WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 7, 1025-1037. Research Article ISSN 2277– 7105

VALIDATED STABILITY INDICATING HPLC METHOD FOR DETERMINATION OF PROCESS RELATED IMPURITIES IN EMPAGLIFLOZIN DRUG SUBSTANCES

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Article Received on
15 May 2017,
Revised on 05 June 2017,
Accepted on 25 June 2017
DOI: 10.20959/wjpr20177-8741

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ABSTRACT

Four process related impurities in Empagliflozin drug substance was detected and quantified using reverse phase high performance liquid chromatographic method. The chromatographic separation was achieved on Inertsil C_8 (250mm×4.6 mm, 5µm) column under gradient elucidation using 0.1% orthophosphoric acid and acetonitrile as mobile phase with UV detection at 230nm and a flow rate of 1.2 ml/min. The column temperature was maintained at 55°C throughout the analysis. Forced degradation study was carried out under acidic, alkaline, oxidative, photolytic, thermal and humidity conditions. The developed method was validated with respect to sensitivity, linearity, precision, accuracy and robustness. It can be implemented for routine quality control analysis and stability testing of Empagliflozin.

KEYWORDS: Empagliflozin, HPLC, Degradation, Process impurity, Validation, ICH guidelines.

INTRODUCTION

Empagliflozin is an orally-active inhibitor of the sodium glucose co-transporter (SGLT2). The empirical formula and molecular weight of empagliflozin are $C_{23}H_{27}ClO_7$ and 450.91 respectively.[1,2] The drug is structurally related to dapagliflozin, posses a tetrahydrofuran moiety in place of ethyl group of ethyl phenyl ether.[3] Empagliflozin is classified as the antidiabetic class drug and used mainly for the treatment of type 2 diabetes.[4] The drug also

controls blood sugar levels by causing the kidneys to get rid of more glucose in the urine of the body and does not help the patients who were insulin-dependent or type1diabetes. Empagliflozin is available in 10 and 25 mg tablet dosage form either individually or combination with other drugs, some of the brands includes Jardiance, Glyxambi, Synjardy, and Synjardy XR.[5-8] However, till date, very few analytical methods were reported for the determination of the drug individually or in combination with other drugs by HPLC[9-16], LC-MS/MS[17], UPLC[18], UV[19] technique. The present work describes the development and validation of a stability-indicating reverse phase high-performance liquid chromatography method for the estimation of process related impurities of empagliflozin, namely impurity-A, impurity-B, impurity-C and impurity-D [Figure 1]. The developed method was validated with respect to specificity, limit of detection and quantification, linearity, precision, accuracy and robustness in accordance with established USP[20] and ICH[21] guidelines. Forced degradation studies were performed to show the stability indicating nature of the method.[22,23]

