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Separation of Empagliflozin and its Impurities by Validated Stability Indicating HPLC Method and LC-MS Characterization of Oxidative Degradation Product

SUNKARA BHAWANI* and T.N.V. GANESH KUMAR

University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Nagarjuna Nagar-522510, India

*Corresponding author: E-mail: banu.sunkara@gmail.com

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The HPLC assay strategy for two impurities (EPN-R-ISO impurity and the EPN-Diol impurity) which are related to empagliflozin synthesis was designed and verified for the reliable measurement of EPN-R-ISO and EPN-Diol impurities in empagliflozin bulk APIs. The chromatographical EPN-R-ISO and the EPN-Diol impurities analysis was done on Discovery C18 stationery column with isocratic type mobile phase exploited was potassium dihydrogenphosphate (0.01 M, pH calibrated to 2.0 by phosphoric acid) and acetonitrile in 70%:30% v/v ratio and injected at a flow measure of 1.0 mL/min. The degradation of empagliflozin under stressful conditions such as acid generated hydrolysis, base generated hydrolysis, peroxide generated oxidation, thermal generated degradation and UV generated hydrolysis was also included. The method established was verified for precision (0.0837% RSD for EPN-R-ISO; 0.1831% RSD for EPN-Diol), sensitivity (LOD: 0.030262 ppm EPN-R-ISO concentration, 0.031873 ppm EPN-Diol concentration; LOQ: 0.092621 ppm EPN-R-ISO concentration, 0.09755 ppm EPN-Diol concentration), linearity (0.1 ppm to 90.0 ppm concentration of both EPN-R-ISO and EPN-Diol) and accuracy (95.07% to 96.27% EPN-R-ISO recovery; 97.72% to 100.03% EPN-Diol recovery).

Keywords: HPLC, Empagliflozin, LC-MS, Degradant characterization.

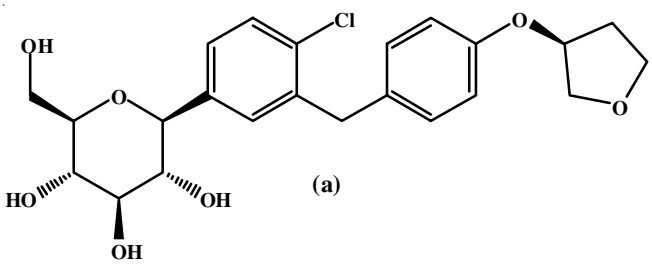
INTRODUCTION

Empagliflozin (Fig. 1a) is a specific inhibitor for sodium glucose cotransporter-2. The antihyperglycemic drug empagliflozin is efficient and typically well tolerated. In addition to other countries across the world, Europe, the USA and Japan have all authorized empagliflozin for the treatment of those people with diabetes type 2 [1,2]. A separate insulin action mechanism underlies empagliflozin treatment. Empagliflozin demonstrated better selectivity in preclinical trials compared to dapagliflozin drug and canagliflozin drug. Studies reveal that empagliflozin is both safe and effective and helps people lose weight and improves their cardiovascular health [3,4].

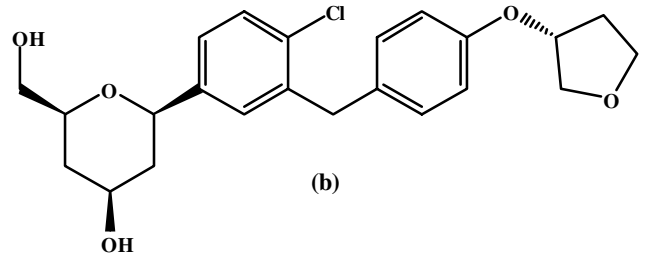
In both industries and researches, the prevalence of impurities in pharmaceuticals is being investigated more frequently. Due to the mutagenic/teratogenic/carcinogenic potential of certain impurities, particularly in pharmaceuticals intended for continuous use, ingesting these undesirable molecules might have a solemn impact on one's healthiness [5,6]. The management and controlling of impurities are the major concern for numerous regulatory bodies. Furthermore, a number of government compendia incorporate specification margins that limit the quantities of impurities present in API molecules and pharmaceutical formulations [7-10].

In this investigation, the EPN-R-ISO impurity (Fig. 1b) and EPN-Diol impurity (Fig. 1b), both linked to empagliflozin drug synthesis, were assessed. The two analyzed impurities (EPN-R-ISO impurity and the EPN-Diol impurity) are the intermediate molecules which were observed in several empagliflozin synthesis paths [11,12]. During the purification phase, all residues of the impurities EPN-R-ISO and EPN-Diol will be rinsed off. However, a restriction of "not more than 0.15%" was placed on these two impurities [8,13].

