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STABILITY INDICATING ASSAY METHOD DEVELOPMENT AND VALIDATION OF EDOXABANTOSYLATE MONOHYDRATE: A COMPREHENSIVE STUDY INVOLVING IMPURITIES ISOLATION, IDENTIFICATION AND DEGRADATION KINETICS DETERMINATION IN VARIOUS CONDITIONS

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Keywords:

Edoxabantosylate monohydrate, Stability indicating assay method, Degradation products, Isolation, Characterization, ESI-MS/MS, ^{1}H-NMR, ^{13}C-NMR

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ABSTRACT: Objective: State of the art, robust and environmentally benign stability indicating assay method (SIAM) has been developed for model drug Edoxabantosylate (EDO) **Methods:** Successful RP-HPLC chromatographic method was accomplished on a CHROMBUDGET® 100-5-C18 column (250 mm × 4.6 mm, 5 µ) column using methanol and acetate buffer in the ratio of 53:47 having 2.9 pH and 10mM buffer strength. The degradation study was performed in the presence of various stressors, and SIAM develop was selective enough to discriminate the EDO peak with the peak of other degradation production (DP) with acceptable resolution. The degradation kinetics study and fragmentation pattern of isolated major DPs were carried out. **Results:** The developed SIAM validated as per ICH Q2(R1) guidelines. Two major DPs *i.e.* DP-I and DP-II, were isolated and characterized thoroughly. Furthermore, the reaction kinetics data revealed that EDO degradation was following first-order degradation kinetics. **Conclusion:** An extensive stress degradation study along with degradation kinetics, were performed. All probable degradation pathways were predicted by using LC-MS/MS and NMR data obtained by characterization of the isolated DPs.

INTRODUCTION: Chemically Edoxaban (EDO) is a N'-(5- chloropyridin-2-yl)-N-[(1S,2R,4S)-4-(dimethylcarbamoyl)- 2- [(5-methyl-6,7-dihydro-4H- [1,3] thiazolo [5,4-c] pyridine-2-carbonyl) amino] cyclohexyl] oxamide [1] and pharmacologically it is used as an oral anticoagulant which acts by selective inhibiting the factor Xa **Fig. 1**.

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It is widely used in Venous thromboembolism as approved by Japan in 2011 [2]. Food and Drug Administration (FDA) has approved EDO in the prevention of stroke and nonvalvular atrial fibrillation in 2015 [3].

Although having a wide application, suitable analytical methods for EDO remain to be explored. Only a few reports for its estimation using HPLC is available in the literature. Reddy *et al.*, has developed an RP-HPLC method for EDO estimation in bulk and dosage form using Hypersil BDS C18 column (250 × 4.6 mm, 5 µm) by applying 0.1M K_2HPO_4: Methanol (65:35, v/v) mobile phase system [4].

Peraman and group have also reported a method for Edoxabantosylate. However, isolation and detailed characterization of its impurities have not been reported so far. A reported UV method is also available [5].

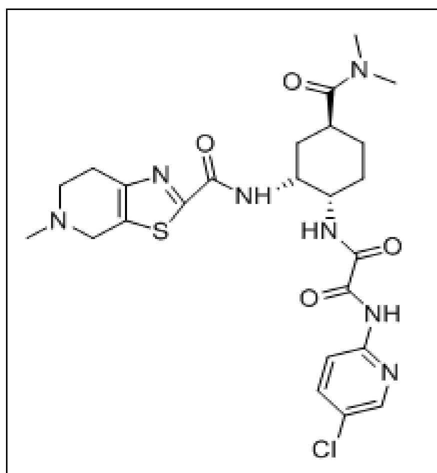


FIG. 1: CHEMICAL STRUCTURE OF EDOXABAN

In this investigation, authors have developed an LC-MS/MS transferrable method for EDO, which would be efficient enough to detect EDO in bulk and in the dosage form in the presence of degradation products. The forced degradation study was employed using different stressors, and the generated degradation products were separated, and the major degradation products (DPs) were isolated. Here, two major and identical DPs were formed in acidic and alkaline hydrolytic conditions designated as DP-I and DP-II; which were isolated using preparative TLC and characterized in depth. Moreover, the peroxide degradation also gave two different DPs, *i.e.* DP-III and DP-IV along with DP-I. The fragmentation pathway prediction and degradation kinetics study were also carried out. The isolation of DPs would help in postulating the nature of EDO, and it would also help predict the favorable storage conditions for EDO. It could be concluded from the investigation that the EDO was extremely labile under alkaline condition, whereas it is labile for acidic and peroxide degradation condition. The presence of the amide group in the structure of EDO could be held responsible for its susceptibility to hydrolysis.

MATERIALS AND METHODS:

Chemicals and reagents: EDO, an active pharmaceutical ingredient (API) was kindly gifted by hetero drugs (Hyderabad, India). Various analytical grade chemicals used in the stress degradation like hydrochloric acid (HCl), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), ammonium acetate and acetic acid were purchased from Loba Chemie Pvt. Ltd. HPLC grade Methanol (MeOH) was procured from Rankem Pvt. Ltd. Double distilled water was used throughout the study. All the solutions were filtered through a 0.2µ filter Ultipor®N66® Nylon membrane filter (Pall Life Sciences, USA).