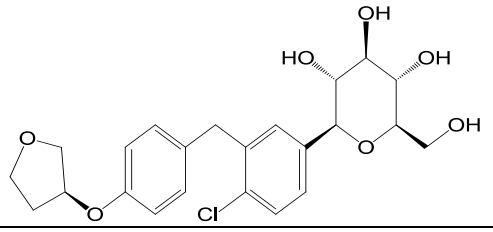
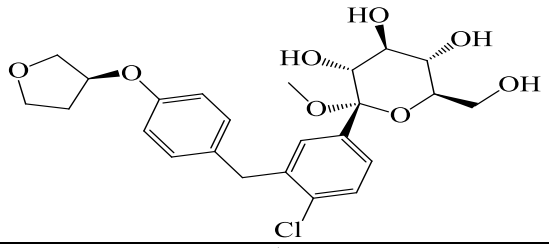
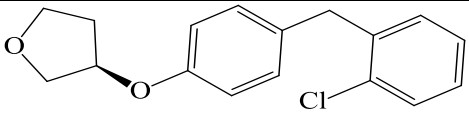
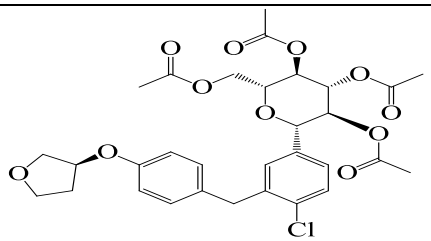
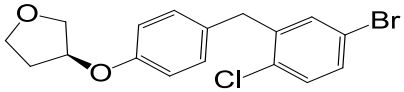
Empagliflozin [(2S,3R,4R,5S,6R)-2-(4-Chloro-3-{4-[(S)-(tetrahydro-furan-3-yl)oxy]-benzyl} phenyl)-6- hydroxymethyl-tetrahydropyran-3,4,5-triol	
Impurity-A (2S,3R,4S,5S,6R)-2-(4-Chloro-3-(4-(((S)-Tetrahydrofuran-3yl)oxy)Benzyl)Phenyl)-6-(Hydroxymethyl)-2-Methoxytetrahydro-2H-Pyran-3,4,5-Triol	
Impurity-B (S)-3-(4-(2Chlorobenzyl) Phenoxy) Tetrahydrofuran	
Impurity-C (2R,3R,4R,5S,6S)-2-(Acetoxymethyl)-6-(4-Chloro-3-(4-(((S)-Tetrahydrofuran3 yl) Oxy)Benzyl)Phenyl)Tetrahydro-2H-Pyran-3,4,5-Triyltriacetate	
Impurity-D (S)-3-(4-(5-Bromo-2-Chlorobenzyl) Phenoxy)-Tetrahydrofuran	

Figure 1: Chemical structure of Empagliflozin and process impurities

MATERIALS AND METHOD

Chemical and Reagents

Empagliflozin working standard, sample and its related process impurity-A (Purity>83.0%) impurity-B, impurity-C and impurity-D (Purity>97.0%) were procured in-house Macleods pharmaceutical LTD, India. Water (HPLC grade), orthophosphoric acid, methanol, acetonitrile, hydrochloric acid and hydrogen peroxide were purchased from Merck, sodium hydroxide from Fisher scientific. All the reagents and chemicals were used as such as received without further any purification.

Instrumentation

The HPLC system consisted of Shimadzu model LC 2010 $C_{HT,}$ UV and PDA detector. The output signals were monitored and integrated using chromeleon software. Sartorius analytical balance and Pico^{+} pH meter were used.

Chromatographic Conditions

The analysis was carried out on Inertsil C_8 column (250mm×4.6mm, 5µm) thermostated at 55°C. The mobile phase A was 0.1% orthophosphoric acid and mobile phase B acetonitrile. Mobile phase filter through a 0.45µm membrane filter and degassed. The flow rate of mobile phase was 1.2 ml/min. The HPLC gradient program was time (min) /%B (v/v): 0/17, 20/20, 45/48, 85/48, 86/17 and 100/17. The injection volume was 10µl. The chromatograms were recorded at 230 nm and the composition of water: acetonitrile in the ratio (30:70 v/v) used as diluent.

Preparation of solutions

Empagliflozin working standard and samples were prepared at 1µg/ml and 1000µg/ml concentrations using diluent and used as same respectively. Solution of impurity-A, impurity-B impurity-C and impurity-D (1.5µg/ml) were prepared by dissolving known amount of the compounds in diluents. These solutions were prepared freshly and diluted further quantitatively to study the validation attribute. The specification limit considered for validation studies was 0.15% for each process related impurities and 0.1% for unknown impurity.

Procedures for forced degradation study

To demonstrate stability indicating power of developed method for the determination of impurities in empagliflozin, liquid state forced degradation (acidic, alkaline and oxidative)

was carried out by treating the 1000 µg/ml solution of empagliflozin with 5N HCl and 1N NaOH at 80°C for five hours. For oxidative degradation the sample exposed with 10% H_2O_2 at 40°C for five hours. Solid state forced degradation (thermal, photolytic and humidity) of empagliflozin was conducted by exposing sample to (1) thermal temperature at 80°C for 24 hours in hot air oven (2) photo stability chamber (1.2 million lux hours and ultraviolet energy 200-W h/m^2) (3) humidity chamber at 90% RH/50°C for 24 hours. All these solutions were analyzed by the developed method.

