Section A-Research paper

Method Development, Validation and Forced Degradation Studies of Empagliflozin and Metformin in Combined Dosage Forms by RP-HPLC

Ms. Anamika Singh, Dr. Tamanna Narsinghani

School of Pharmacy, Devi Ahilya Vishwavidyalaya, Indore, India

Corresponding Author: Dr. Tamanna Narsinghani

Email ID: kashishnarsinghani@rediffmail.com

ABSTRACT:

A simple, sensitive, robust, precise, and efficient RP-HPLC approach for the simultaneous determination of Empagliflozin and Metformin in combination. As per ICH Q2 (R1) guidelines, the final chromatographic conditions were optimized with a mobilephase ratio of (30:70% v/v) in Water containing 0.1% formic acid: Methanol containing 0.1% formic acid at a flow rate of 1 mL/min, column temperature of 35°C, injection volume of 20µL, Interstil C18 analytical column. Empagliflozin and Metformin reported retention times of 1.73 min and 2.35 min, respectively. Validation of a method was found to be linear in the range of 0.5-1.5 µg/ml for Empagliflozin and 0.63-1.88 µg/mL for Metformin. The % Recovery for Empagliflozin was discovered tobe 99.913-100.718%, while for Metformin, it was found to be 99.964-100.188%. The Precision results for both drugs were within the limits while expressed Intraday and Interday. For Empagliflozin, the LOD and LOQ were reported to be 0.106 µg/mLand 0.323 µg/mL, respectively, and for Metformin, 0.129 µg/mL and 0.389 µg/mL. As per ICH Q1A (R2) guidelines, thecombination was subjected to acid, base, oxidation, thermal, andphotolysis stress conditions.

KEYWORDS: RP-HPLC, Empagliflozin, Metformin, Forced Degradation, ICH guidelines

INTRODUCTION

Diabetes is a long-term condition in which the body'sability to produce or respond to the hormone insulinis disrupted, resulting in incorrect carbohydrate metabolism and high blood glucose levels. To maintain a tight check on your blood sugar levels, you should use a combination of medicines, exercise, andfood and keep them within a range set by your doctor.By paying close attention to what and when you eat,you can minimize or avoid the "seesaw effect" of rapidly shifting blood sugar

levels, which can need quick adjustments in prescription dosages, especially insulin, by paying close attention to what and when you eat.

Empagliflozin is a sodium-glucose cotransporter 2 (SGLT2) inhibitor and metformin hydrochloride is a biguanide, indicated as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus when treatment with both empagliflozin and metformin hydrochloride is appropriate [1-6]. For the simultaneous determination of EMPA and MET, several analytical techniques have been published for single drugs but not for this combination. UV spectrophotometric methods [7-11]and HPLC [12-20] were among the methods used.

Analytical method validation ensures that different HPLC analytical procedures provide consistent and repeatable results; it is a crucial stage in the development of a method from a synthetic mixture since it provides information on precision, linearity, detection, and quantitation limits. "The goal of validation of an analytical method is to demonstrate that it is suitable for its intended purpose," according to ICH standards. Validation data must now be provided to the appropriate authorities during the pharmaceutical development process. The ICH has validation requirements for analytical procedures [21]. This study developed a new sensitive and quick RP- HPLC technique for identifying Empagliflozin and Metformin in combination, which was then validated as per ICH Q2 (R1) regulations. The ICH Q1A (R2) recommendations were used to conduct forced degradation tests in which the combination was subjected to acid, base, oxidation, heat, and photolysis stress conditions [22].

MATERIALS AND METHODS

Drug Identification: The identification of Metformin and Empagliflozin standard API for experimental work had done for confirmation of its identity, standard, quality and purity. The identification was done by taking IR, solubility, melting point determination and mass spectra.

Melting Point Determination: Melting point of Metformin and Empagliflozin has been determined using Capillary Method. Drug was taken in capillary and capillary is placed into melting point apparatus. Result of determination is shown in Table 1.

Solubility Study: The solubility of Metformin and Empagliflozin was practically determined as per Indian pharmacopoeia. Solubility was determined by taking 100.0 mg of in 100 ml volumetric flasks, adding required quantity of solvent at room temperature and shaken for few minutes. Solubility data for each study was observed and recorded in Table 2.

IR spectra and Structure Interpretation: IR spectra of Metformin and Empagliflozin drug were taken for structure interpretation from % transmission at specified wave numbers. Direct reflectance

method was used. Initially background scan is taken. A suitable amount of sample is placed on the ATR crystal. Then a pressure is applied between ATR crystal and sample to ensure contact between ATR and sample. Then the sample is scan for appropriate minute.

Mass spectra and its Fragmentation: Mass spectra of Metformin and Empagliflozin drug were taken by electrospray ionization technique (ESI MS) to identify and characterized its molecular mass and its fragmentation by plotting a graph of intensity versus m/z ratio. The drug solution is infused into mass spectrometer by syringe method and its fragmentation is identified by MRM scan (Q1→Q3).

Selection of Chromatographic Condition: Proper selection of the LC-MS/MS method depends upon the nature of the sample (ionic or neutral molecules), its molecular weight, pK_a and solubility. LC-MS/MS was selected for the initial separation based on literature survey and its simplicity and suitability. To optimize the chromatographic conditions the effect of chromatographic variables such as mobile phase, pH, flow rate and solvent ratio were studied.

Procedure for Solution Preparation

Preparation of Diluent: The diluent containing water and methanol is prepared in the ratio of 70:30.

Preparation of Standard Stock Solution of Metformin: Accurately weighed separately quantity of 12.5 mg Metformin API were transferred into 100 ml volumetric flask and dissolved in diluent using ultra sonication and diluted up to mark to give a stock solution having concentration of 125µg/ml Metformin.

Preparation of Standard Stock Solution of Empagliflozin: Accurately weighed separately quantity of 10 mg Empagliflozin API were transferred into 100 ml volumetric flask and dissolved in diluent using ultra sonication and diluted up to mark to give a stock solution having concentration of 100µg/ml Empagliflozin.

Preparation of Working Standard Solution of Metformin: From above Standard Stock Solution of Metformin, 1 ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 1.25 µg/ml of Metformin.

Preparation of Working Standard Solution of Empagliflozin: From above Standard Stock Solution of Empagliflozin, 1 ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 1 µg/ml of Empagliflozin.

Combine Preparation of Working Standard Solution of Metformin and Empagliflozin: Take 1ml from Metformin stock solution and 1ml from Empagliflozin stock solution into 100ml volumetric flask and make up the volume with diluent to get 1.25 µg/ml of Metformin and 1 µg/ml of Empagliflozin.

Preparation of Mobile Phase: Prepare 0.1% Formic Acid in water and Methanol in the ratio of 30:70.Mix well and degas by sonication.

Preparation of Sample Stock Solution of Metformin and Empagliflozin: The average weight of 10 tablets was determined and was ground in a mortar. Stock solution was prepared by dissolving tablet powder equivalent to 12.5 mg of Metformin and 10.0 mg of Empagliflozin was transferred to 100ml volumetric flask. Then 50 ml diluent was added and sonicated for 5 mins to ensure complete solubilization of drug. After sonication, volume was made up to the mark with diluent. Filter the stock solution with 0.45µ Millipore filter and the final filtrate is collected as sample stock solution.

Preparation of Sample Working Solution of Metformin and Empagliflozin: From above Sample Stock Solution of Metformin and Empagliflozin, 1 ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 1.25 µg/ml of Metformin and 1.0 µg/ml Empagliflozin.

Chromatographic Separation: Standard solutions of Metformin and Empagliflozin were injected in column with 20 µl micro-syringe. The chromatogram was run for appropriate minutes with mobile phase. The chromatogram was stopped after separation achieved completely. Data related to peak like area, mass, retention time etc. were recorded using software.

VALIDATION OF LC-MS/MS METHOD

Specificity: The blank solution, working standard solution and working sample solution of Metformin and Empagliflozin is injected in to the LC-MS/MS system. The chromatogram of standard and sample has no interference with the chromatogram of blank.

Linearity and Range: The linearity for Metformin and Empagliflozin were assessed by analysis of standard solution in range of 0.6-1.8 µg/ml for Metformin and 0.5-1.5µg/ml Empagliflozin respectively. 0.5, 0.75, 1.0, 1.25, 1.50 ml solutions were pipette out from the Stock solution of Metformin and Empagliflozin and transfer to 100 ml volumetric flask and make up with diluent to obtain 0.63, 0.94, 1.25, 1.56 and 1.88 µg/ml for Metformin and 0.5, 0.75, 1.0, 1.25 and 1.50 µg/ml for Empagliflozin respectively. In term of slope, intercept and correlation co-efficient value is obtained. The graph of peak area obtained verses respective concentration was plotted.

Precision

Repeatability: Standard solution containing Metformin (1.25 µg/ml) and Empagliflozin (1.0 µg/ml) was injected six times and areas of peaks were measured and % R.S.D. was calculated.

Intraday Precision: Standard solution containing (0.625, 1.25, 1.875µg/ml) of Metformin and (0.50, 1.0, 1.5 µg/ml) Empagliflozin were analyzed three times on the same day and % R.S.D was calculated.

Interday Precision: Standard solution containing (0.625, 1.25, 1.875µg/ml)of Metformin and (0.50, 1.0, 1.5 µg/ml) Empagliflozin were analyzed three times on different day and % R.S.D was calculated.

Accuracy: 0.5 µg/ml drug solutions were taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 100ml. The area of each solution peak was measured. The amount of Metformin and Empagliflozin was calculated at each level and % recoveries were computed.