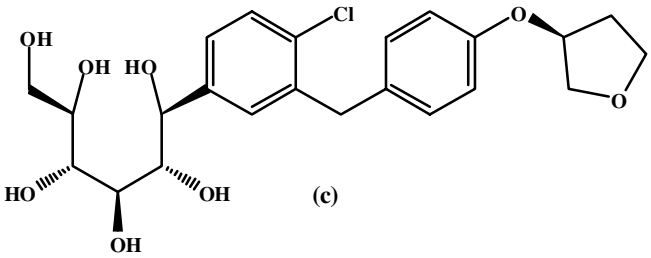
Several chromatographic methods and detection mechanisms have been reported [14-19] to measure the impurities pertaining to empagliflozin drug in empagliflozin API formulation. However, the analysis of EPN-R-ISO and EPN-Diol impurities isn't addressed in these studies. Since there are no studies is reported to assess of these compounds (EPN-R-ISO and EPN-Diol impurities), thus, we stress the significance of this study



(2S,3R,4R,5S,6R)-2-[4-Chloro-3-[[4-[(3S)-oxolan-3-yl]oxy phenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol



(2S,3R,4R,5S,6R)-2-[4-Chloro-3-(4-(((R)-tetrahydrofuran--3-yl)oxy)benzyl)phenyl)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-3,4,5-triol



(2S,3R,4R)-1-(4-Chloro-3-(4-(((S)-tetrahydrofuran-3-yl) oxy)benzyl)phenyl)hexane-1,2,3,4,5,6-hexaol

Fig. 1. (a) EPN drug, (b) EPN-R-ISO impurity, (c) EPN-Diol impurity

in present study to evaluate the impurities, which are associated with the EPN synthesis. The intent of this study is to present an HPLC/UV technique for the EPN-R-ISO and EPN-Diol impurities separation and quantification that is simplistic, rapid, sensitive and thoroughly validated. Additionally, unknown oxidative stress-related impurities produced were investigated by LC-MS techniques.

EXPERIMENTAL

Instruments like HPLC 2695 system (Waters), PDA detector (Waters), Empower software (Waters), UV-VIS spectrophotometer (PG Instruments T60), UV win 6 Software (PG Instruments T60), ultra sonicator (Labman), Hot Air Oven (Serve well Instrument Pvt Ltd) were used in EPN-R-ISO and EPN-Diol impurities analysis. Instruments like HPLC e2695 system (Waters) coupled to QTRAP 5500 MS detector (Waters) furnished with electrospray ionization (Waters) and SCIEX software were used in degradants characterization.

"Merck, India" supplied the milli-Q type water, methanol and acetonitrile whereas Rankem supplied potassium dihydrogen phosphate, triethylamine, phosphoric acid and sodium dihydrogen phosphate. The drug empagliflozin, EPN-R-ISO impurity and EPN-Diol impurity were gifted from MSN company, India.

HPLC conditions: The column exploited in EPN-R-ISO and EPN-Diol assay was a Discovery C18 stationery column (length measure of 250 mm; identification measure of 4.6 mm; particle measure of 5 µM). Isocratic type mobile phase exploited in EPN-R-ISO and EPN-Diol assay was potassium dihydrogen phosphate (0.01 M, pH calibrated to 2.0 by phosphoric acid) and acetonitrile in 70%:30% v/v ratio. At a column and injection port temperatures of 30 ºC and 5 ºC, respectively, the mobile phase was injected at a flow measure of 1.0 mL/min. For the EPN-R-ISO and EPN-Diol analysis, 10 µL volume sized solutions were loaded into the Discovery C18 stationery column. By fixing the wavelength at 220 nm, the chromatography of EPN-R-ISO and EPN-Diol eluting was set down. The samples (EPN/EPN-R-ISO/EPN-Diol) dissolved in water/acetonitrile (5:5, v/v ratio) mixed solution.

EPN-R-ISO and EPN-Diol solutions: The EPN-R-ISO and EPN-Diol stock was made in water/acetonitrile (5:5, v/v ratio) mixed solution with 600 ppm concentration each. The EPN-R-ISO and EPN-Diol standard (60 ppm concen-tration each) was also prepared in water/acetonitrile (5:5, v/v ratio) mixed solution by dilution of EPN-R-ISO and EPN-Diol stock (50 ppm concentration each). The linearity EPN-R-ISO and EPN-Diol standards with 0.1, 15, 30, 45, 60, 75 and 90 ppm concentrations each were acquired through EPN-R-ISO and EPN-Diol stock (600 ppm concentration each) dilution appropriately in in water/acetonitrile (5:5, v/v ratio) mixed solution.

Empagliflozin sample: Empagliflozin sample stock was also prepared in water/acetonitrile (5:5, v/v ratio) mixed solution with 600 ppm empagliflozin concentration. Empagliflozin standard (60 ppm concentration) was again prepared in water/acetonitrile (5:5, v/v ratio) mixed solution by dilution of empagliflozin sample stock (600 ppm concentration).