Method validation

Validation of the chromatographic method was carried out with reference to specificity, linearity, limit of Detection, limit of Quantitation, precision, accuracy, solution stability and robustness.

RESULTS AND DISCUSSION

Method Development

The method was developed by considering the main parameters like selection of wavelength, HPLC column, mobile phase, column oven temperature, flow rate, injection volume and diluent. The solubility was checked for empagliflozin and all process impurities in water, methanol, acetonitrile and the combination of water: methanol, water: acetonitrile, acidic and basic buffer in different ratios. All compounds had a good solubility in water and acetonitrile in ratio (30:70 v/v) than others diluent. Hence, this composition of water and acetonitrile was selected as diluent.

The selection of wavelength was carried out by prepared a empagliflozin standard solution and all process impurities in diluent at the specification limit. The prepared solution injected into the HPLC system with PDA detector and spectra was recorded. The chromophoric structure of all the compounds almost similar and all compounds were found to have optimum UV absorption at 230 nm. Therefore the 230 nm wavelength was chosen for the study and quantification of Empagliflozin and it’s impurities.

The selection of HPLC column carried out by conducted trials on various packaging material of ODS, C8 and C18 in different length, internal diameter, particle size and pore size manufactured by different industries. After performing trials on column the decisively separation was accomplish on GL sciences manufactured HPLC column i.e. Inertsil C8 (250 x 4.6mm) 5µm.

The selection of mobile phase was carried out on isocratic condition by prepared water as mobile phase A and acetonitrile as mobile phase B in the ratio 50:50 v/v and the empagliflozin standard solution and all impurity solution was injected. The result was observed that the all the impurities peak was not eluted within 100 minutes. The trials were continued by applied gradient condition with same mobile phase result was observed that the empagliflozin peak and impurity-A was co-eluted with noisy baseline. After performing many trials with experimental data the chromatographic separation was finalized by the following gradient program was fixed as (Time/% Mobile Phase B) (0.0/17), (20/20), (45/48), (85/48), (86/17) and (100/17) by using buffer (0.1% orthophosphoric acid) and acetonitrile were as Mobile Phase A and B. The result was satisfied that all compound are separated with each other and having the resolution >2.

The column temperature was selected by taking many trials with different column oven temperature (20°C to 60°C). The empagliflozin and impurities peak was well separated and the reproducible result were obtained at 55°C.

The flow rate of the mobile phase was optimized from 0.5-2.0 ml/min for separation of analyte peak from blank and impurities peaks. It was found from the experiments that 1.2 ml/min flow rate was ideal for the successful elution of the compound in a reasonable time.

The empagliflozin and all process impurities solution was injected from 5 µl to 50 µl injection volume into HPLC system. Based on the response and shape of the peak 10 µl injection volume was selected.

Result of forced degradation study

When empagliflozin was subjected to liquid state forced degradation (acidic, alkaline and peroxide), the drug molecule degrades upto 0.27% in acidic hydrolytic condition resulting unknown impurity along with high level impurities at RRT 1.25 (0.13%). No significant degradation was observed the drug molecule exposed under alkaline condition. While in the oxidative stress of the drug molecule, unknown impurity is formed and total degradation is 0.11 %. No significant degradation was observed drugs under solid state forced degradation (thermal, humidity and photo stability). The unknown degradant produced in forced degradation were separated well from empagliflozin and all process impurities. The empagliflozin and all process impurities peak were found to be spectrally pure and no co-eluation was observed due to blank and impurity peak.