Limit of Detection and Limit of Quantitation: The LOD and LOQ were estimated from the set of 3 calibration curves used to determination method linearity.

Robustness: Following parameters were changed one by one and their effect was observed on system suitability for standard preparation.

1. Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min.
2. Ratio of Mobile phase was changed (±2) Buffer: Methanol (32:68) and Buffer: Methanol (28:72).

Analysis of Market Formulation: Take tablet powder equivalent to 12.5 mg Metformin and 10.0mg of Empagliflozin was transferred to a 100 ml volumetric flask, shake for 15 minutes and made up volume up to the mark with diluent. The solution was filtered through 0.45µ Millipore filter and first few drops of filtrate were discarded. 1 ml of this solution was diluted to 100 ml with diluent. The solution was injected 20 µl into the LC-MS/MS system. The areas of resulting peak were measured.

RESULTS AND DICUSSION

Drug Identification: The identification of drugs was carried out by performing melting point determination, solubility study and taking IR and mass spectra as preliminary work which showed into following results.

Melting Point Determination: Melting point of Metformin and Empagliflozin has been determined using Capillary Method.

Table: Melting Point of Drugs

Drug Name	Reported (°C)	Observed (°C)
Metformin	222-226^{o}C	221-223^{o}C
Empagliflozin	151-153 ^{o}C	151-152 ^{o}C

Observation: Melting point of Metformin and Empagliflozin was found to be in the range of acceptance criteria as shown in above table.

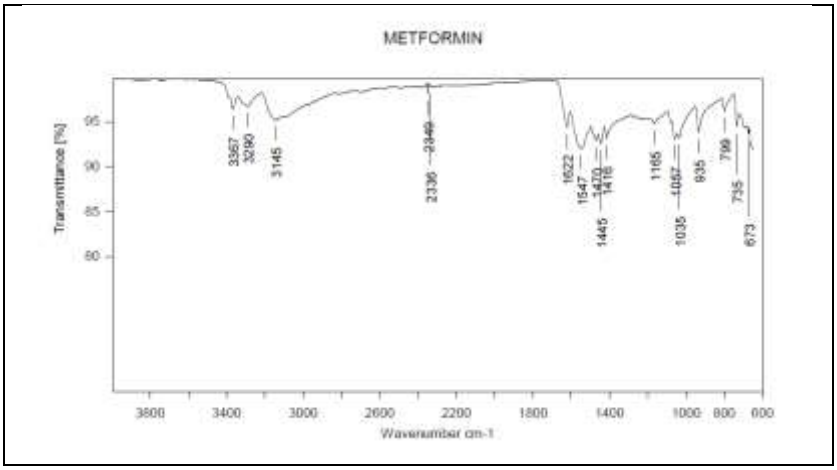
Solubility Study

Table: Solubility Data of Metformin and Empagliflozin

Solvent	Solubility of Metformin	Solubility of Empagliflozin
Water	Very slightly soluble	slightly soluble
Acetonitrile	Freely soluble	Soluble
Methanol	Soluble	Freely soluble

Identification by IR Spectroscopy

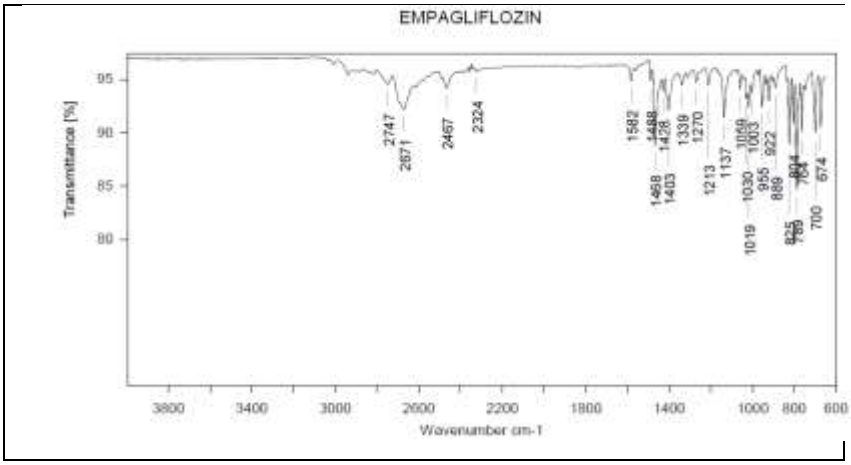
1) Metformin



Functional Group	Frequency (cm^{-1})
N-H stretching	3145-3367
C-N stretching	1165
O-H bending	1622

Fig: IR Spectra of Metformin

2) Empagliflozin



Functional Group	Frequency (cm^{-1})
C-CL stretching	600-800
C=C stretching	1428-1582
O-H stretching	2671

Fig: IR Spectra of Empagliflozin

Conclusion: From the IR interpretation data it can be concluded that major functional group peak are observed in IR spectra of the drug samples. So it reveals that the given sample is of Metformin and Empagliflozin drug.

METHOD DEVELOPMENT

- **Chromatographic condition:**

Column	:	Intertsil ODS, C18, (100mm x 2.1mm), 1.6µm			
Flow rate	:	1.0 mL/min	Injection volume	:	20 µL
Column oven temperature	:	35 °C	Run time	:	9 min
Column oven compartment	:	Ambient	Mode	:	Isocratic
Metformin R.T	:	About 1.73 min			
Empagliflozin R.T	:	About 2.35 min			

Chromatographic Trials

Table: Effect of different mobile phase compositions on the separation of Metformin and Empagliflozin

Mobile Phase	Ratio (v/v)	Retention Time	Remarks
Metformin and Empagliflozin in Water containing 0.1% formic acid: Methanol containing 0.1% formic acid	50:50	2.38 (Metformin) 6.04(Empagliflozin)	Both the peak is observed but Empagliflozin peak shape is not proper.
Metformin and Empagliflozin in Water containing 0.1% formic acid: Methanol containing 0.1% formic acid	40:60	2.17 (Metformin) 3.47(Empagliflozin)	Both the peak is observed but both peak shapes is not proper.
Metformin and Empagliflozin in Water containing 0.1% formic acid: Methanol containing 0.1% formic acid	30:70	1.73 (Metformin) 2.35 (Empagliflozin)	Both the peak is observed with good separation between them.

After considering the varying combinations of various mobile phases, Water containing 0.1% formic acid: Methanol containing 0.1% formic acid was finalized as it was showing good peak shape with less retention time

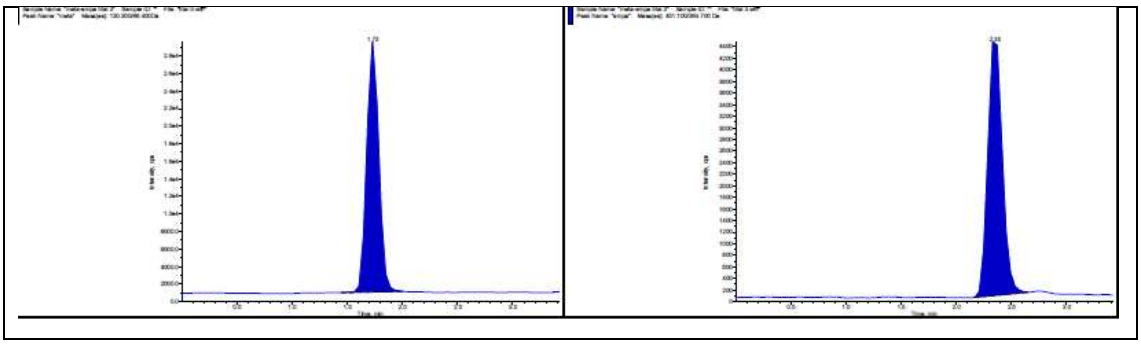


Fig : Chromatogram of Metformin and Empagliflozin in Water: Methanol Water: Methanol: 0.1% formic acid (30:70v/v)

Table: LC-MS/MS Chromatographic Condition

Instrument	Liquid chromatography Mass spectrometer (API-2000) equipped with auto sample, auto injector, column oven, ion source ESI electron spray ionizer with Q1 and collision energy.		
Ion Source setting		***Scan setting***	
Ion source	ESI	Polarity	Positive ion
Curtain Gas	20psi	Scan type	MRM
Ion Spray Voltage	5000	Scan time	1-4 min
Temperature	400°C	Declustering Potential	50V
Ion Source Gas(GS1)	50psi	Focusing Potential	400V
Ion Source Gas(GS2)	60psi	Entrance Potential	10V
Scan type	Metformin	MRM:(Q1)130.300 Da and (Q3)86.400 Da	
	Empagliflozin	MRM:(Q1)451.100 Da and (Q3)364.700 Da	

- **Chromatographic condition:**

Column	**:**	Intertsil ODS, C18, (100mm x 2.1mm), 1.6µm			
Flow rate	**:**	1.0 mL/min	Injection volume	**:**	20 µL
Column oven temperature	**:**	35 °C	Run time	**:**	9 min
Column oven compartment	**:**	Ambient	Mode	**:**	Isocratic
Metformin R.T	**:**	About 1.73 min			
Empagliflozin R.T	**:**	About 2.35 min			

FORCED DEGRADATION STUDY

Metformin and Empagliflozin standard was injected under various stress conditions. The optimized degradation condition is shown below.