Linearity EPN-R-ISO and EPN-Diol curves: Standard EPN-R-ISO and EPN-Diol solutions were loaded in a series of 0.1, 15, 30, 45, 60, 75 and 90 ppm concentrations each to ascertain the EPN-R-ISO and EPN-Diol linearity range. From each chromatogram, the EPN-R-ISO and EPN-Diol peak area was worked out and linearity EPN-R-ISO and EPN-Diol curves were developed related to the corresponding EPN-R-ISO and EPN-Diol concentrations. The regression coefficient as well as a regression colinear equation containing the slope estimates and intercept estimates, were applied to compute the EPN-R-ISO and EPN-Diol concentrations in samples.

Assessment of EPN-R-ISO and EPN-Diol in EPN bulk API: The prepared empagliflozin sample (60 ppm) was loaded into Discovery C18 stationery column and examined with the proposed conditions. From chromatograms, the EPN-R-ISO and EPN-Diol peak areas in test EPN sample was worked out and exercised to compute EPN-R-ISO and EPN-Diol contents using linearity EPN-R-ISO and EPN-Diol curves or their correlated regression colinear equation.

Degradation studies: The purpose of these forced degradation analysis on 0.5 mg/mL sample of empagliflozin drug was to establish definitively that the method used to investigate EPN-R-ISO and EPN-Diol impurities in empagliflozin drugs is indicative of its stability [20]. The experiments involved in evaluating the effects of acid (2.0 N HCl, 24 h reflux at 60 ºC),

alkali (2 N NaOH, 24 h reflux at 60 ºC), hydrogen peroxide (30% H_2O_2, 24 h reflux at 60 ºC), thermal (24 h exposure at 105 ºC) and photo (7 days exposure to UV) on empagliflozin sample.

LC-MS based degradants characterizing conditions: In positive ion electrospray ionization interface mode, the MS was operated. The other MS parameters tuned included: Drying gas temperature: 120-250 ºC; collision energy: 15 V; Source temperature: 550 ºC; Ion spray voltage: 5500 V; collision gas: nitrogen; drying gas flow stream: 5 L/min; entrance potential: 10 V; declustering potential: 40 V; exit potential: 7 V; and dwell time: 1 s.

RESULTS AND DISCUSSION

Stream line of HPLC based EPN-R-ISO and EPN-Diol impurities assaying conditions: For the analysis of EPN-R-ISO and EPN-Diol impurities simultaneously using the HPLC assay, different amounts of solvents and columns such as methanol: 0.1% phosphoric acid (50:50, v/v; Kromasil 250 column), acetonitrile: 0.01 M KH_2PO_4 (50:50, v/v; Phenomenon 150 column), acetonitrile: 0.1% phosphoric (50:50, v/v; ODS 150 column), acetonitrile: 0.01 M KH_2PO_4 (80:20, v/v; ODS 250 column), acetonitrile: 0.01M KH_2PO_4 (70:30, v/v; phenomenon 150 column) and acetonitrile: 0.01 M KH_2PO_4 (70:30, v/v; discovery 250 column) were studied as mobile phase system solvents for the occurrence of an satisfactory peaks for the quantitative investigation of EPN-R-ISO and EPN-Diol impurities in empagliflozin samples. The acetonitrile: 0.01 M KH_2PO_4 (70:30, v/v; discovery 250 column) combination in isocratic flow (1.0 mL/min) mode elution appeared to present a completely separated chromatogram of EPN-R-ISO R_t at 9.603 min, EPN-Diol R_t at 5.756 and EPN R_t at 11.926 with an agreeable peak properties as shown in Fig. 2.

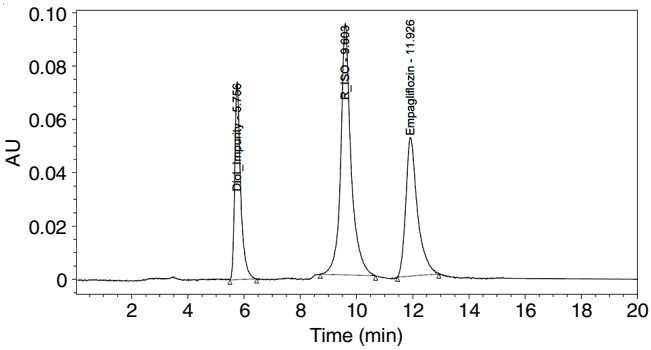


Fig. 2. EPN drug (Rt – 11.926 min), EPN-R-ISO impurity (Rt – 9.603 min) and EPN-Diol impurity (5.756 min min) chromatogram

During the entire trails, the parameters were column temperature (30 ºC), injection port temperature (5 ºC) and flow rate (1.0 mL/min) were kept constant. For the HPLC experiment, the maximal chromatographic response of EPN-R-ISO, EPN-Diol and EPN was observed at 220 nm. Hence, 220 nm was used throughout the measurements of EPN-R-ISO and EPN-Diol.