Result of method validation studies

System suitability

The solution of empagliflozin spiked with impurities was analyzed during validation studies. From the data collected for RRT, Resolution between successive pairs and tailing factors of the compounds for all the validation experiments, it is found that the resolution value more than 2 and tailing factor not more than 2 to all compound. The % RSD of six replicate injections for standard solution at 1µg/ml is found with the acceptance criteria not more than 5% as per USP.

Specificity

To determine the specificity of the method Diluent (Blank), impurity-A, impurity-B, impurity-C and impurity-D, sample as such and spiked sample with impurities at the specified limit were initially injected under the proposed chromatographic condition to determine the individual retention time of these impurities with respect to the empagliflozin peak. Based on the result obtained there is no interference observed due to blank and empagliflozin at the retention time of any known impurity and unknown impurity. All the known impurities peaks are sharp well resolved from each other and have clear baseline with the retention time for empagliflozin, 11.51 min, for impurity-A, 15.03 min, for impurity-B 53.44 min, for impurity-C 57.85 min and for impurity-D 67.32 min. The empagliflozin peak was subjected to peak purity assessment test using photodiode array detector and it indicates that the peak is found spectrally pure and no co-elution was observed (Figure 2).

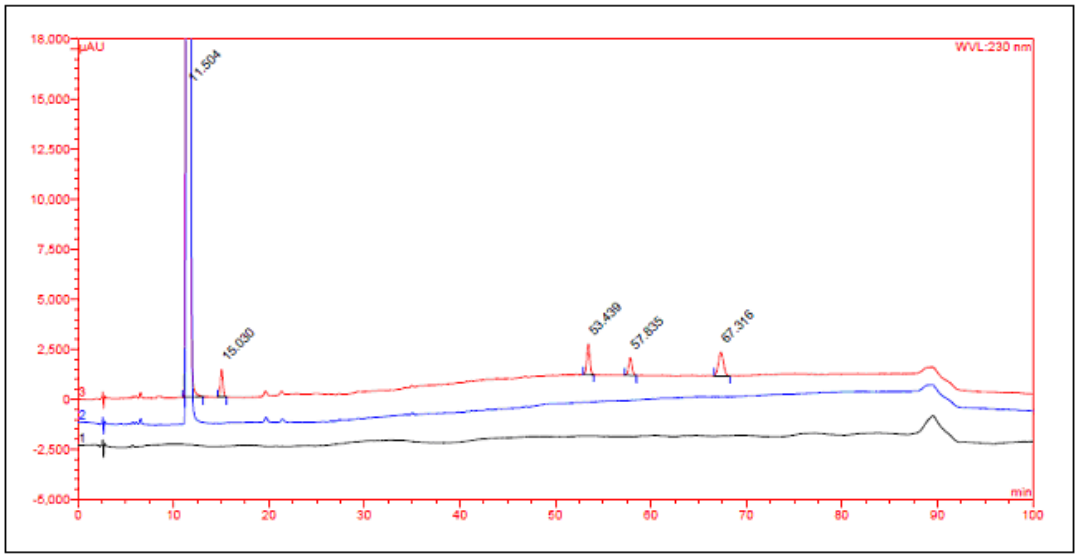


Figure 2: Overlay specificity chromatograms: (1) Blank, (2) Sample solution, (3) Spiked sample solution

Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ for empagliflozin and all four process impurities was estimated through signal-to- noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions having a known concentrations. LOD is defined as the lowest concentration that can be detected. LOQ is the lowest concentration that can be quantified with acceptable precision and accuracy. The low values of LOD and LOQ indicates adequate sensitivity of the method (Table 1). Precision study was also carried out at LOQ level by injecting six individual preparations and calculating the % RSD of the area. Overlay chromatogram of LOD and LOQ presented in figure 3.