Table 6.7: Different Degradation Conditions for Empagliflozin & Metformin

Sr. No.	**Stress Type**	**Empagliflozin**	**Metformin**
		1 N HCl at 80 ^{o}C for 4 hr.	**Stress Condition**
1	Acid Degradation	1 N NaOH at 80 ^{o}C for 24 hr.	1 N HCl at 80 ^{o}C for 24 hr.
2	Base Degradation	30.0 % H_2O_2 at 80 ^{o}C for 5 hrs.	1 N NaOH at 80 ^{o}C for 4 hr.
3	Oxidative Degradation	105 ^{o}C for 3 days	30.0 % H_2O_2 at 80 ^{o}C for 6 hrs.
4	Thermal Degradation	UV for 3 days	105 ^{o}C for 3 days
5	Photolytic Degradation	1 N HCl at 80 ^{o}C for 4 hr.	UV for 3 days

METFORMIN ACID DEGRADATION PATHWAY
Metformin (130.300 Da)

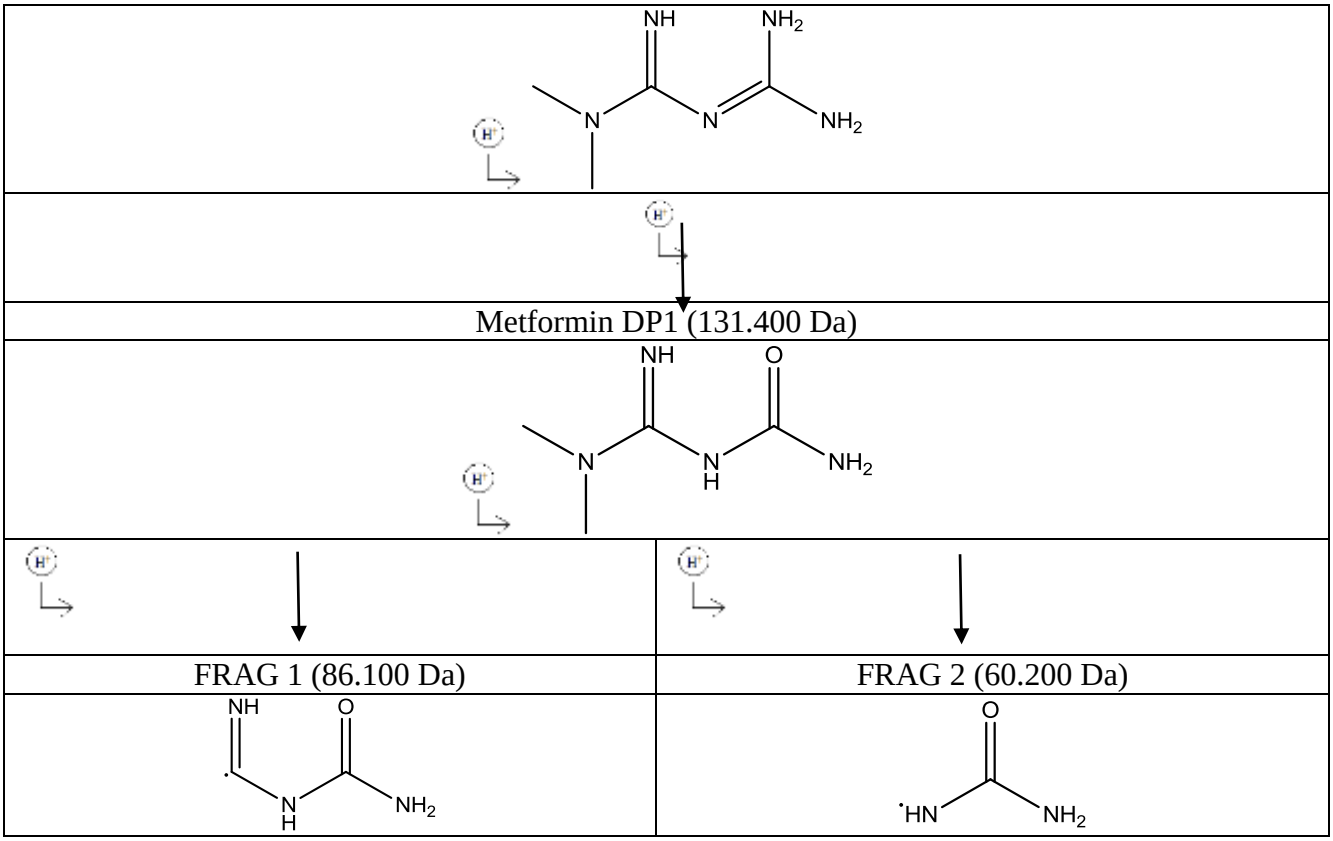


Fig: Metformin acid Degradation Pathway

Observation

- The acidic degradation solution of Metformin is infused into the mass spectrometer to identified and characterized acidic degradation product (i.e DP1) of Metformin.
- From the above mass spectra and fragmentation pathway, it is found that, Metformin is degraded under acid condition.
- The ESI-MS/MS spectra shows basic degradation product DP1 whose m/z ratio is obtained around 131.400 Da and its fragment ion peak is found whose m/z ratio is obtained around 86.100 Da and 60.200 Da
- For MRM scan, Metformin basic degradation product (DP1) molecular mass (Q1) is 131.400 Da and its fragment mass (Q3) is 86.100 Da is selected

ESI-MS/MS Spectra and Fragmentation Pattern of basic Degradation Solution of Metformin: Mass spectra of Metformin under basic degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.

METFORMIN BASE DEGRADATION PATHWAY
Metformin (130.300 Da)

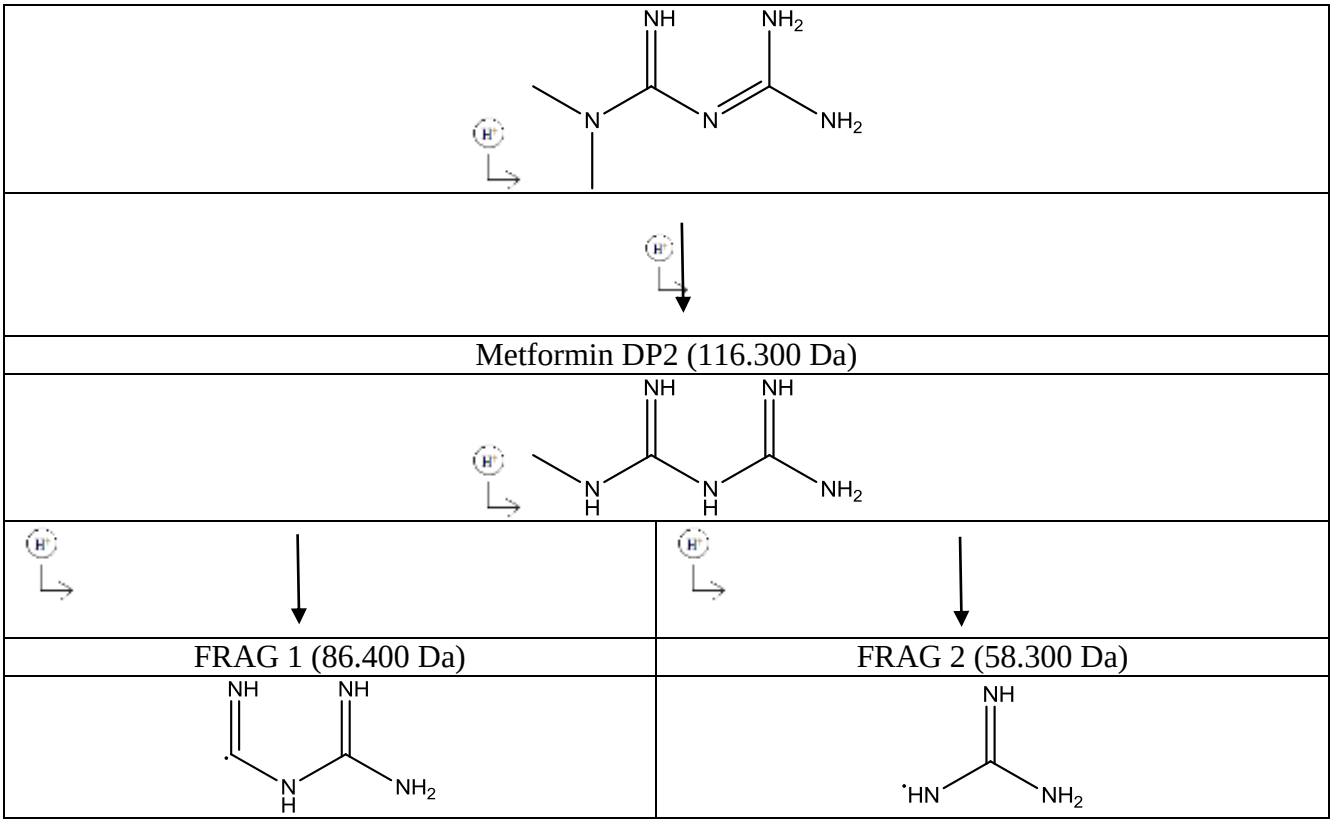


Fig: Metformin basic Degradation Pathway

Observation

- The basic degradation solution of Metformin is infused into the mass spectrometer to identified and characterized basic degradation product (i.e DP2) of Metformin.
- From the above mass spectra and fragmentation pathway, it is found that, Metformin is degraded under basic condition.
- The ESI-MS/MS spectra shows basic degradation product DP1 whose m/z ratio is obtained around 116.300 Da and its fragment ion peak is found whose m/z ratio is obtained around 86.400 Da and 58.300 Da
- For MRM scan, Metformin basic degradation product (DP1) molecular mass (Q1) is 58.300 Da and its fragment mass (Q3) is 86.400 Da is selected

ESI-MS/MS Spectra and Fragmentation Pattern of oxidative Degradation Solution of Metformin: Mass spectra of Metformin under oxidative degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.