Validation: The HPLC based EPN-R-ISO and EPN-Diol impurities assay approach was authenticated in accordance with ICH regulations [21].

Selectivity: Loaded (10 µL) water/acetonitrile (5:5, v/v ratio) mixed solution, EPN-R-ISO and EPN-Diol standard (60 ppm concentration each), EPN-R-ISO and EPN-Diol spiked EPN sample (60 ppm concentration each) and empagliflozin standard (60 ppm concentration) into Discovery C18 stationery column and examined with proposed conditions. Interference was not detected due to blank water/acetonitrile (5:5, v/v ratio) mixed solution and EPN at the retention times of EPN-R-ISO and EPN-Diol. For EPN-R-ISO and EPN-Diol, the retention time from the standard EPN-R-ISO and EPN-Diol solution and EPN-R-ISO and EPN-Diol spiked EPN sample solution were nearly matched. Fig. 3 exhibits the result data of specificity analysis.

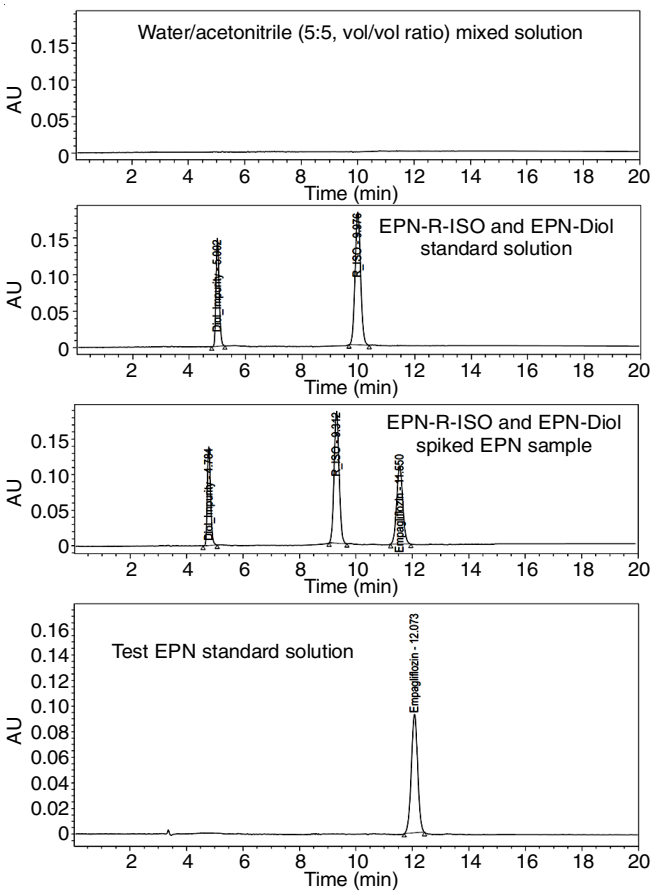


Fig. 3. Chromatograms of specificity test

LOD and LOQ: Solutions with defined decreasing EPN-R-ISO and EPN-Diol concentrations were loaded (10 µL) into Discovery C18 stationery column to assess the LOD and LOQ measurements for EPN-R-ISO and EPN-Diol. The LOD and LOQ were later gauged by evaluating the minimal quantity size where the EPN-R-ISO and EPN-Diol can be facilely detected (signal/noise relation: 3:1) and also quantified (signal/noise relation: 10:1). LOD of EPN-R-ISO and EPN-Diol were 0.030262 ppm and 0.031873 ppm, respectively while LOQ were 0.092621 ppm and 0.09755 ppm, respectively (Fig. 4)

Linearity: Linearity of EPN-R-ISO and EPN-Diol from 0.1 ppm concentration to 90.0 ppm concentration was verified. Linear regression information analysis was drawn on to find