Table 1: LOD and LOQ of Empagliflozin and impurities

Compound	LOD (%)	S/N ratio	LOQ (%)	S/N ratio	% RSD at LOQ level
Empagliflozin	0.010	3.52	0.03	12.54	1.09
Impurity-A	0.013	3.48	0.04	13.28	2.31
Impurity-B	0.011	3.64	0.03	11.98	1.98
Impurity-C	0.014	4.28	0.04	14.26	2.00
Impurity-D	0.007	4.54	0.02	12.32	5.09

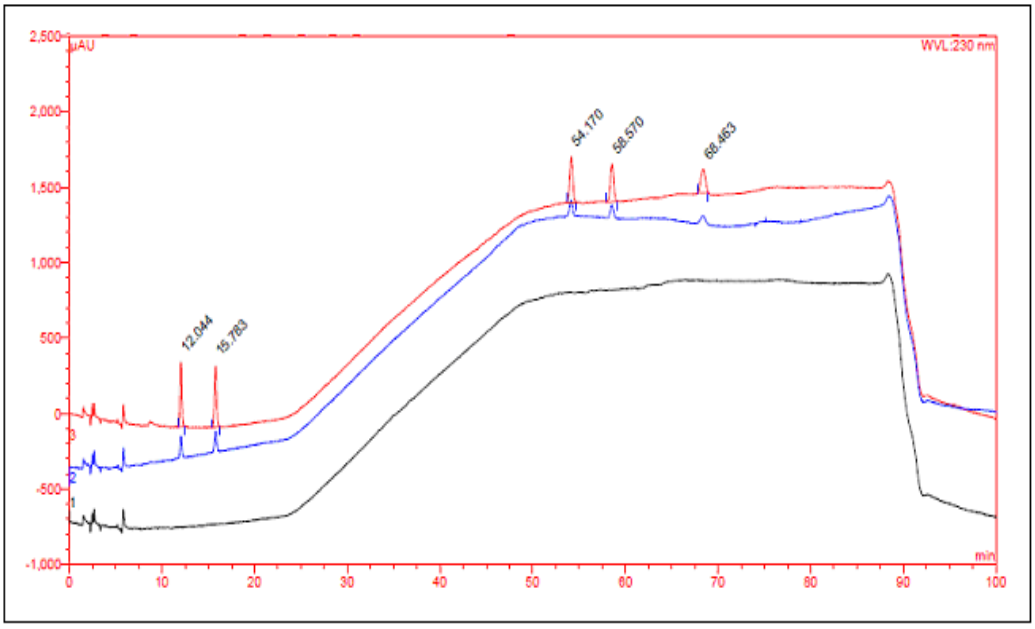


Figure 3: Overlay chromatograms: (1) Blank, (2) LOD solution, (3) LOQ solution

Linearity and Relative response factor

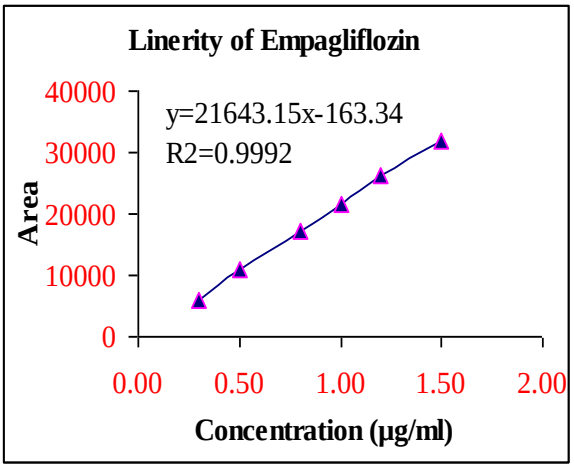
The linearity of empagliflozin and all four process impurities were evaluated by using six levels ranging from LOQ to 150% with respect to sample concentration at the specified limit. A calibration curve was established between the response and concentration of analyte. The