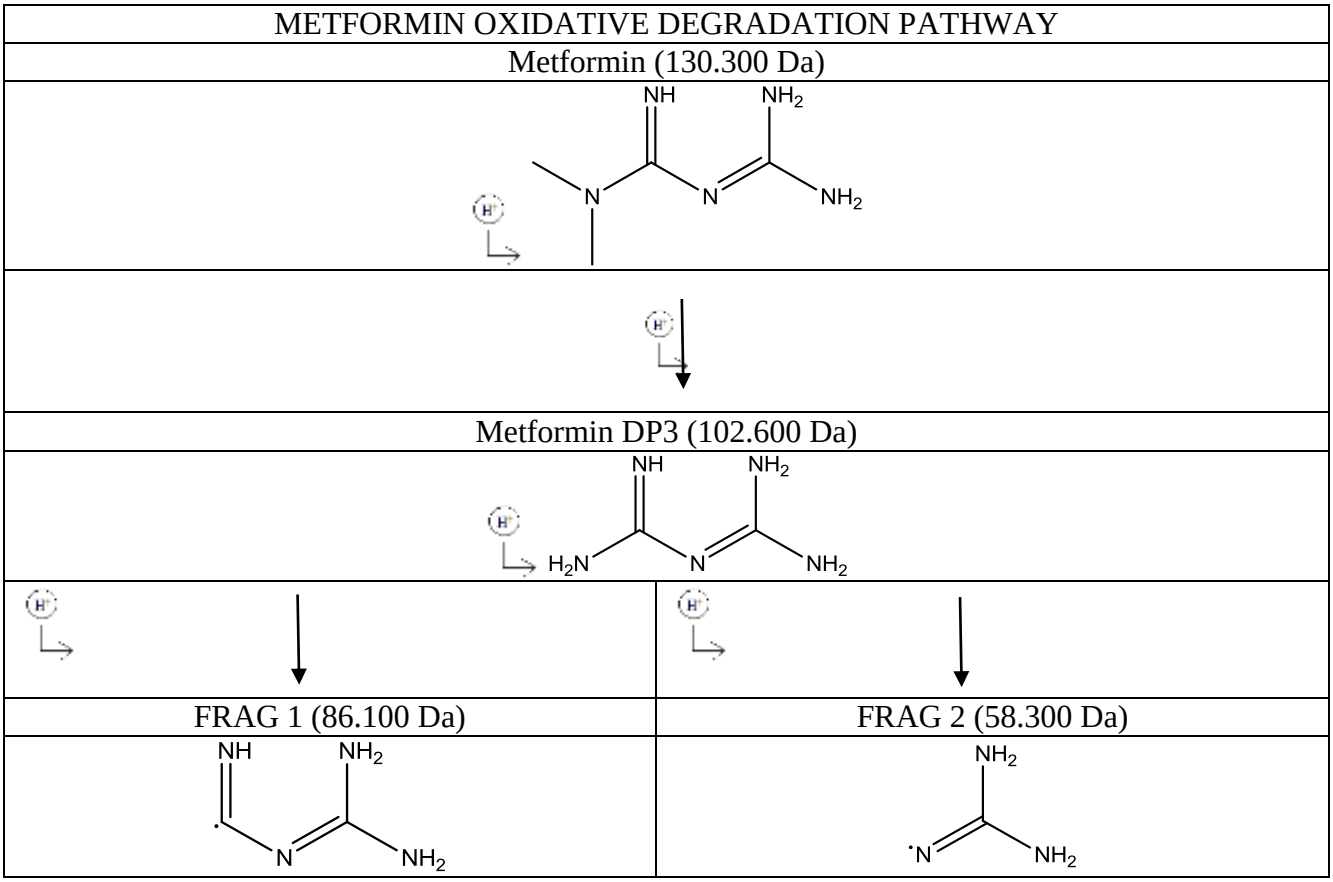


Fig: Metformin oxidative Degradation Pathway

Observation

- The oxidative degradation solution of Metformin is infused into the mass spectrometer to identified and characterized basic degradation product (i.e DP1) of Metformin.
- From the above mass spectra and fragmentation pathway, it is found that, Metformin is degraded under oxidative condition.
- The ESI-MS/MS spectra shows oxidative degradation product DP1 whose m/z ratio is obtained around 102.600 Da and its fragment ion peak is found whose m/z ratio is obtained around 86.100 Da and 58.300 Da
- For MRM scan, Metformin oxidative degradation product (DP1) molecular mass (Q1) is 102.600 Da and its fragment mass (Q3) is 86.100 Da is selected.

ESI-MS/MS Spectra and Fragmentation Pattern of thermal Degradation Solution of Metformin: Mass spectra of Metformin under thermal degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.

Observation

- The thermal degradation solution of Metformin is infused into the mass spectrometer to

identified and characterized thermal degradation product (i.e DP1) of Metformin.

- From the above mass spectra, it is found that, Metformin is not degraded under thermal condition.
- Hence, it was found that Metformin was stable under thermal conditions.

ESI-MS/MS Spectra and Fragmentation Pattern of photolytic Degradation Solution of Metformin: Mass spectra of Metformin under photolytic degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.

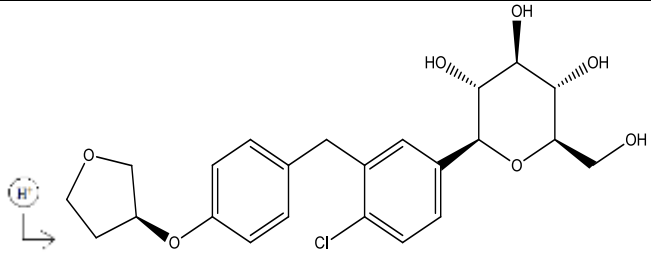
Observation

- The photolytic degradation solution of Metformin is infused into the mass spectrometer to identified and characterized photolytic degradation product (i.e DP1) of Metformin.
- From the above mass spectra, it is found that, Metformin is not degraded under photolytic condition.
- Hence, it was found that Metformin was stable under photolytic conditions.

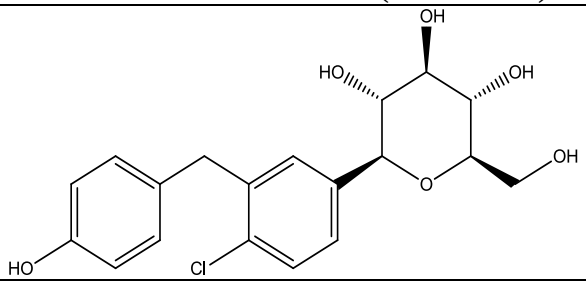
ESI-MS/MS Spectra and Fragmentation Pattern of acidic Degradation Solution of Empagliflozin: Mass spectra of Empagliflozin under basic degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.

EMPAGLOFLOZIN ACID DEGRADATION PATHWAY

EMPAGLOFLOZIN (451.100 Da)



EMPAGLOFLOZIN DP1 (381.500 Da)



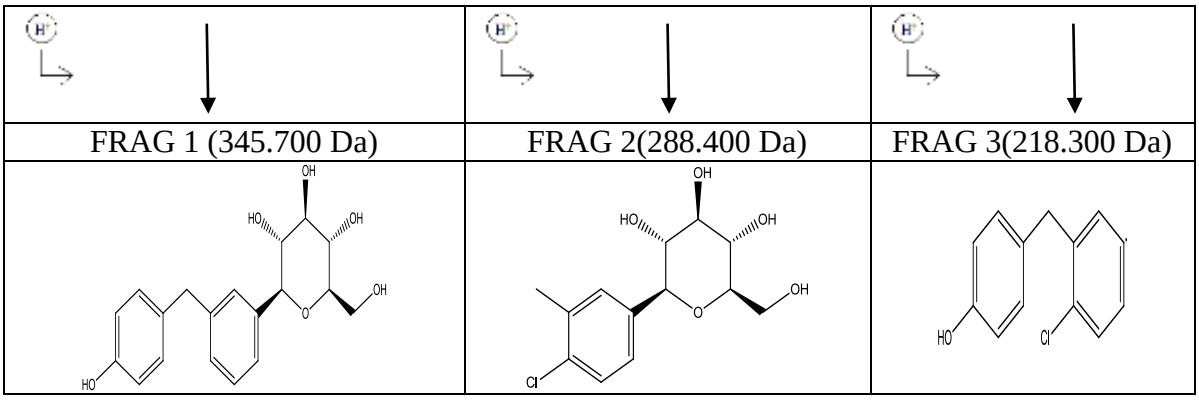
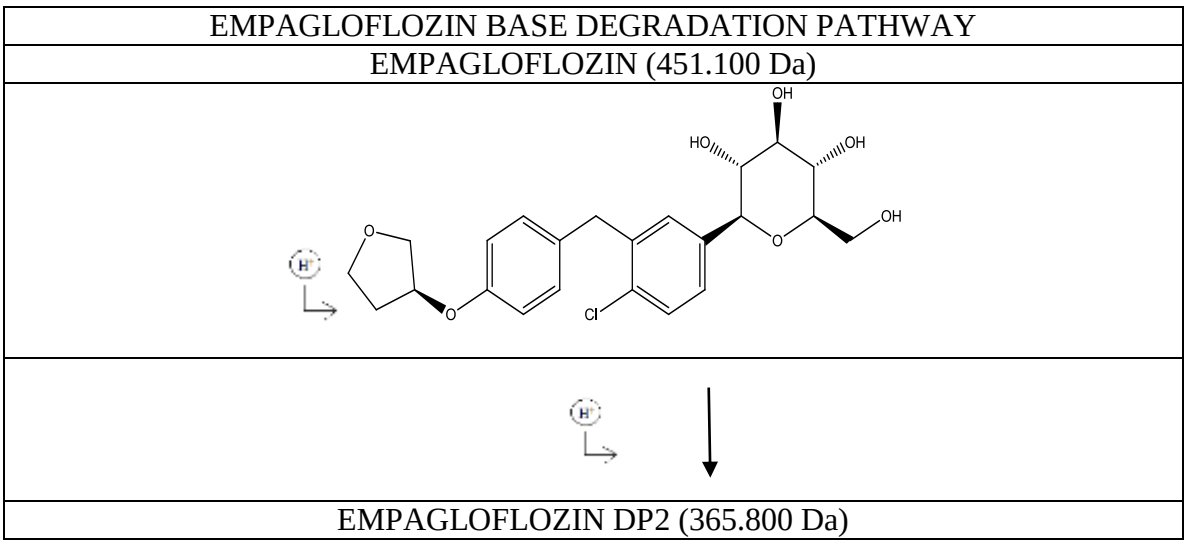


