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STABILITY INDICATING HPLC METHOD FOR THE QUANTIFICATION OF (R)-ISOMER IN EMPAGLIFLOZIN DRUG SUBSTANCE

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ABSTRACT
A simple sensitive isocratic normal phase chiral HPLC Method was developed for the chiral purity of Empagliflozin, a new anti-diabetic drug substance. The enantiomers of Empagliflozin and its (*R*)-enantiomer were resolved on new immobilized cellulose tris (3,5- dichlorophenyl carbamate) stationary phase**,** Chiralpak IC column using a mobile phase consisting of n-Hexane: Isopropyl alcohol : ethanol: Methyl tert- butyl ether and trifluoro acetic acid in the ratio of (650:200:100:50:1 v/v/v/v) at 25 °C column oven temperature with the flow rate of 1.0 mL min^{-1} and detection on UV/VIS detector wavelength at 224 nm. The USP resolution between both the Enantiomers was not less than 1.5 in this method. Limit of detection (LOD) and Limit of Quantitation (LOQ) for (*R*)-Empagliflozin were 0.01 %w/w and 0.030%w/w respectively. The accuracy of the method was in the range of 90.5 to 95.9%. The developed method was validated as per International Conference on Harmonisation (ICH) guidelines in terms of Specificity, Limit of detection (LOD), Limit of Quantitation (LOQ), Precision, Linearity, Accuracy, Solution stability and Robustness. This method is useful to control the enantiomeric impurity in routine use.

KEYWORDS: Empagliflozin; HPLC method; Chiral method; (*R*)-Isomer; Method Validation.

INTRODUCTION
Empagliflozin (EMP) is chemically known as (2*S*,3*R*,4*R*,5*S*,6*R*)-2-(3-(4-((*S*)-tetrahydrofuran-3-yloxy)benzyl)-4-chlorophenyl)-tetrahydro-6-(hydroxymethyl)-2*H*-pyran-3,4,5-triol (**Figure 1)** is a Sodium-glucose co-transpoter2 (SGLT-2) inhibitor for Type-2 diabetes mellitus. It lowers the blood glucose level by reducing renal reabsorption from kidney it lead to increased urinary glucose excretion thus it reduces the plasma glucose level. Empagliflozin is also used in combination with Metformin or Linagliptin to treat type-2 diabetes mellitus.[1-5]

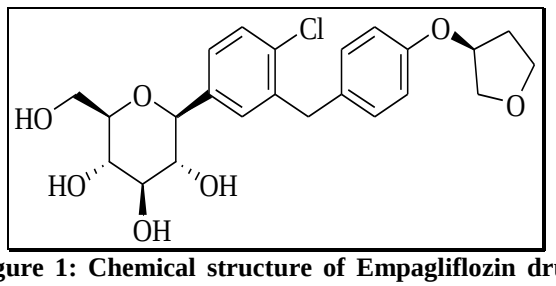

Figure 1: Chemical structure of Empagliflozin drug substance.

Separation of enantiomers in a chiral drug substance is necessary in pharmaceuticals. In addition, undesired enantiomer may have different or less therapeutic activity than the desired enantiomer. Moreover, these enantiomers show variances in pharmacokinetics and pharmacodynamics as well. Thus, separation and quality control of undesired enantiomer is a vital challenge in pharmaceutical analytical chemistry. In Empagliflozin drug substance, (R)-isomer is an undesired isomer (**Figure 2**).[6] A literature survey revealed few HPLC methods for the determination of Empagliflozin, its enantiomer and its related substances.[7-13] Among these analytical methods, chiral HPLC is the most useful technique with wide range of chiral stationary phases available for choosing the molecular functionality.[6]

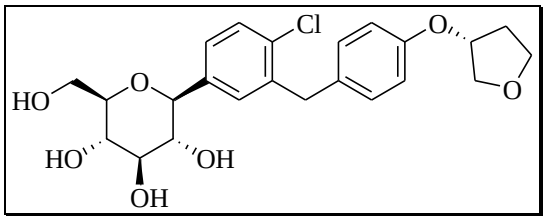

Figure 2: Chemical structure of (*R*)-isomer of empagliflozin drug substance.