Chromatographic System and Conditions: The chromatographic method was developed on a Shimadzu LC system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV detector (Kyoto, Japan) using CHROMBUDGET® 100-5-C18 column (250 mm × 4.6 mm, 5 µm). Samples were injected through a Rheodyne 7725 injector valve with a fixed loop at 20µL, and the data acquisition and integration were executed on Spinchrome software® (Spinco Biotech, Vadodara). A stress degradation study was carried out using a silicon oil bath, having a temperature adjustable knob facility. The pH meter (Lab India, India) was used to check the pH of all solutions. Other equipment used was sonicator (ePEI ultrasonic generator), electronic analytical balance (A×120, Shimadzu), hot air oven (Tempo Instrument & Equipment Pvt Ltd, India.) and auto pipettes (Eppendorf, Hamburg, Germany). Furthermore, the photolytic study was carried out using photo chamber. The LC-MS/MS experiment was performed on a 3200 QTRAP LC-MS/MS instrument (AB Sciex instruments,) equipped with a binary pump LC system, Ekspert 100-XL Autosampler, Ekspert 100 Pump, Ekspert 100 Column Oven, and Quadrupole mass analyzer.

Preparation of Mobile Phase: Varied number of trials were executed utilizing different buffer systems (ammonium acetate, potassium dihydrogen phosphate, Ammonium Formate) in different pH range employing different organic modifier (MeOH and ACN). The final optimized RP-HPLC method was having the mobile phase composition of ammonium acetate buffer and MeOH: 47:53. Where 10 mM ammonium acetate having a pH of 2.9 adjusted with acetic acid was prepared. Finally, the solution was filtered through a 0.2 µ Nylon membrane filter. This aqueous phase was mixed with the organic phase (MeOH) in the ratio of 47:53.

Preparation of EDO Calibration Curve Solutions: Accurately weighed 10 mg of EDO was transferred into a 10 mL volumetric flask containing 1 mL methanol. The API was dissolved after sonicating the drug solution, and volume was made up to the mark using methanol to obtain 1000 µg/mL EDO stock solution. The standard solution of 1000 µg/mL was utilized further, to prepare calibration curve solutions ranging from 1, 5, 20, 40, 80 and 120 µg/mL by withdrawing 0.01, 0.05, 0.2, 0.4, 0.8 and 1.2 mL respectively, followed by analysis at λ_{max} 289 nm in HPLC.

Forced Degradation Study of EDO: Stress degradation involves the application of different stressors *viz*, acidic, basic, and oxidative to study the impurities formed a [6-8] and different pathway of these degradation products formation. The major application of this study is in formulation development and stability [9-10].

Hydrolytic Degradation Study: The 10 mg EDO API and its formulation equivalent to 10 mg EDO were exposed to acid and base hydrolytic conditions in the presence of a different concentration of 0.1, 0.5 and 1N HCl and 0.001, 0.005 and 0.01N NaOH. Identical treatment was given to the marketed formulation of EDO. Both API, as well as its marketed formulation, were subjected to acidic and basic stress condition at 60 ºC, and periodically samples were withdrawn and neutralized with opposite stressor before injecting into HPLC after appropriate dilution.

A similar procedure was employed to study the forced degradation behavior to study neutral hydrolysis where water was taken as a neutral stressor. The identical experiment was followed to study neutral hydrolysis in the presence of water.

Peroxide Degradation Study: To perform peroxide degradation, EDO and its formulation was subjected to 3% H_2O_2, and 10% H_2O_2 condition at room temperature and 60 ºC and the aliquots were withdrawn periodically and analyzed by HPLC after proper dilutions.

Thermal Degradation Study: To explore the stability profile of EDO at a different temperature, 10 mg pure API and its formulation equivalent to 10 mg EDO were spread uniformly on the Petri plates individually, and exposed to a harsher dry heat condition, *i.e.* at 80 ºC for 10 days in a hot air oven. The samples were withdrawn at 2 and 10 days, followed by HPLC analysis.

Photolytic Degradation Study: 10 mg of API and formulation (equivalent to 10mg EDO) were exposed to 5382 LUX and 144UW/cm^2 for 10 days, and the degradation samples were subjected to analysis after suitable dilutions.

Development of Stability Indicating Assay Method (SIAM): Above mentioned forced degradation samples were diluted appropriately to get the final concentration of 100 µg/mL individually and analyzed separately to study degradation patterns of EDO in various harsher conditions and the %degradation was found out using below equation 1. Furthermore, an equal volume of each forced degradation solutions was mixed and diluted to 1 mL. This mixture of stress solution was injected in HPLC to check the efficiency of the developed method to separate all the DPs selectively having a resolution of more than 2 along with least asymmetry.

