**	99.2	0.21%
Analyst-2		99.5	

Table No. 11: Results of Empagliflozin.

		%Assay	%RSD
Analyst-1	**EMPAGLIFLOZIN**	100.1	0.28%
Analyst-2		99.7	

RESULT

The %RSD assay values between two analysts was calculated, this indicates the method was rugged.

Table No. 12: Summary of Metformin.

S.No	Parameter	Result	Acceptance criteria
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 2700 0.99 2.626 0.08	 Not less than 2000 Not more than 2.0 Not more than 2.0
2	Specificity	Specific	Specific
3	Method precision(%RSD)	0.73	Not more than 2.0%
4	Linearity Range(mcg/ml) Correlation coefficient(r2)	1700.0- 5100.0 0.9992	 Not less than 0.990
5	Accuracy (Mean % recovery) 50% 100% 150%	 99.4 99.2 99.3	 97 - 103%
6	Robustness	All the system suitability parameters are within the limits.	

*RSD = Relative standard deviation.

Table No. 13: Summary of Empagliflozin.

S.No	Parameter	Result	Acceptance criteria
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 8393 0.99 4.845 0.15	 Not less than 2000 Not more than 2.0 Not more than 2.0
2	Specificity	Specific	Specific
3	Method precision(%RSD)	0.75	Not more than 2.0%
4	Linearity Range(mcg/ml) Correlation coefficient(r^2)	25.0- 75.0 0.9994	 Not less than 0.990
5	Accuracy (Mean % recovery) 50% 100% 150%	 99.3 99.7 99.5	 97 - 103%
6	Robustness	All the system suitability parameters are within the limits.	

CONCLUSION

From the above experimental results it was concluded that, this newly developed method for the simultaneous estimation of METFORMIN and EMPAGLIFLOZIN was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories.

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