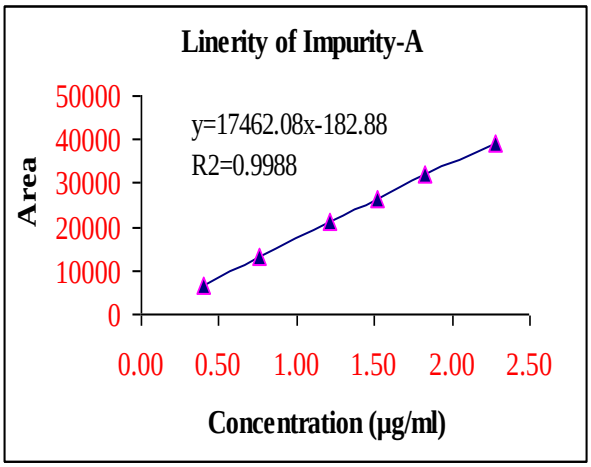
slope, intercept, correlation coefficient of the regression line and residual sum of square were calculated. A correlation was achieved more than 0.99 and the data presented in table 2. A figure 4 shows the linearity graph of empagliflozin and its related process impurities.

The Relative response factor (RRF) for all impurities with respect to empagliflozin were determined from slope values obtained from the linearity curve and result have been given in table 2.These RRF will be used while calculating the levels of these known impurities in the sample analysis and all other impurities will be calculated using the diluted standard solution of empagliflozin.

Table 2: Linearity of Empagliflozin and impurities

Empagliflozin		**Impurity-A**		**Impurity -B**		**Impurity-C**		**Impurity- D**	
Conc. (µg/ml)	Area	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
0.30	6050.584	0.40	6603.188	0.30	7042.203	0.41	6310.371	0.21	6292.719
0.50	10853.882	0.76	13065.485	0.75	17986.074	0.75	11248.655	0.76	24294.941
0.80	17107.812	1.21	21025.549	1.20	28734.934	1.20	18569.059	1.22	38441.205
1.00	21610.196	1.52	26364.638	1.51	35776.281	1.50	22863.653	1.52	46066.395
1.20	26156.318	1.82	32292.875	1.81	43229.170	1.80	28312.425	1.83	57578.482
1.50	31949.901	2.28	39073.032	2.26	52926.125	2.25	34866.771	2.28	69718.700
Slope	21643.15	17462.08		23494.46		15665.09		30663.58	
Intercept	-163.34	-182.88		288.86		-289.98		433.87	
Correlation coefficient	0.9996	0.9994		0.9998		0.9997		0.9993	
Residual sum of squares	379571.86	840000.88		541141.66		370059.43		3413070.98	
RRF	--	0.81		1.09		0.72		1.42	