In this context, we developed a simple normal phase chiral HPLC method and further validated for the determination (*R*)-isomer of Empagliflozin in Empagliflozin drug substance.

MATERIALS AND METHODS

Chemicals, reagents, standards and samples

The investigated samples of Empagliflozin drug substance and its (R)-iosmer were received as a gift from APL Research Centre-II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). And the other GR grade chemicals such as n-Hexane, Methyl tert-butyl ether, Isopropyl alcohol, Ethanol, Trifluoro acetic acid and methanol and AR grade chemicals including hydrochloric acid (~37%), sodium hydroperoxide, hydrogen peroxide (~30%), m-chlorobenzoic acid of AR grade were purchased from Merck Research Laboratories, India.

Instrumentation

The HPLC analysis was carried out using Alliance-waters 2695 separations module with 2996 PDA detector and 2489 UV detector and empower software; Alliance-waters e2695 separations module with 2998 PDA detector and empower software; Shimadzu prominence with PDA detector and Empower software; Shimadzu-LC-2010cht and empower software.

Preparation of Mobile Phase

Mobile phase was prepared by dissolving a degassed mixture of *n*-Hexane, Isopropyl alcohol, ethanol, Methyl tert-butylether and trifluoroacetic acid in the ratio of 650:200:100:50:1 v/v/v/v/v.

Preparation of Diluent

Diluent was prepared a degassing mixture of *n*-Hexane and Ethanol in the ratio of 50:50 v/v.

Chromatographic Conditions

Column	:	Chiralpak IC, 250 mm x 4.6 mm, 5µm
Pump mode	:	Isocratic
Flow rate	:	1.0 ml / min
Detection	:	UV, 224 nm
Column oven Temp	:	25°C
Injection Volume	:	10 µl
Data acquisition time	:	40 min

Preparation of Solutions

System suitability solution

Accurately weighed and transferred about 5 mg of Empagliflozin enriched [containing (R)- Empagliflozin] reference sample into a 5 mL volumetric flask, add 0.5 mL of methanol. Sonicated to dissolve and made up to volume with diluent.

Blank solution

Transferred 5 mL of methanol into a 50 mL dry volumetric flask and made upto volume with diluent.

Sample Solution

Weighed and transferred about 50 mg of sample into a 50 ml clean, dry volumetric flask, added 5 mL of methanol and sonicated to dissolve and made up to volume with diluent.

Evolution of System suitability

The column efficiency obtained from the USP resolution between (R)- Empagliflozin and Empagliflozin is not less than 1.5.

METHOD VALIDATION

Identification

Empagliflozin standard and Empagliflozin drug substance solutions were prepared as per test method and injected into HPLC. The retention times were described in **Table 1**.

Table 1: Retention time of Empagliflozin standard and Empagliflozin drug substance.

Sample	Retention time (min)	
Empagliflozin	Standard	Drug substance
	19.471	19.463

From the above results, it was concluded that as retention time obtained for Empagliflozin standard and Empagliflozin drug substance is similar to that of standard.

- **SPECIFICITY**

The solutions of diluent, Empagliflozin drug substance, Empagliflozin drug substance spiked with (*R*)-isomer of Empagliflozin (control sample) and Empagliflozin drug substance spiked with all known related substances including (R)-isomer of Empagliflozin (spiked sample) were injected in to HPLC to confirm any co-elution with (R)-isomer of Empagliflozin peak from any known related substances. Peak purity for (R)-isomer of Empagliflozin was established by using waters empower software. The specificity results were presented in **Table 2**.

Table 2: Specificity results.

Sample name	Retention Time (min)	RRT	Peak purity	
			Purity angle	Purity threshold
Control sample				
(R)-isomer of Empagliflozin	17.051	0.87	1.524	3.739
Empagliflozin	19.493	1.00	0.049	0.241
Spiked sample				
(R)-isomer of Empagliflozin	17.063	0.88	1.390	2.635
Empagliflozin	19.482	1.00	0.033	0.238

From the above results, peak purity of (R)-isomer of Empagliflozin was passed by purity angle which was less than the purity threshold**.**

- **FORCED DEGRADATION STUDY**

The stability indicating nature of the method was verified by the forced degration studies of Empagliflozin drug substance subjected to acid, base hydrolysis, oxidative condition, thermal, photolytic and humidity stress conditions. Empagliflozin drug substance was stressed with the following mentioned conditions and solutions were prepared with respective stressed samples and each solution was injected into HPLC. The forced degradation studies were presented in **Table 3**.