Fig: Empagliflozin acidic Degradation Pathway

Observation

- The acidic degradation solution of Empagliflozin is infused into the mass spectrometer to identified and characterized acidic degradation product (i.e DP1) of Empagliflozin
- From the above mass spectra and fragmentation pathway, it is found that, Empagliflozin is degraded under acidic condition.
- The ESI-MS/MS spectra shows acidic degradation product DP1 whose m/z ratio is obtained around 381.500 Da and its fragment ion peak is found whose m/z ratio is obtained around 345.700 Da, 288.400 Da and 218.300 Da.
- For MRM scan, Empagliflozin acidic degradation product (DP1) molecular mass (Q1) is 381.500 Da and its fragment mass (Q3) is 288.400 Da is selected

ESI-MS/MS Spectra and Fragmentation Pattern of basic Degradation Solution of Metformin:
Mass spectra of Empagliflozin under basic degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.



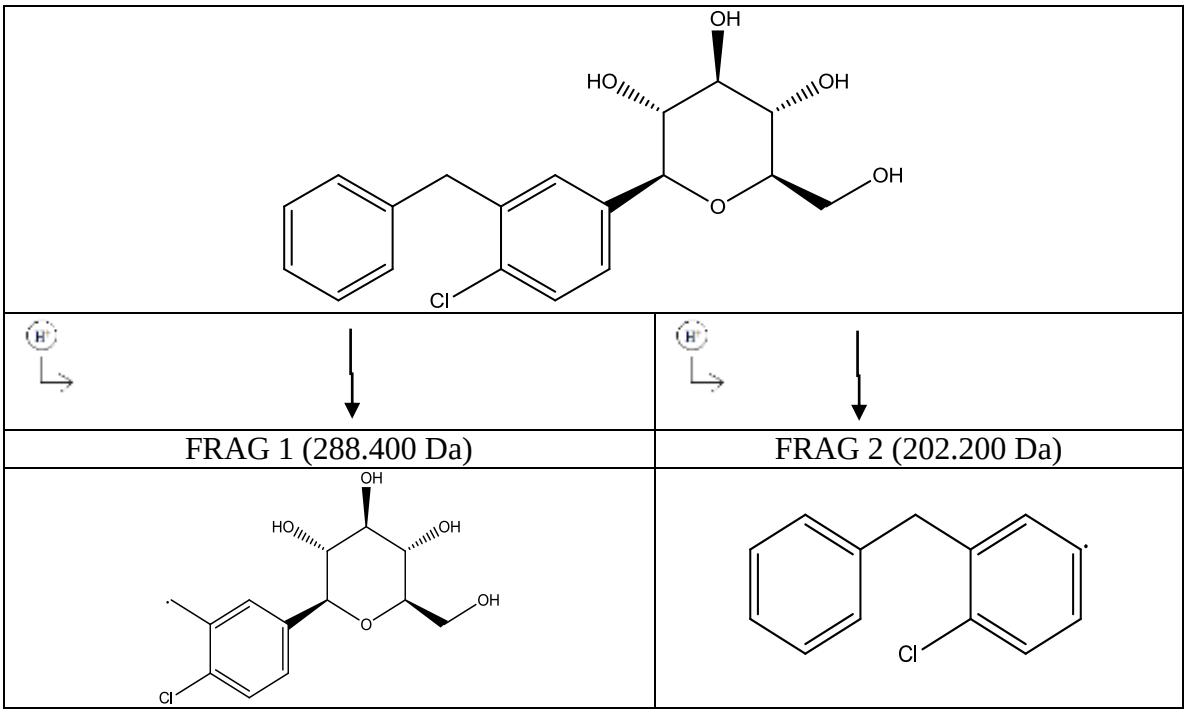


Fig: Empagliflozin basic Degradation Pathway

Observation

- The basic degradation solution of Empagliflozin is infused into the mass spectrometer to identified and characterized basic degradation product (i.e DP1) of Metformin.
- From the above mass spectra and fragmentation pathway, it is found that, Empagliflozin is degraded under basic condition.
- The ESI-MS/MS spectra shows acidic degradation product DP1 whose m/z ratio is obtained around 365.800 Da and its fragment ion peak is found whose m/z ratio is obtained around 288.400 Da and 202.200 Da.
- For MRM scan, Empagliflozin acidic degradation product (DP1) molecular mass (Q1) is 365.800 Da and its fragment mass (Q3) is 288.400 Da is selected

Hence, it was found that Metformin was stable under basic conditions

ESI-MS/MS Spectra and Fragmentation Pattern of oxidative Degradation Solution of Empagliflozin: Mass spectra of Empagliflozin under oxidative degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.

Observation

- The oxidative degradation solution of Empagliflozin is infused into the mass spectrometer to identified and characterized oxidative degradation product (i.e DP1) of Empagliflozin
- From the above mass spectra and fragmentation pathway, it is found that, Empagliflozin is not degraded under oxidative condition.

ESI-MS/MS Spectra and Fragmentation Pattern of thermal Degradation Solution of Metformin: Mass spectra of Empagliflozin under thermal degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.

Observation

- The thermal degradation solution of Empagliflozin is infused into the mass spectrometer to identified and characterized thermal degradation product (i.e DP1) of Metformin.
- From the above mass spectra, it is found that, thermal is not degraded under thermal condition.

Hence, it was found that Empagliflozin was stable under thermal conditions

ESI-MS/MS Spectra and Fragmentation Pattern of photolytic Degradation Solution of Metformin: Mass spectra of Metformin under photolytic degradation conditions were taken and its fragmentation pattern was observed using ESI-MS/MS.

Observation

- The photolytic degradation solution of Empagliflozin is infused into the mass spectrometer to identified and characterized photolytic degradation product (i.e DP1) of Metformin.
- From the above mass spectra, it is found that, photolytic is not degraded under photolytic condition.

Hence, it was found that Empagliflozin was stable under photolytic condition

Chromatographic Conditions for LC-MS/MS for Forced Degradation Studies

Table: Chromatographic Conditions of LC-MS/MS for Forced Degradation Studies

Instrument	Liquid chromatography Mass spectrometer (API-2000) equipped with auto sample, auto injector, column oven, ion source ESI electron spray ionizer with Q1 and collision energy.		
Ion Source setting		***Scan setting***	
Ion source	ESI	Polarity	Positive ion
Curtain Gas	20psi	Scan type	MRM
Ion Spray Voltage	5000	Scan time	1-4 min
Temperature	400°C	Declustering Potential	50V
Ion Source Gas(GS1)	50psi	Focusing Potential	400V
Ion Source Gas(GS2)	60psi	Entrance Potential	10V
Scan type	Metformin	MRM:(Q1)130.300 Da and (Q3)86.400 Da	
	Empagliflozin	MRM:(Q1)451.100 Da and (Q3)364.700 Da	
	Meta DP1	MRM:(Q1)131.400 Da and (Q3)86.100 Da	
	Meta DP2	MRM:(Q1)116.300 Da and (Q3)86.400 Da	
	Meta DP3	MRM:(Q1)102.600 Da and (Q3)86.100 Da	
	Empa DP1	MRM:(Q1)381.500 Da and (Q3)288.400 Da	
	Empa DP 2	MRM:(Q1)365.800 Da and (Q3)288.400 Da	

- **Chromatographic condition:**

Column	:	Intertsil ODS, C18, (100mm x 2.1mm), 1.6µm			
Flow rate	:	1.0 mL/min	Injection volume	:	20 µL
Column oven temperature	:	35 °C	Run time	:	4 min
Column oven compartment	:	Ambient	Mode	:	Isocratic
Metformin R.T	:	About 1.73 min			
Empagliflozin R.T	:	About 2.03 min			
Meta DP1 R.T	:	About 1.18 min			
Meta DP2 R.T	:	About 1.57 min			
Meta DP3 R.T		About 5.62 min			
Empa DP1 R.T	:	About 1.13 min			
Empa DP2 R.T	:	About 1.78 min			