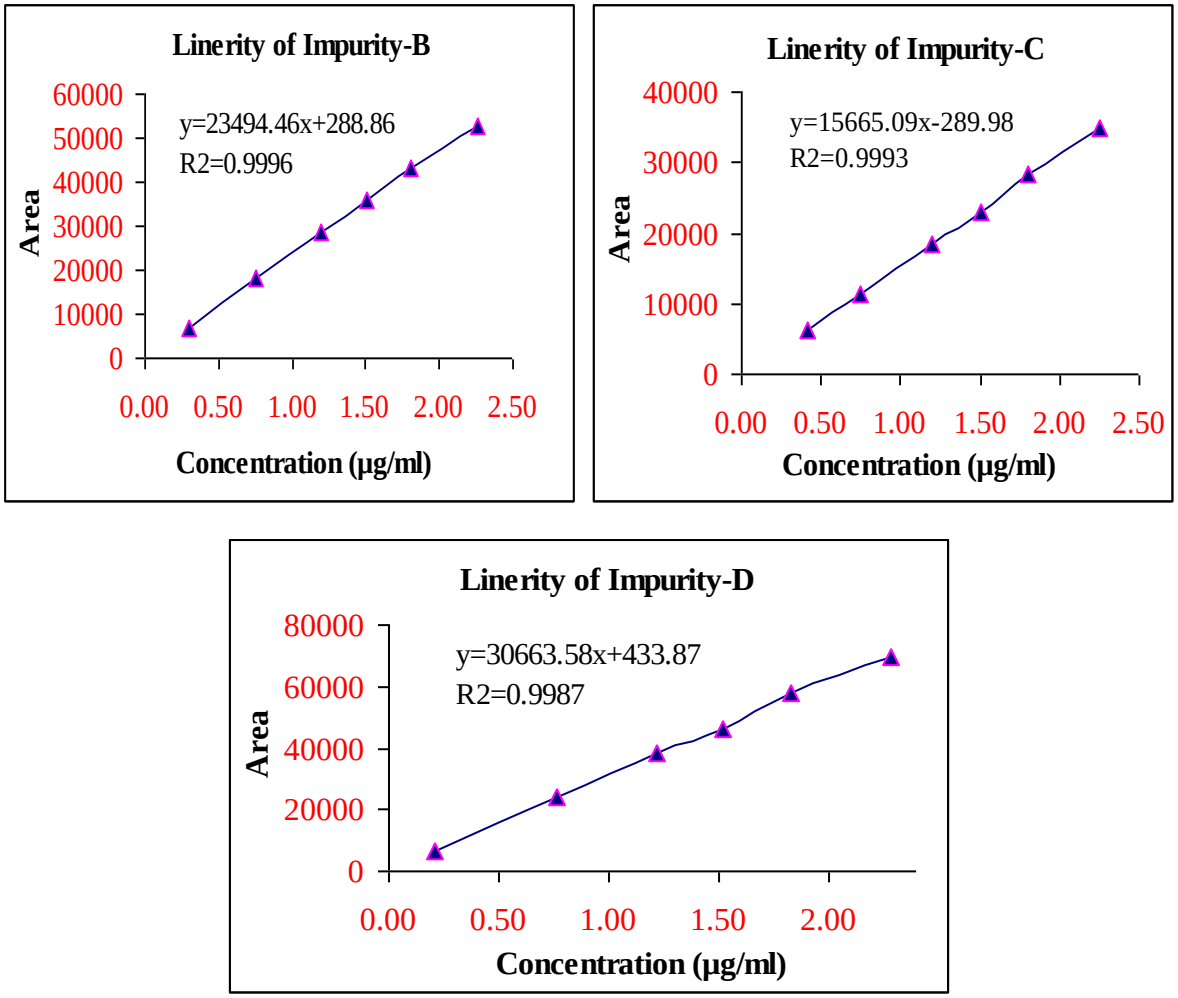


Figure 4: Linearity graph of Empagliflozin, Impurity-A, Impurity-B, Impurity-C and Impurity-D

Precision

Precision of the method was studied for repeatability and intermediate precision. Method precision was demonstrated by analyzing six different preparation of empagliflozin sample spiked with all the impurities at specification level. All the sample was analyzed in a single session. The method was found to be precise with a RSD of NMT 15%. To determine the intermediate precision of the method six different preparation of sample of the same lot analyzed by spiking the impurities at specification level by a different scientist using different instrument with different lot of column on a different day. The comparative data of the analysis by two different analysts is described in table3.

Table 3: Impurity (%) after spiked sample of Empagliflozin in method precision and intermediate precision

Parameter	Impurity (%)	Prep-1	Prep-2	Prep-3	Prep-4	Prep-5	Prep-6	Mean	%RSD
Method Precision	Impurity-A	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.0
	Impurity-B	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.0
	Impurity-C	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.0
	Impurity-D	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.0
	Unknown Max	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.0
	Total Impurities	0.63	0.63	0.63	0.63	0.63	0.62	0.63	0.65
Intermediate Precision	Impurity-A	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.0
	Impurity-B	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.0
	Impurity-C	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.0
	Impurity-D	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.0
	Unknown Max	0.02	0.03	0.02	0.03	0.03	0.02	0.03	0.0
	Total Impurities	0.62	0.63	0.62	0.63	0.63	0.63	0.63	0.82

Accuracy

Accuracy of the method was determined by spiking all the impurities at four different concentration levels of LOQ, 50, 100 and 150% each in triplicate of the specified limit. The recovery of all these impurities was found to be within the pre-defined criteria of 80-120%. The RSD for all level within the 10% and the data is presented in table 4.