Table 3: Force degradation results.

Degradation mechanism	Degradation condition	(R)-Isomer of Empagliflozin		
		Observed (% area)	Purity angle	Purity threshold
	Not degraded	Not detected	Not applicable	Not applicable
Acid degradation	0.5M ethanolic HCl/85°C/2 hours	Not detected	Not applicable	Not applicable
Base degradation	0.05M ethanolic NaOH/85°C/2 hours	Not detected	Not applicable	Not applicable
Oxidative degradation	0.5% *m*-CPBA/85°C/2 hours	Not detected	Not applicable	Not applicable
Thermal degradation	105°C/120 hours	Not detected	Not applicable	Not applicable
Photolytic degradation	White fluorescent light,1.2 million lux hours and UV light, 200 watt hours/ meter square	Not detected	Not applicable	Not applicable
Humidity degradation	92.5%RH/RT/120 hours	Not detected	Not applicable	Not applicable

Form the above data, it was concluded that, there was no co-eluting peaks were observed at the retention time of (R)-Isomer of Empagliflozin peak. Peak purity for (R)-isomer of Empagliflozin was passed by purity angle which was less than purity threshold. From the above studies, it was identified that, there was no enhancement in (R)-isomer of empagliflozin under these stress conditions. Based on the above information, the test method is specific and stability indicating for the determination of (R)-isomer of Empagliflozin content in Empagliflozin drug substance.

- **LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)**

The limit of detection and limit of quantification values of (R)-isomer of Empagliflozin were determined from based on signal to noise ratio of analyte. The predicted concentrations of Limit of detection (LOD) and Limit of quantification (LOQ) for Empagliflozin and (R)-isomer of Empagliflozin were verified for precision by preparing the solutions containing Empagliflozin and (R)-isomer of Empagliflozin at above these predicted concentrations. The samples were injected six times into the HPLC and analysed the results. The LOD and LOQ results are tabulated in **Table 4**.

Table 4: Results of Limit of detection (LOD) and Limit of quantification (LOQ).

Injection ID	Area of (R)-isomer of Empagliflozin		Area of Empagliflozin	
	LOD	LOQ	LOD	LOQ
1	2135	6794	2167	7136
2	2543	6728	1613	7074
3	2295	6487	1889	6650
4	2625	6604	2052	7881
5	1979	6246	2636	6731
6	2383	7328	2176	7338
Statistical analysis				

Mean	2327	6698	2089	7135
SD	244	365	341	447
% RSD	10.5	5.4	16.3	6.3
Conc.(µg/mL)	0.099	0.301	0.099	0.299
Conc.(%w/w)	0.010	0.030	0.010	0.030

The above obtained LOQ results indicated that, (R)-isomer of Empagliflozin was well below 50% of specification level. Hence, the test method is precise for the quantification of the (R)-isomer of Empagliflozin in Empagliflozin drug substance.

- **LINEARITY**

A series of solutions were prepared using (R)-isomer of Empagliflozin at concentration levels from LOQ to 150% of specification level and each solution was injected into HPLC as per methodology. From the data, the linearity has been deduced from LOQ level to 150% of specification level and presented below. The linearity results were stated in **Table 5** and **Table 6**. And the linearity plot of Empagliflozin and Empagliflozin (R)-isomer is shown in **Figure 3** and **Figure 4** respectively.

Table 5: Linearity results of Empagliflozin.

Empagliflozin			
Concentration (µg/mL)	**Area**	**Statistical analysis**	
0.299	7136	Slope	26063
0.374	9408		
0.748	18116	Intercept	848
1.122	28217		
1.496	37459	Residual sum of squares	1357376
1.870	48381		
2.244	57841	Correlation of coefficient	0.9996

Table 6: Linearity results of Empagliflozin (R)-isomer.