Fig.: Chromatogram for Acid Degradation of Metformin

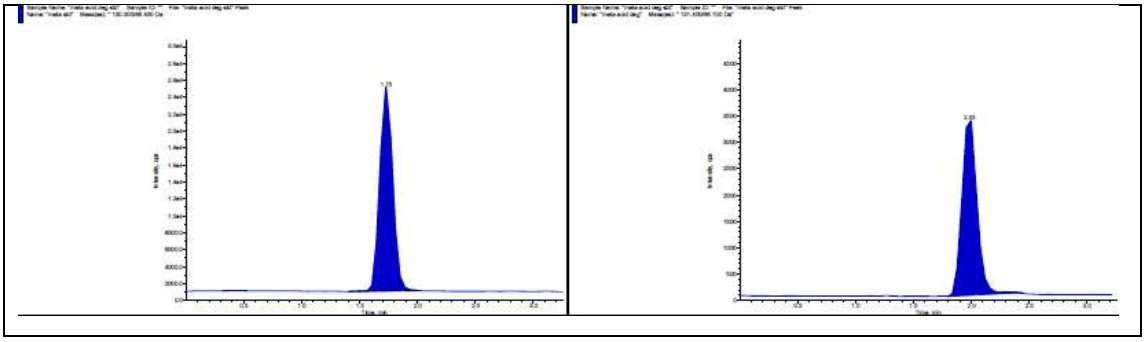


Fig.: Chromatogram for base Degradation of Metformin

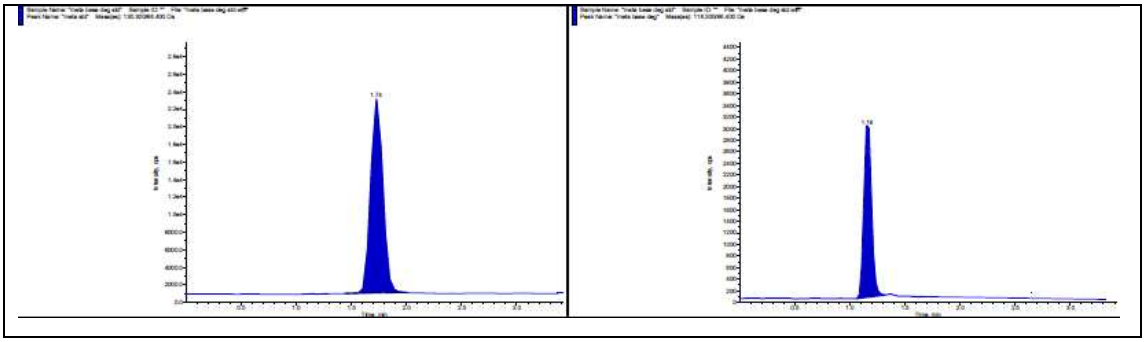


Fig.: Chromatogram for oxidative Degradation of Metformin

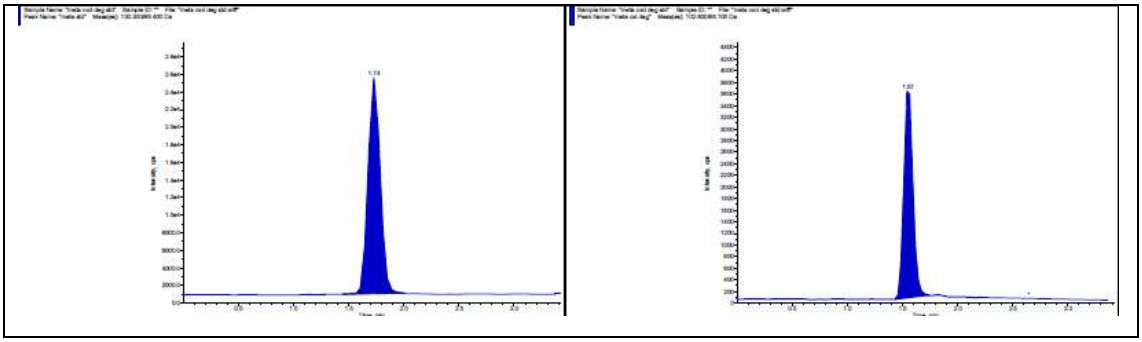


Fig.: Chromatogram for acidic Degradation of Empagliflozin

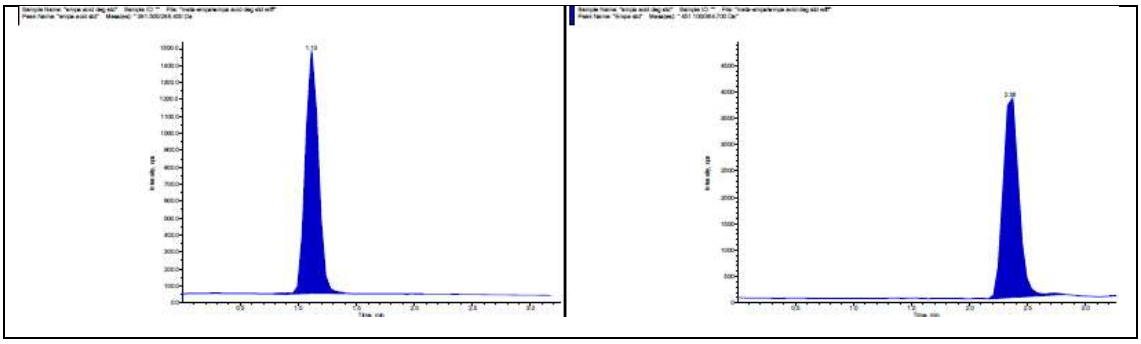
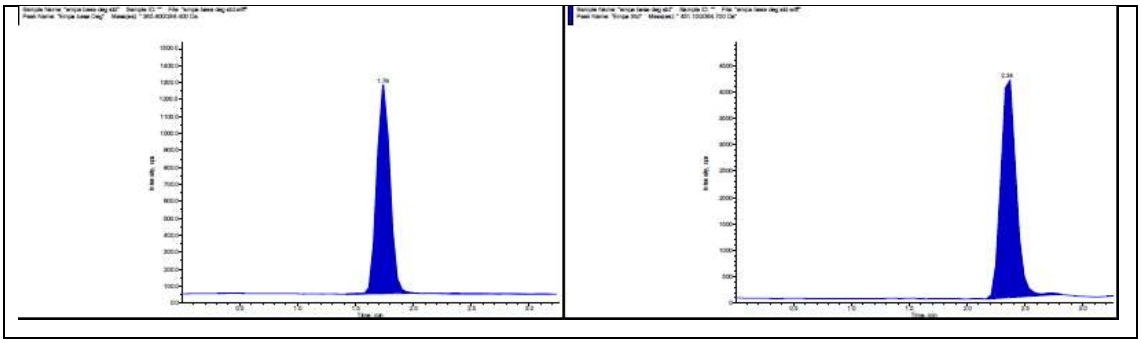


Fig.: Chromatogram for base Degradation of Empagliflozin



METHOD VALIDATION

Specificity

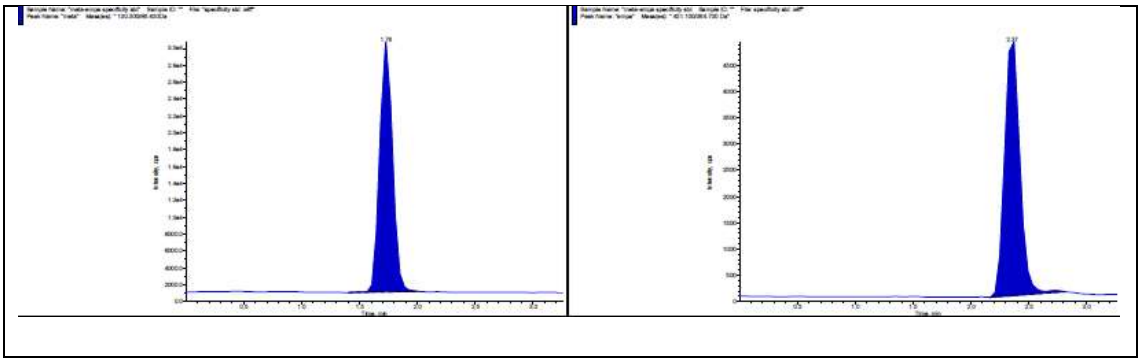


Fig: Chromatogram of Metformin and Empagliflozin Standard

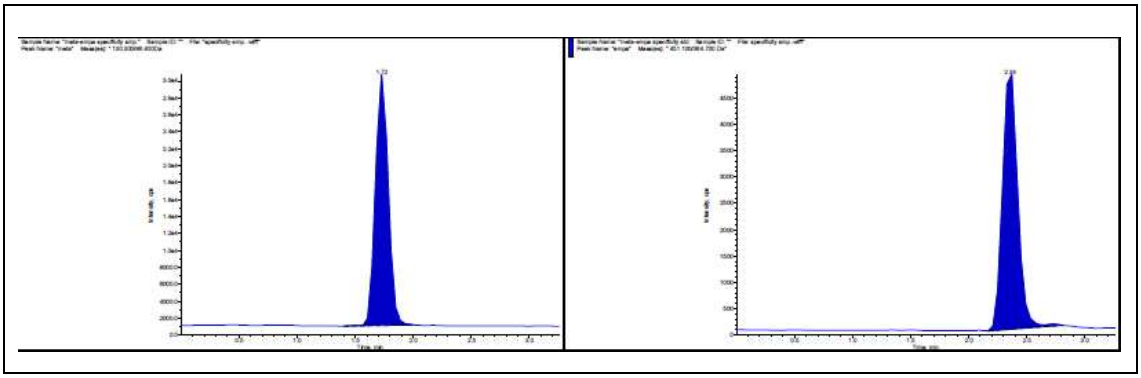


Fig.: Chromatogram of Metformin and Empagliflozin Sample

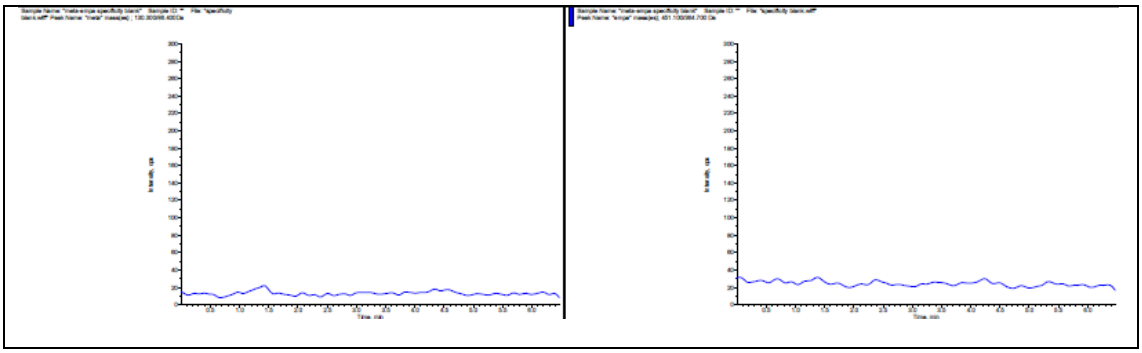


Fig.: Chromatogram of Metformin and Empagliflozin Blank

The Chromatograms of Metformin and Empagliflozin standards and Metformin and Empagliflozin sample show no interference with the Chromatogram of Metformin and Empagliflozin Blank, so the Developed method is Specific.