Table 4: Recovery data at LOQ to 150 %

Level	Compound	Impurity-A	Impurity-B	impurity-C	Impurity D
LOQ Level	Added (%)[b]	0.039135	0.029505	0.039642	0.019887
	Found (%)[b]	0.038828	0.029743	0.040915	0.020622
	Recovery (%)[c]	99.2 ±2.47	100.8 ±4.25	103.2 ±0.92	103.7 ±3.97
50 % of specification[a]	Added (%)[b]	0.075531	0.073739	0.073450	0.073632
	Found (%)[b]	0.075531	0.076296	0.77069	0.075432
	Recovery (%)[c]	102.5 ±0.54	103.5±0.1.00	104.9 ±2.56	102.4 ±2.20
100 % of specification[a]	Added (%)[b]	0.147428	0.147599	0.147020	0.147385
	Found (%)[b]	0.152067	0.153511	0.151294	0.151198
	Recovery (%)[c]	103.1±0.37	104.0±0.22	102.9±0.20	102.6±0.55
150 % of specification[a]	Added (%)[b]	0.221142	0221399	0.220530	0.221077
	Found (%)[b]	0.230597	0.230182	0.228669	0.228971
	Recovery (%)[c]	104.3 ±0.83	104.0 ±0.68	103.7 ±0.78	103.6±0.96

a: Specification level 0.15 % for all impurities. b: n=3,average of the three determinations. c: Average±RSD.

Robustness

The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters because, the described method was designed for

future application in the routine drug analysis by pharmaceutical laboratories and other quality control laboratories. Robustness of the proposed method was evaluated by changing column temperature (altered by ± 5°C), flow rate (altered by ± 0.2 ml/min). The method exhibited good robustness with respect to variability in all robust condition (Table 5). Because the change made in chromatographic condition did not influence the analytical result.

Table 5: Robustness of Method

Parameters	**Control**	**Flow rate**		**Column temperature**	
Change in parameter	**Condition**	**1.0 ml/min**	**1.4 ml/min**	**50 °C**	**60 °C**
Retention time of Empagliflozin	11.52	13.96	10.13	11.83	11.29
Resolution	8.28	8.87	8.45	8.29	8.70
RRT of Impurity-A	1.31	1.30	1.31	1.30	1.31
RRT of Impurity-B	4.64	4.20	5.02	4.67	4.67
RRT of Impurity-C	5.02	4.53	5.44	5.04	5.06
RRT of Impurity-D	5.85	5.42	6.25	5.96	5.84
%RSD of standard injection	0.85	0.63	0.64	0.72	0.49
Tailing factor	1.08	1.06	1.07	1.07	1.12

Stability of analytical solution

To study the stability of the empagliflozin in the solution, a sample was studied for the individual and total impurities at every 4 hours to 24 hours against a freshly prepared spiked sample. It was found that the there is no change in the impurity level of this sample against a freshly prepared sample. The solution is stable upto 24 hours under the proposed experiment conditions.

CONCLUSION

A gradient RP-HPLC method was developed and validated for quantitative determination of process related impurities of empagliflozin drug substances. The method has higher sensitivity towards the determination of impurities and was found to be specific, sensitive, precise, linear, accurate, and robust. Thus, this method can be implemented for routine testing as well as stability analysis of empagliflozin drug substances.

ACKNOWLEDGEMENT

The authors wish to thank the management of Macleods pharmaceutical Ltd., India for supporting this work.

CONFLICT OF INTERESTS

Declared none.

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