(*R*)-isomer of Empagliflozin			
Concentration (µg/mL)	**Area**	**Statistical analysis**	
0.301	6794	Slope	21995
0.376	8498	Intercept	105
0.752	16291	Residual sum of squares	3100255
1.127	24481	Correlation of coefficient	0.9990
1.503	32526		
1.879	40154	Response factor (or) Correction factor	1.18
2.255	50728		

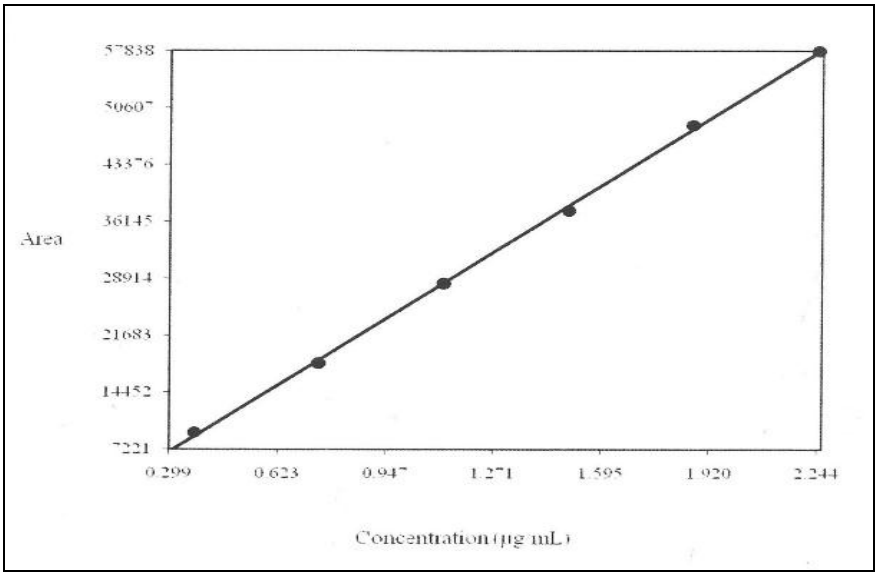

Figure 3: Linearity plot of Empagliflozin.

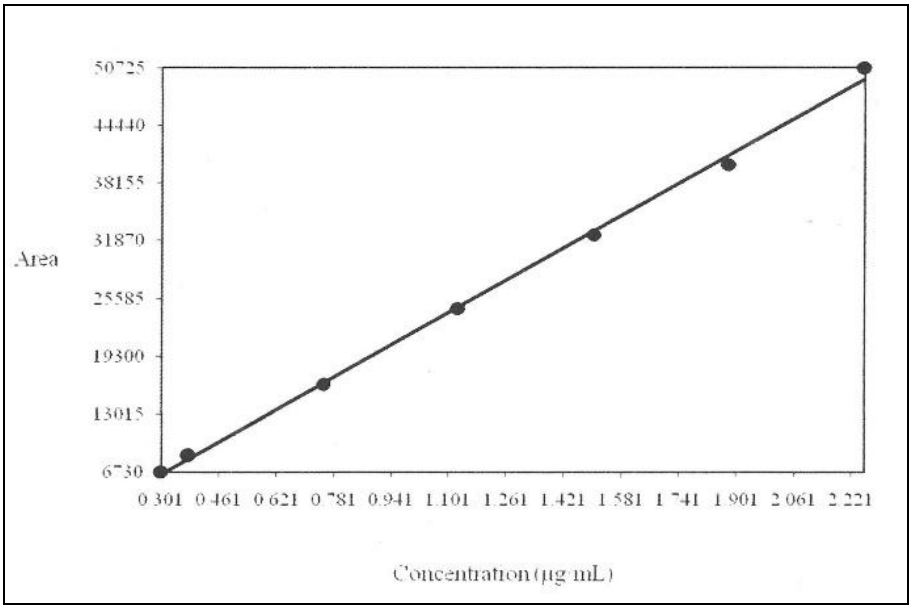

Figure 4: Linearity plot of Empagliflozin (R)-isomer.

- **PRECISION**

System precision: Standard solution was prepared at 0.10% level and injected six times into HPLC.