Linearity and Range: The linearity for Metformin and Empagliflozin were assessed by analysis of standard solution in range of 0.63-1.88 µg/ml and 0.5-1.5 Metformin and Empagliflozin respectively. Correlation co-efficient for calibration curve Metformin and Empagliflozin was found to be 0.998 and 0.998 respectively.

The regression line equation for Metformin and Empagliflozin are as following:

For Metformin and Empagliflozin: y = 80.48x - 19.12 and y = y = 16588x + 15.821

Linearity Data for Metformin

Sr. No	Concentration (µg/ml)	Area
1	0.63	11568.218
2	0.94	16828.746
3	1.25	22276.095
4	1.56	25937.998
5	1.88	32229.647

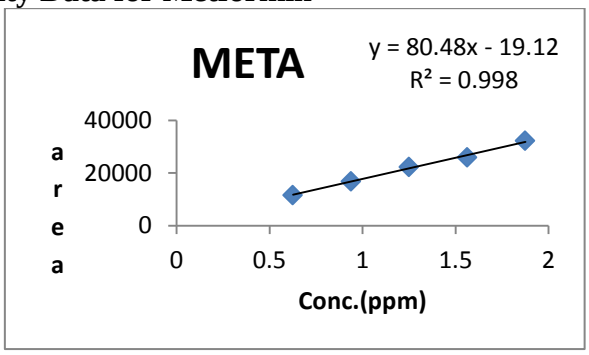


Fig.: Calibration Curve of Metformin (0.63-1.88µg/ml)

Linearity Data for Empagliflozin

Sr. No	Concentration (µg/ml)	Area
1	0.5	8321.779
2	0.75	12879.432
3	1	16215.327
4	1.25	20214.776
5	1.5	25389.546

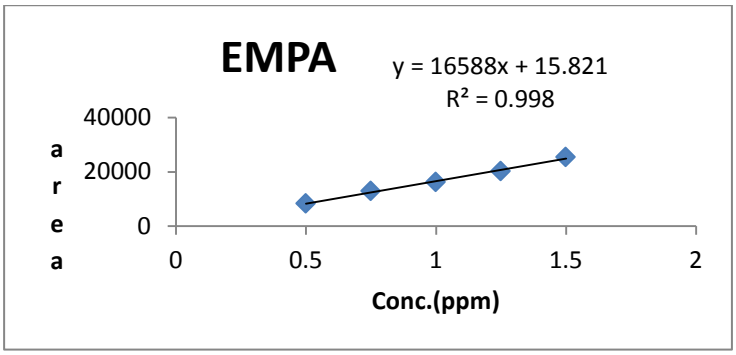


Fig.: Calibration Curve of Empagliflozin (0.5-1.5 µg/ml)

Precision

Repeatability: The data for repeatability of peak area measurement for Metformin and Empagliflozin , based on six measurements of same solution of Metformin and Empagliflozin are depicted in table 6.12 and 6.13 respectively. The % RSD for Metformin and Empagliflozin was found to be 1.329 and 1.768 respectively.

Table: Repeatability Data for Metformin

Metformin (1.25 µg/ml)				Empagliflozin (1 µg/ml)		
Sr. No.	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1.	22219.224	22625.259±322.482	1.425	16554.311	16334.831 ±221.025	1.353
	22540.113			16118.229		
	22881.589			16339.202		
	23101.298			16532.296		
	22619.671			16443.667		
	22389.656			16021.279		

Intraday precision: The data for intraday precision for Metformin and Empagliflozin is shown in table 6.14 and 6.15 respectively. The % R.S.D. for Intraday precision was found to be 0.821-1.548 for Metformin and 0.381-1.749 for Empagliflozin .

Table: Intraday precision data for Estimation of Metformin

Sr. No.	**Metformin**		
	Conc. (µg/ml)	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	0.625	11906.417 ± 111.719	0.938
2	1.250	22660.611 ± 350.844	1.548
3	1.875	32773.779 ± 368.943	0.821

Table: Intraday precision data for Estimation of Empagliflozin

Sr. No.	**Empagliflozin**		
	Conc. (µg/ml)	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	0.500	8386.215 ± 31.989	0.381
2	1.00	16223.706 ± 383.817	1.749
3	1.500	25439.452± 395.083	1.553

Interday precision: The data for intraday precision for Metformin and Empagliflozin is shown in table 6.16 and 6.17 respectively. The % R.S.D. for interday precision was found to be 0.651 to 0.976 for Metformin and 0.605-1.662 Empagliflozin .

Table: Interday Precision data for Estimation of Metformin

Sr. No.	Metformin		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	0.625	11942.737± 77.741	0.651
2	1.250	22884.733 ± 200.103	0.874
3	1.875	32723.268± 319.310	0.976

Table: Interday Precision data for Estimation of Empagliflozin

Sr. No.	Empagliflozin		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	0.500	8367.476 ± 50.615	0.605
2	1.00	16534.374± 274.749	1.662
3	1.500	25742.832± 290.115	1.127

Accuracy: Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. The results are shown in table 6.18 and 6.19 respectively. Percentage recovery for Metformin and Empagliflozin was 99.964-100.188% and 99.913-100.718 respectively.

Table: Recovery Data for Metformin

Sr. No.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	0.625	0.5	0.496	99.178	1.747
2		0.625	0.5	0.489	97.756	
3		0.625	0.5	0.506	101.210	
4	100 %	0.625	0.625	0.622	99.503	1.148
5		0.625	0.625	0.632	101.173	
6		0.625	0.625	0.619	98.976	
7	120 %	0.625	0.75	0.749	99.932	1.624
8		0.625	0.75	0.761	101.400	
9		0.625	0.75	0.736	98.163	

Table: Recovery Data for Empagliflozin

Sr. No.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	0.5	0.4	0.403	100.808	2.006
2		0.5	0.4	0.399	99.832	
3		0.5	0.4	0.388	96.979	
4	100 %	0.5	0.5	0.485	96.969	1.808
5		0.5	0.5	0.492	98.457	

6		0.5	0.5	0.503	100.521	
7	120 %	0.5	0.6	0.583	97.242	2.246
8		0.5	0.6	0.600	100.034	
9		0.5	0.6	0.610	101.667	

LOD and LOQ: Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

Limit of Detection

Table: Limit of Detection Data for Metformin and Empagliflozin

Metformin	Empagliflozin
LOD = 3.3 x (SD / Slope) = 3.3 x (628.520/16138.275) = 0.129 µg/ml	LOD = 3.3 x (SD / Slope) = 3.3 x (535.041/16588.351) = 0.106 µg/ml

Limit of Quantitation

Table: Limit of Quantitation Data for Metformin and Empagliflozin

Metformin	Empagliflozin
LOQ = 10 x (SD / Slope) = 10 x (628.520/16138.275) = 0.389 µg/ml	LOQ = 10 x (SD / Slope) = 10 x (535.041/16588.351) = 0.323 µg/ml

Robustness: The effect of changes was found to be within the acceptance criteria as shown in table 6.22 and 6.23 respectively. The % RSD should be less than 2%.

Table: Robustness data for Metformin

Sr No.	Area at Flow rate (- 2.0 ml/min)	Area at Flow rate (+ 2.0 ml/min)	Area at Mobile phase(-2)	Area at Mobile phase(+2)	Area at Flow rate (- 2.0 ml/min)	Area at Flow rate (+ 2.0 ml/min)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	25043.226	20136.504	26033.766	21341.663	18043.539	13325.405	12360.306	12160.369
2	25315.664	20453.776	26431.776	21435.335	18443.227	13554.154	12342.443	12342.443
3	25711.098	20741.114	26214.225	22002.512	18512.779	13761.554	12611.89	12160.369
% R.S.D	1.324	1.479	0.760	1.656	1.381	1.610	1.211	1.545

Analysis of marketed formulation by developed method: Applicability of the proposed method was tested by analyzing the commercially available Tablet formulation Remo-V. The results are shown in table.

Table: Analysis of Marketed Formulation

Tablet	Label claim	Assay (% of label claim*) Mean ± S. D.

	Metformin Empagliflozin	**Empagliflozin**	**% Metformin Empagliflozin**	**% Empagliflozin**
Remo-V	1000mg	12.5 mg	100.66 ± 1.234	101.35± 1.587

The assay results were comparable to labeled value of each drug in combined dosage form. These results indicate that the developed method is accurate, precise, simple and rapid. It can be used in the routine quality control of dosage form in industries.