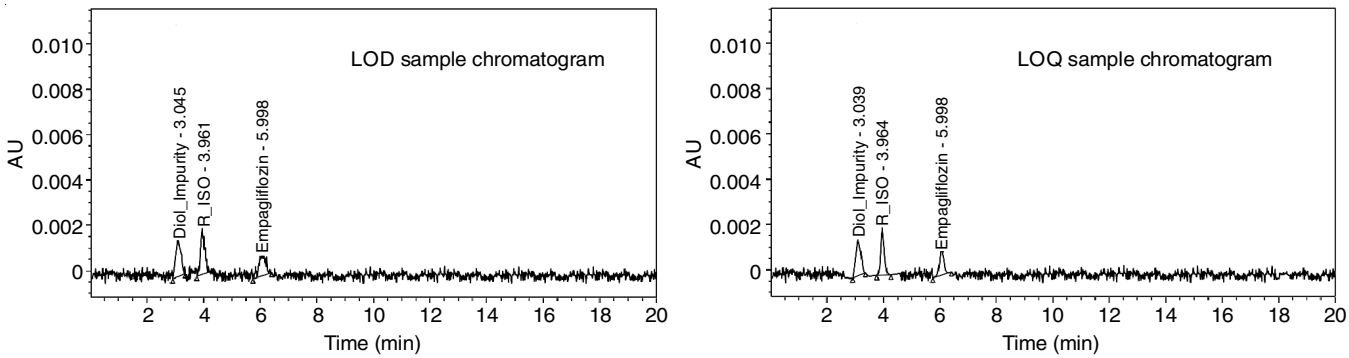


Fig. 4. Chromatograms of sensitivity test

out the intercept (4178.2 for EPN-Diol and 10498.0 for EPN-R-ISO), slope (3076.7 for EPN-Diol and 6604.1 for EPN-R-ISO) and correlation coefficient (0.9992 for EPN-R-ISO and 0.9992 for EPN-Diol) values (Fig. 5). In EPN-R-ISO and EPN-Diol concentration range (0.1 ppm to 90.0 ppm) evaluated, a value of > 0.99 for correlation coefficient presented that the method is linear.

Method precision: For method precision verification, the percentage of RSD for EPN-R-ISO and EPN-Diol impurities found content was calculated from six duplicate infusions of EPN-R-ISO and EPN-Diol spiked empagliflozin sample (60 ppm concentration each). The characteristics of method precision are listed in Table-1 and the values are within limits in method precision investigations.

TABLE-1
PRECISION TEST INFORMATION OF EPN-R-ISO AND EPN-DIOL IMPURITIES

EPN-R-ISO content found (ppm)		EPN-Diol content found (ppm)	
60.60	Average content:	59.70	Average content:
60.00	60.13	60.31	60.45
60.31	SD of contents:	60.60	SD of contents:
59.72	0.33963	60.90	0.41355
59.81	RSD of content:	60.60	RSD of content:
60.32	0.565	60.61	0.684

Accuracy: The accuracy was verified by loading (10 µL) 3 sample empagliflozin solutions appended with EPN-R-ISO and EPN-Diol impurities at three separate EPN-R-ISO and EPN-Diol concentrations (50% of specified quantity limit: 30 ppm EPN-R-ISO and 30 ppm EPN-Diol, 100% specified quantity limit: 60 ppm EPN-R-ISO and 60.0 ppm EPN-Diol and 150% of specified quantity limit: 90 ppm EPN-R-ISO and 90 ppm EPN-Diol). The characteristics of accuracy are listed in Tables 2 and 3. The accuracy has been supported by measurement of recovery results for EPN-R-ISO and EPN-Diol impurities, which are within the limits.

TABLE-2
ACCURACY TESTS INFORMATION OF EPN-R-ISO IMPURITY

Preparation	EPN-R-ISO impurity ppm			RSD of recovery
	Included	Found	Recovery (%)	
50% of specified quantity limit – 0.500 ppm EPN-R-ISO				
Prep-1	30.00	28.75	95.77	
Prep-2	30.00	28.29	94.08	95.07
Prep-3	30.00	28.65	95.37	
Specified quantity limit – 1.00 ppm EPN-R-ISO				
Prep-1	60.00	57.78	96.21	
Prep-2	60.00	57.39	95.55	95.64
Prep-3	60.00	57.04	95.16	
150% of specified quantity limit – 1.50 ppm EPN-R-ISO				
Prep-1	90.00	86.45	96.09	
Prep-2	90.00	86.96	96.60	96.28
Prep-3	90.00	86.58	96.14	

TABLE-3
ACCURACY TESTS INFORMATION OF EPN-DIOL IMPURITY

Preparation	EPN-Diol impurity ppm			RSD of recovery
	Included	Found	Recovery (%)	
50% of specified quantity limit – 0.492 ppm EPN-Diol				
Prep-1	30.00	29.35	97.78	
Prep-2	30.00	29.26	97.65	97.72
Prep-3	30.00	29.34	97.72	
Specified quantity limit – 0.984 ppm EPN-Diol				
Prep-1	60.00	59.17	98.62	
Prep-2	60.00	58.66	97.86	98.52
Prep-3	60.00	59.49	99.08	
150% of specified quantity limit – 1.476 ppm EPN-Diol				
Prep-1	90.00	90.65	100.69	
Prep-2	90.00	89.36	99.33	100.03
Prep-3	90.00	90.07	100.08	