Method precision: Six sample solutions were prepared individually using single batch of Empagliflozin drug substance spiked with (R)-isomer of Empagliflozin at specification level and injected each solution into HPLC as per methodology.

Intermediate precision (Ruggedness): Sample (Sample batch used in method precision) solution of same sets were prepared individually as described under method precision spiked with (*R*)-isomer of Empagliflozin at specification level and injected each solution into HPLC as per the methodology by another analyst, on a different day using different system and different column. The results are shown in **Table 7**.

Table 7: Results of System precision.

Injection ID	Area of (R)-isomer of Empagliflozin	Statistical analysis	
1	27204	Mean	27188
2	26452	SD	906
3	25917	%RSD	3.3
4	27239		
5	27978	95% confidence interval (±)	951
6	28336		

RSD for peak areas of Empagliflozin from six replicate injections was 3.3% and it was passed the acceptance criteria that are not more than 10.0%.

- **PRECISION**

Method precision

Acceptance criteria

RSD for the value of (*R*)-isomer of Empagliflozin content from the analysis of six preparations should be not more than 10.0%. The method precision results are **Table 8**.

Table 8: The method precision results.

(*R*)-isomer of Empagliflozin	Method Precision (Set-I)(% area)					
	Sample-1	Sample-2	Sample-3	Sample-4	Sample-5	Sample-6
	0.168	0.163	0.177	0.170	0.157	0.152
Statistical analysis						
Sample Name	Mean	SD	%RSD	95% confidence interval (±)		
(*R*)-isomer of Empagliflozin	0.165	0.009	5.5	0.009		

The above test results showed that the test method is precise.

- **INTERMEDIATE PRECISION (RUGGEDNESS)**

Acceptance criteria

RSD for the value of (*R*)-isomer of Empagliflozin content from the analysis of six preparations should be not more than 10.0%. Overall RSD for the value of (R)-isomer of Empagliflozin from the analysis of six preparations of method precision (set-1) and six preparations of intermediate precision (Set-II) should be not more than 10.0%. The results of precesion results are shown in **Table 9**.

Table 9: Method precision and intermediate precision results.

Intermediate precision (Ruggedness) (Set-I) (% area)						
(*R*)-isomer of Empagliflozin	0.156	0.157	0.158	0.158	0.158	0.158
Statistical analysis						
(R)-isomer of Empagliflozin	Mean	SD	%RSD	95% confidence interval (±)		
	0.158	0.001	1.0	0.001		

Sample Name	**Method precision (Ruggedness) (Set-I) (% area)**					
(*R*)-isomer of Empagliflozin	0.168	0.163	0.177	0.170	0.157	0.152
Intermediate precision (Set-II) (%area)						
(*R*)-isomer of Empagliflozin	0.156	0.157	0.158	0.158	0.158	0.158
Overall statistical analysis (Set-1 and Set-II)						
Name	Overall mean	Overall SD	Overall (%)RSD	Overall 95% confidence interval (±)		
(*R*)-isomer of Empagliflozin	0.161	0.007	4.3	0.001		

The results (Method precision and intermediate precision) indicated that the method is rugged for the determination of (*R*)-isomer of Empagliflozin content in Empagliflozin drug substance with respect to analyst, day to day, system to system and column to column variations.

- **ACCURACY (RECOVERY)**

Emapgliflozin sample solutions were prepared in triplicate by spiking with (*R*)-isomer of Empagliflozin at levels of LOQ, 50%, 100% and 150% of specification limit as per test concentration and injected each solution into HPLC as per methodology. The results are described in **Table 10**.

Acceptance criteria: % Recovery should be between 80.0 and 120.0 for LOQ level and 85.0 and 115.0 for other levels.

(*R*)-isomer of Empagliflozin (At LOQ level)							
Concentration/ Sample ID		**Amount added (% area)**		**Amount found (% w/w)**		**% Recovery**	
LOQ level sample 1		0.0299		0.0271		90.6	
LOQ level sample 2		0.0298		0.0284		95.3	
LOQ level sample 3		0.0298		0.0281		94.3	
Mean (% Recovery)	93.4	SD	2.48	%RSD	2.7	95% confidence interval (±)	6.2

- **ACCURACY (RECOVERY)**

Table 10: Accuracy results.