CONCLUSION

For routine analysis of Empagliflozin and Metformin, a simple, precise, accurate, and fast simultaneous estimate approach has been devised and validated. The developed approach is suggested for regular and manufacturing standards analysis of the combination of Empagliflozin and Metformin. A stability-indicating RP-HPLC technique was devised and validated for the determination of Empagliflozin and Metformin in synthetic combinations. All method validation parameters meet the ICH Q2 (R1) guideline's acceptance criteria. As a result, we can conclude that the procedure is selective, linear, accurate, and accurate. As a result, it can be used to routinely analyze Empagliflozin and Metformin in combination. There was no indication of any degradation in the major peak, and the results were found to be within acceptable limits. As a result, the proposed stability-indicating RPHPLC assay method may be used to estimate Empagliflozin and Metformin in the mixture.

REFERENCE

1. Mendham J., Denney R.C., Barnes J.D. and Thomas M.J., 2002, *Chemical analysis* in *Vogel's Textbook of Quantitative Chemical Analysis*, 6th edition, Pearson Education Asia, Singapore.
2. Beckett A.H., Stenlake J.B. and A. Davidson, 2002, *Instrumental Method in the development and use of Medicines; Practical Pharmaceutical Chemistry*, 4th edition Volume 2, CBS Publishers and Distribution, New Delhi.
3. Willard H.H., Merritt L.L., Dean J.A. and Settle F.A., 2002, *HPLC Theory and Instrumentation; In Instrumental Methods of Analysis*, 8th edition, CBS Publishers and Distributors, New Delhi.
4. Scoog D.A., Holler F.M. and Nieman T.A., 2005, *Principles of Instrumental Analysis*, 5th edition, Brooks/Coles publication.
5. Snyder L.R., Kirkland J.J. and Glajch L.J., 1997, *Basics of Separation; Practical HPLC Method Development*, 2nd Edition, John Willey and Sons, Inc, New York.
6. Kenneth C.A., 2002, *A Text book of Pharmaceutical Analysis*, 3rd edition, A Wiley Interscience Publication, New York.
7. Ahuja S., Scypinski S. and Crowther J.B., 2001, *Validation of Pharmaceutical Text Method; Handbook of Modern Pharmaceutical Analysis*, Academic Press, London.

8. Watson D.G., 2000, *Pharmaceutical Analysis* in *A textbook of pharmacy students and pharmaceutical chemists*, Churchill Livingstone, London, p. 17-46, 195-204, 237-274.
9. Skoog D.A., West D.M., Holler F.J., Crouch S.R., 2008, *Fundamentals of Analytical Chemistry*, 8th Edition, Brooks/Cole publication.
10. Code Q1A R (2) - Stability Testing of new Drug Substances and Products, *ICH Harmonized Tripartite Guideline*, 1994.
11. Code Q1B- Photo Stability Testing of new Drug Substances and Products, *ICH Harmonized Tripartite Guideline*, 1994.
12. Code Q1C - Stability Testing Requirements for New Dosage Forms, *ICH Harmonized Tripartite Guideline*, 1998.
13. Blessy M., Patel R.D., Prajapati P.N., Agrawal Y.K., 2014, Development of forced degradation and stability indicating studies of drugs- A reviw, *J. Pharm. Anal.*4, 159-165.
14. Banker and Rhodes, 2002, *Stability Studies*, Marcel Dekker, New York, 4th Edition.
15. Code Q1F – Stability data package for registration application in climatic zone III and iv, *ICH Harmonized Tripartite Guideline*, 1994.
16. Code Q3B R (2) - Impurities in new drug products, *ICH Harmonized Tripartite Guideline*, 1994.
17. Code Q6A - Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products, *ICH Harmonized Tripartite Guideline*, 2000.
18. Code Q6A R- Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances, *ICH Harmonized Tripartite Guideline*, 1994.
19. Code Q2A-Text on Validation of Analytical Procedure Step-3 Consensus Guideline, *ICH Harmonised Tripartite Guideline*, 1994.
20. Code Q2B- Validation of Analytical Procedure Methodology Step-4 Consensus Guideline, *ICH Harmonised Tripartite Guideline*, 1994.
21. Bommadevara P., 2014, Validated stability indicating RP - HPLC method for the simultaneous determination of Ofloxacin, Ornidazole, Clobetasol Propionate, Terbinafine Hydrochloride, Methyl Paraben, Propyl Paraben in bulk and pharmaceutical dosage form, *Int. J. Pharm and Ana Res.* 3, 301-318.
22. Cione A.P.P., Liberale M.J., Silva P.M., 2010, Development and Validation of an HPLC method for stability evaluation of Nystatin, *Braz. J. Pharm. Sci.* 46, 305- 310.
23. Tiwari R.N., Shah N., Bhalani V., Mahajn A., LC, 2015, MS^n and LC- MS/MS studies for the characterization of degradation products of Amlodipine, *J. Pharm. Ana.* 5, 33- 42.

24. Yuan *et al*, 2018, Study of forced degradation behavior of Pramlintide Acetate by HPLC and LC- MS, *Journal of Food and Drug Analysis* 26, 409- 415.
25. Alamein A.M., 2012, Validated stability indicating methods for the determination of Zafirlukast in the presence of its alkaline hydrolysis degradation product, *Bulletin of Faculty of Pharmacy* 50, 111- 119.
26. Pires S.A. *et al*, 2015, LC- MS characterization of Valsartan degradation products and comparison with LC- PDA, *Braz. J. Pharm. Sci.* 51, 839- 845.
27. Bansal G, Singh M., Jindal K., Singh S., 2008, LC and LC- MS study on Establishment of Degradation Pathway of Glipizide Under Forced Decomposition Conditions, *Journal of Chrom. Sci.* 46, 510- 517.
28. Talluri M.V.N., Khatoon L., Kalariya P., Chavan B., Ragampeta S., 2015, LC-MS-MS characterization of Forced Degradation Products of Fidarestat, a Novel Aldose Reductase Inhibitor: Development and Validation of a Stability- Indicating RP- HPLC Method, *J. Chrom. Sci.* 53, 1588- 1596.
29. Kaushik D., Bansal G., 2015, Four new degradation products of doxorubicin: An application of forced degradation study and hyphenated chromatographic techniques, *J. Pharm. Ana.* 5, 285-295.
30. Pawar S.M., Khatal L., Gabhe S., Dhaneshwar S., 2012, LC- UV and LC- MS evaluation of stress degradation behavior of desvenlafaxine, *J. Pharm. Ana.* 2, 264- 271.
31. Rakibe U., Tiwari R., Mahajan A., Rane V., Wakte P., 2018, LC and LC- MS/MS studies for the identification of degradation products of acebutanol, *J. Pharm. Ana.*, 357-365.
32. www.usfda.gov.in
33. Rajan V and Patil SP, 2018, Reversed Phase High Performance Liquid Chromatography Method for Determination of Assay and Forced Degradation Study of Apremilast from Active Pharmaceutical Dosage Form, *J. Chem. Pharm.* 10, 139-144
34. Murali D. andRao P., 2018, Stability Indicating RP-HPLC Method for the Simultaneous Estimation of Imipenem and Cilastatin in Injection Formulations, *World J. Pharm. Res.* 8, 898-913.
35. Reddy B., Radhakrishnan P. Alam I., Kiran P., 2019, A Validated Stability Indicating RP- HPLC Method Development for Anticancer Drug Enzalutamide in Bulk and Pharmaceuticals, *Int. J. Pharm. Sci. Drug Res.* 11, 85-90.
36. Bhole R., Zombade T., Bonde C., Zambare Y., 219, Identification and Characterization of Degradation Products by using MS-MS Studies for Developed and Validated Stability Indicating

HPTLC Method for Estimation of of Nintedanib Esylate in Pharmaceutical Dosage Form, *Eurasian J. Anal. Chem.* 14, 60-70.

37. Prakash V., Suresh J., Konari S., 2018, RP-HPLC Forced Degradation Studies of Aztreonam in Pharmaceutical Dosage Form, *Pharm Methods* 9, 40-44.
38. El-ghobashy M., Yehia A., Helmy A., Youssef N., 2018, Forced degradation of Gliquidone and development of validated stability-indicating HPLC and TLC methods, *Braz. J. Pharm. Sci.* 54, 3-6.
39. Sversu R., Vieira J., Kassab N., Silva D., Salgado H., 2019, Forced Degradation behaviour of two drug combinations: Isolation and Characterization of major degradation products by LC-MS, *Microchem. J.* 150, 1-10.
40. Kamal A., Marie a., Hammad S., 2020, Stability Indicating RP- HPLC method for simultaneous determination of Omeprazole and Aspirinin the presence of Salicylic Acid as Degradation Product, *Microlab J.* 152, 1-10.
41. Phechkrajang C., Quynh P., Suntornsuk L., 2017, Forced Degradation Studies of Candesartan Cilexetil and Hydrochlorthiazide using a Validated Stability Indicating HPLC- UV Method, *Pharm. Chem. J.* 51, 416-424.
42. Gousuddin M., Sengupta P., Chatterjee B., Das S.K., 2017, Stability Indicating RP- HPLC Method for Simultaneous Quantitation of Tramadol and Aceclofenac in presence of their major degradation products: Method Development and Validation, *J. Liq. Chromatogr. R. T.*40, 887-893.
43. Nadella N.P., Ratnakaram V.N., Srinivasu N., 2018, Development and Validation of UPLC method for simultaneous quantification of Carvedilol and Ivabradine in the presence of degradation products using DoE concept, *J. Liq. Chromatogr. R. T.* 41, 143-153.