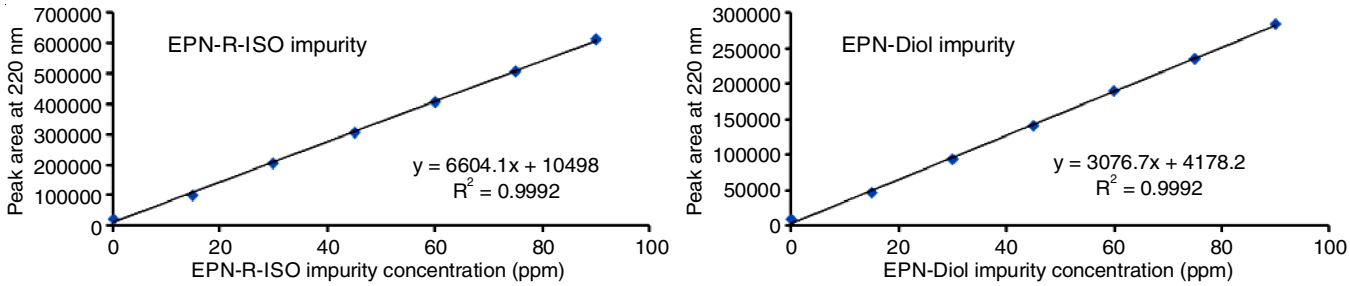


Fig. 5. Linearity curve figures EPN-R-ISO and EPN-Diol

System suitability: For system suitability verification, the percentage of RSD for EPN-R-ISO and EPN-Diol impurities found peak characteristics (retention period, area counts, plate counts, tailing and resolution) was calculated from six duplicate infusions of EPN-R-ISO and EPN-Diol spiked EPN sample (60 ppm concentration each). The characteristics of system suitability are listed in Table-4. The values are within limits in system suitability investigations.

Forced degradation studies: Evaluated the effects of acid (2 N HCl, 24 h reflux at 60 ºC), alkali (2 N NaOH, 24 h reflux at 60 ºC), hydrogen peroxide (30% H_2O_2, 24 h reflux at 60 ºC), thermal (24 h exposure at 105 ºC) and photo (7 days exposure to UV) on EPN sample (0.5 mg/mL). The %degradation results for EPN-Diol impurity were gauged in all conditions tested (Table-5). The impurities EPN-R-ISO and EPN-Diol was not recognized in any of the tested conditions.

Specificity: All the samples exposed to acid (2 N HCl, 24 h reflux at 60 ºC), alkali (2 N NaOH, 24 h reflux at 60 ºC), hydrogen peroxide (30% H_2O_2, 24 h reflux at 60 ºC), thermal (24 h exposure at 105 ºC) and photo (7 days exposure to UV) were imperilled to a peak purity valuation during the specificity analysis. The peak purity, which assumes the degraded empagliflozin components formed are spectrally distinct from empagliflozin, is an examination of spectral discrepancies. Empagliflozin purity angle and empagliflozin threshold angle numerical values were ascertained to execute the empagliflozin peak purity investigation. The empagliflozin peak was deemed pure if the minimal empagliflozin purity index had a positive value and empagliflozin threshold angle numerical values exceeded the empagliflozin peak purity numerical values. The empagliflozin purity angle and empagliflozin threshold angle numerical values confirmed homogeneity of empagliflozin peak (Fig. 6). The investigative

TABLE-4
SYSTEM SUITABILITY TESTS INFORMATION OF EPN-R-ISO AND EPN-DIOL IMPURITIES ASSAY

Preparation	EPN-Diol impurity					EPN-R-ISO impurity				
	RT measure	Area count	Plate counts	Tailing measures	Resolution measures	RT measure	Area count	Plate counts	Tailing measures	Resolution measures
Prep-1	4.766	188607	8557	1.23	–	9.312	402533	12143	1.07	16.6
Prep-2	4.784	188637	8509	1.22	–	9.324	408920	12855	1.09	15.8
Prep-3	4.807	186323	8207	1.23	–	9.407	401555	12726	1.08	16.4
Prep-4	4.915	184122	8446	1.23	–	9.405	409998	12714	1.08	17.2
Prep-5	4.937	188384	8579	1.22	–	9.408	408415	12663	1.07	17.0
Prep-6	5.002	186168	8351	1.21	–	9.476	403345	12618	1.07	17.4
Mean found	4.869	187040	8442	1.223	–	9.389	405794	12620	1.077	16.733
SD found	0.09603	1822.1652	141.4238	0.00816	–	0.06110	3712.5726	246.8517	0.00816	0.58878
RSD found	1.972	0.974	1.675	0.667	–	0.651	0.915	1.956	0.758	3.519