(R)-isomer of Empagliflozin (50% to 150% level)							
% Level/ Sample ID		**Amount added (% area)**		**Amount added (% area)**	**% Recovery**	**Statistical analysis**	
50% level sample-1		0.074		0.071	95.9	Mean	92.3
50% level sample-2		0.074		0.067	90.5	SD	3.12
50% level sample-3		0.074		0.067	90.5	% RSD	3.4
100% level sample-1		0.15		0.138	92.0	Mean	91.8
100% level sample-2		0.149		0.136	91.3	SD	0.40
100% level sample-3		0.15		0.138	92.0	% RSD	0.4
150% level sample-1		0.224		0.206	92.0	Mean	93.6
150% level sample-2		0.224		0.211	94.2	SD	1.40
150% level sample-3		0.223		0.211	94.6	% RSD	1.5
Overall statistical analysis							
Mean	92.6	SD	1.90	%RSD	2.1	95% confidence interval (±)	1.5

The recovery results indicated that the test method has an acceptable level of accuracy for the determination of (R)-isomer of Empagliflozin content in Empagliflozin drug substance from LOQ to 150% of specification level.

- **ROBUSTNESS**

Standard solution and sample solution spiked with (*R*)-isomer of Empagliflozin at specification level were prepared as per test method and injected into HPLC at different deliberately varied conditions to evaluate the system suitability and methods ability to remain unaffected.

The varied conditions include change in flow rate by ±10% detection wavelength by ±3nm, composition of mobile phase (±2% absolute for Isopropyl alcohol, Ethanol and Methyl tert-butylether with respect to n-Hexane) different batch number of column and column oven temperature by ±5°C from methodology values. The robustness results are stated in **Table 11**.

Table 11: Robustness results.

Parameter	Variation	System suitability
		USP resolution
STP	-	1.8
Flow rate	-10%	1.9
	+10%	1.7
Wavelength	-3 nm	1.8
	+3 nm	1.8
Temperature	-5°C	1.8
	+5°C	1.8
Mobile phase Composition variation	-2% Absolute Isopropyl alcohol	1.9
	+2% Absolute Isopropyl alcohol	1.7
	-2% Absolute Ethanol	1.8
	+2% Absolute Ethanol	1.8
	-1% Absolute Ethanol	1.8
	+1% Absolute Ethanol	1.8
	-2% Absolute Methyl *tert*-butylether	1.9
	+2% Absolute Methyl *tert*-butylether	1.8
STP	-	1.8

Parameter	Variation	Spiked Sample
		RRT of (R)-isomer Empagliflozin
Flow rate	-10%	0.88
	+10%	0.88
Wavelength	-3nm	0.88
	+3nm	0.88
Temperature	-5C	0.86
	+5C	0.89
Mobile phase Composition variation	-2% Absolute Isopropyl alcohol	0.87
	+2% Absolute Isopropyl alcohol	0.88
	-2% Absolute Ethanol	0.87
	+2% Absolute Ethanol	0.88
	-1% Absolute Ethanol	0.88
	+1% Absolute Ethanol	0.88
	-2% Methyl *tert*-butylether	0.87
	+2% Methyl *tert*-butylether	0.88

The system suitability results at each of the varied conditions were complied with the requirements as per the test procedure. And also it was observed from the above results of Empagliflozin drug substance spiked with (R)-isomer of Empagliflozin at specification level obtained from the different robustness conditions that there was not much variation in the RRTin comparatively with that of STP condition. At composition of mobile phase ± absolute (with respect to, Isopropyl alcohol and ethanol variation), in system suitability significant retention time variation between (R)-isomer of Empagliflozin and Empagliflozin was observed. So, it was required to maintain the amount of Isopropyl alcohol and Ethanol in the mobile phase composition as per test procedure.

CONCLUSION

A simple and validated HPLC method for the determination of (*R*)-isomer of empagliflozin in empagliflozin drug substance was described. The results

of various validation parameters proved that the method is specific, sensitive, precise and accurate and the method can be introduced into routine testing.

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