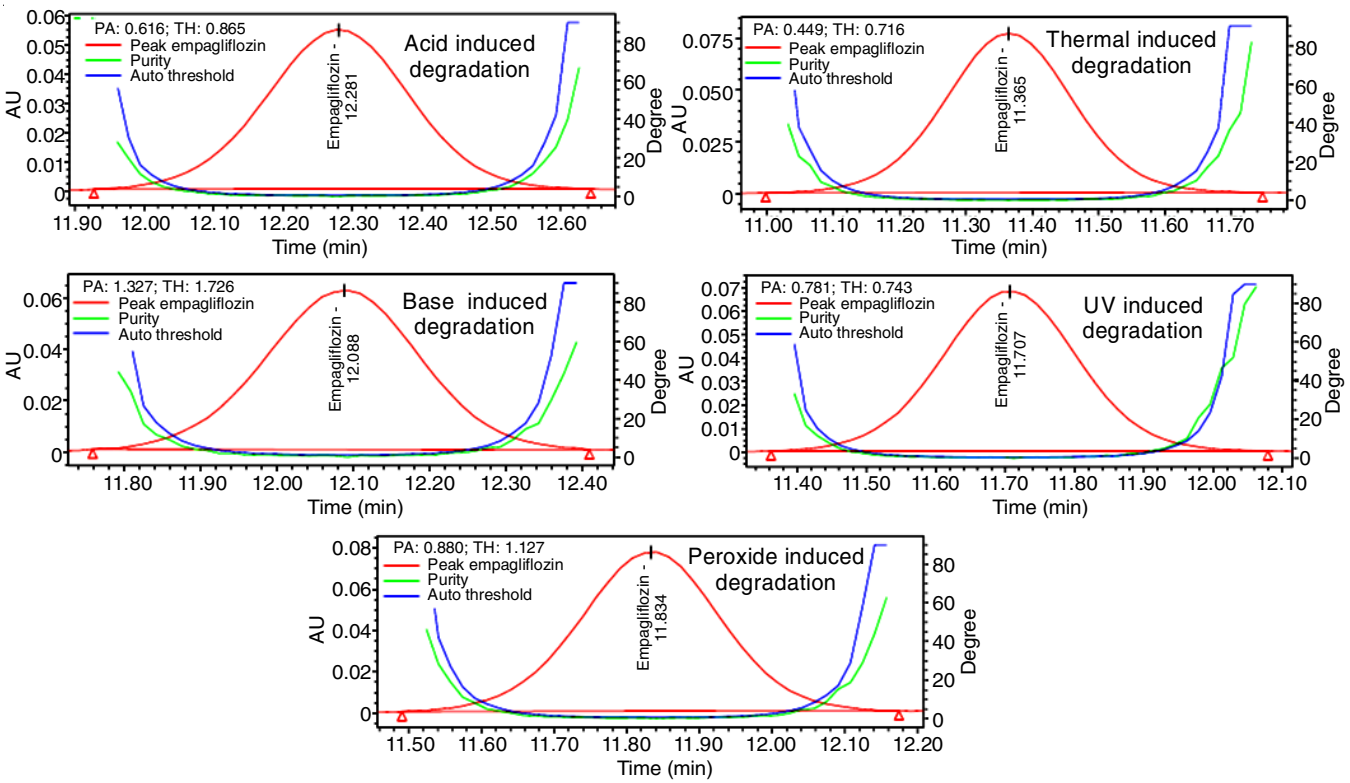


Fig. 6. EPN's peak purity plots under stresses applied (PA = EPN purity angle and TH = EPN threshold angle)

TABLE-5
DEGRADATION INFORMATION OF EPN

Degradation condition	EPN (%) found	EPN (%) degraded	EPN-R-ISO (%) found	EPN-Diol (%) found
2.0 N HCl, 24 h reflux at 60 °C	95.534	4.466	Not found	Not found
30% H_2O_2, 24 h reflux at 60 °C	96.824	3.176	Not found	Not found
2.0N NaOH, 24 h reflux at 60 °C	95.865	4.135	Not found	Not found
24 h exposure at 105 °C	95.027	4.973	Not found	Not found
7 days exposure to UV	97.456	2.544	Not found	Not found

technique for EPN-R-ISO and EPN-Diol in empagliflozin drug is deemed as stability indicating and skilled of distinguishing degradants from empagliflozin signal peaks.

LC-MS characterization: Empagliflozin samples exposed hydrogen peroxide (30% H_2O_2, 24 h reflux at 60 ºC) was analyzed with proposed MS conditions. Two peaks with retention time of 3.123 min and 3.675 min were obtained. The SCIEX program was deployed to analyze the peaks with the retention time of 3.123 min and 3.675 min and identify the degradation compounds. Two peaks at *m/z* 516.53 and 515.51 were found (Fig. 7). The two identified compounds are considered as adduct of potassium. This proved that empagliflozin remains stable to exposed hydrogen peroxide (30% H_2O_2, 24 h reflux at 60 ºC) medium.

Conclusion

The HPLC technique was successfully implemented in the design of an analytical method for EPN-R-ISO and EPN-Diol impurities estimation in empagliflozin bulk API is presented. This method is capable of efficient separation and determination of EPN-R-ISO and EPN-Diol impurities in empagliflozin bulk API. The method established was verified for accuracy, precision, sensitivity, linearity and accuracy. When empagliflozin bulk API was degraded, EPN-R-ISO and EPN-Diol impurities peak was not observed in related degradation chromatograms. It has become proven to be fit for the intended purpose.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

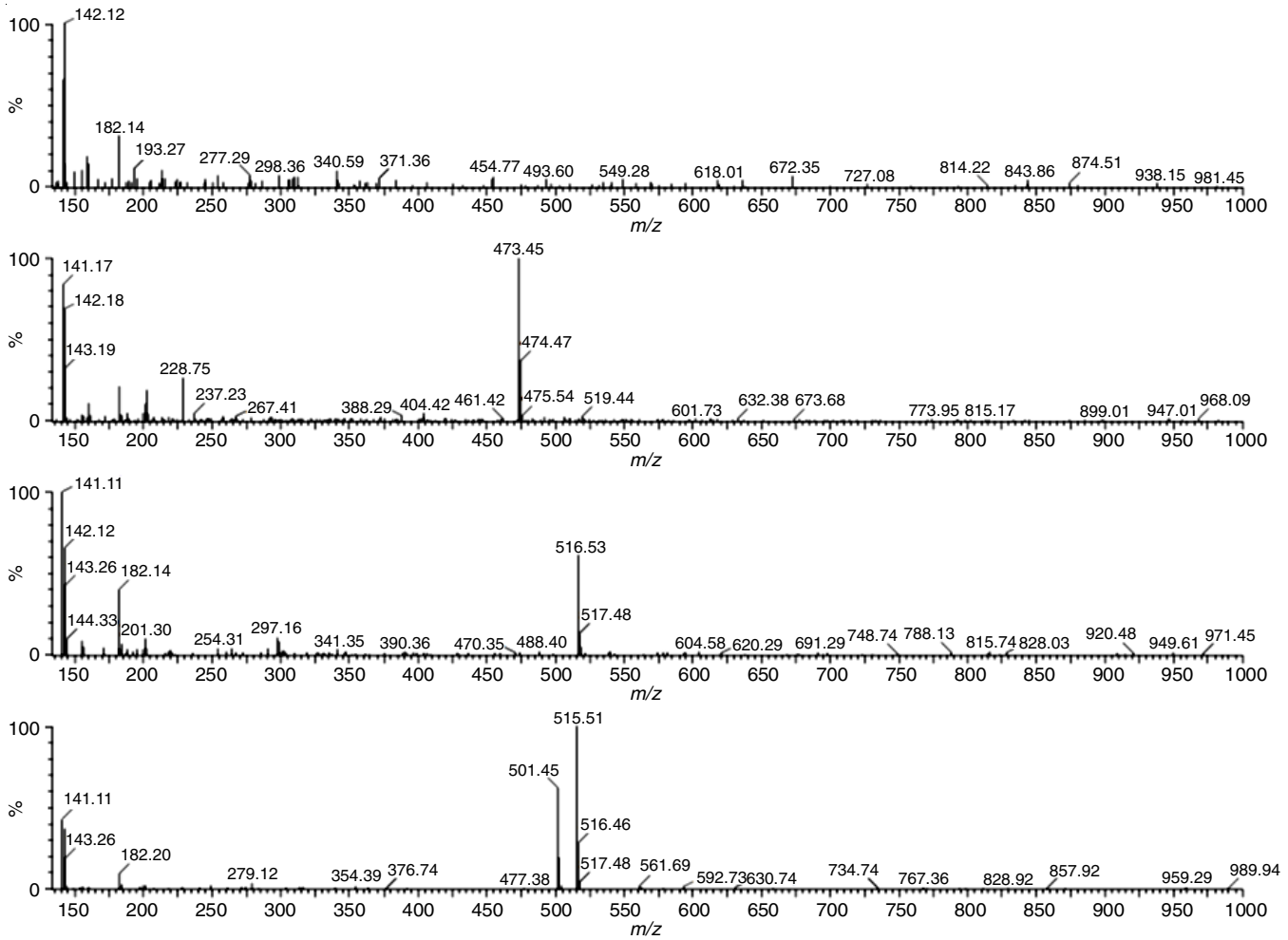


Fig. 7. Mass spectra of EPN samples exposed hydrogen peroxide (30% H_2O_2, 24 h reflux at 60 °C)

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