% Drug degraded = Area of the unstressed sample - Area of stressed sample × 100 / Area of unstressed sample ……… (1)

Method Validation: Developed and optimized RP-HPLC method was validated successfully as per ICH guideline Q2(R1) for different parameters. Linearity was carried out taking different concentration ranging from 1 to 120 µg/mL. A regression equation was generated, and the regression coefficient (R^2) value was calculated. The standard addition method was employed to express the accuracy of the method by incorporating the known amount of EDO corresponding to 80, 100 and 120% to a fixed concentration of EDO formulation and the results were expressed in terms of standard deviation (SD) and percent relative standard deviation (% RSD). Additionally, the precision of the method was studied at three different levels, *i.e.* at lower quality control (LQC), medium quality control (MQC), and higher quality control (HQC) level. Limit of detection (LOD) and quantitation (LOQ) was figured out based on the standard deviation of the response and slope method considering the equation 2 and 3.

LOD: 3.3 σ/S…………(2)

LOQ: 10 σ/S…………(3)

Where, σ stands for the SD of response and S for the SD of slope. Moreover, Specificity was recognized by determining drug peak from its nearest resolved peak. Selectivity of the method was mentioned in terms of peak purity of drug peak and the peaks of DPs as well. The system suitability study was conducted injecting a 100 µg/mL EDO solution for six times and SD and %RSD was calculated for different parameters like retention time, theoretical plates, and asymmetry.

Reaction Kinetics Study: This study was performed to determine the order of degradation kinetics under different stress conditions. 100 µg/mL of EDO solution was stressed in 1, 1.5, and 2N HCl at 40, 50, and 60 ºC. Whereas, similar temperature condition was provided for studying the degradation kinetics profile in 0.001, 0.01, and 0.05N alkaline condition at 40, 50, and 60 ºC as well. The degradation reaction kinetics study was also performed at temperatures above in 3%, 10%, and 15% H_2O_2. Thus, the reaction order was determined by performing degradation kinetics at higher stressor concentration and temperature range. Samples were withdrawn at a predefined time interval and analyzed by RP-HPLC method. Finally, the plots of %drug remained *vs*. time (for zero order reaction kinetics), and %log drug remained *vs*. time (for first-order reaction kinetics) were plotted individually, and R^2 values were calculated to decide the order of degradation kinetics.

Analysis of Formulation: EDO formulation was analyzed to obtain % assay value. EDO tablet was crushed using mortar and pestle, and the content was emptied into MeOH to extract the EDO. This was followed by filtration, and the filtrate was collected. Eventually, the 100 µg/mL EDO solution was prepared and analyzed by HPLC. The results, *i.e.* HPLC area for 100 µg/mL EDO formulation obtained was compared with 100 µg/mL EDO API solution. The experiment was repeated for six times, and % assay was calculated using following equation 5.

% Assay = Area of EDO in formulation ×100 / Area of EDO in API solution …………(5)

Isolation of Impurities: The acid and base hydrolysis generated degradation productions, *i.e.* DP-I and DP-II were isolated using preparative TLC plates following the selective enrichment of the DPs. The TLC plates were run in the mobile phase having 50:50 composition of chloroform: isopropyl alcohol. On the completion of the mobile phase run, the bands of DP-I and DP-II were visualized in UV chamber and scraped. The scraped DPs were solubilized in MeOH, followed by rotary evaporation of the solvent and finally, the solid DPs were isolated and further characterized by RP-HPLC, LC-MS/MS, ^{1}H-NMR and ^{13}C-NMR.

Prediction of Probable DPs and Degradation Pathway: DPs generated in different stressed conditions were subjected to LC-MS/MS analysis equipped with photodiode array (PDA) detector. The study was conducted on the Chrombudget® 100-5- C18 column (150 × 4.6 mm; 3µ) RP-HPLC column with the mobile phase of ammonium acetate (10mM; pH 2.9). Solutions of each DPs were injected individually, scanned for the entire wavelength range from 200 to 400 nm, and molecular weight with its fragmentation product was obtained. This data was utilized to predict the probable degradation pathway by studying its degradation mechanism to predict possible structures of DPs.

RESULTS AND DISCUSSION:

RP-HPLC Method Development: RP-HPLC method was developed and optimized, implementing one factor at a time (OFAT) strategy. The results obtained after extensive trials taken using different buffers and organic modifier favored a combination of ammonium acetate buffer having a pH of 2.9 and MeOH as mobile phase composition in ration of 47:53 which satisfied all criteria *viz.,* optimum retention time which could separate the DPs with higher resolution having least tailing factor and higher number of theoretical plates.

TABLE 1: OPTIMIZED RP-HPLC METHOD

Parameters	Values
Column type	Chrombudget® 100-5- C18 column (150 × 4.6 mm; 3µ)
Buffer type	Ammonium acetate (10mM; pH:2.9)
Mobile phase composition	Ammonium acetate:MeOH::47:53
Flow rate (mL/min)	1
Retention time (min)	15.1
Asymmetry factor	1.2
Theoretical plates	5982

This method showed a retention time of 15.1 min with a theoretical plate of 5982 having asymmetry of 1.2, as displayed in following **Fig. 2** and summarized in **Table 1**.

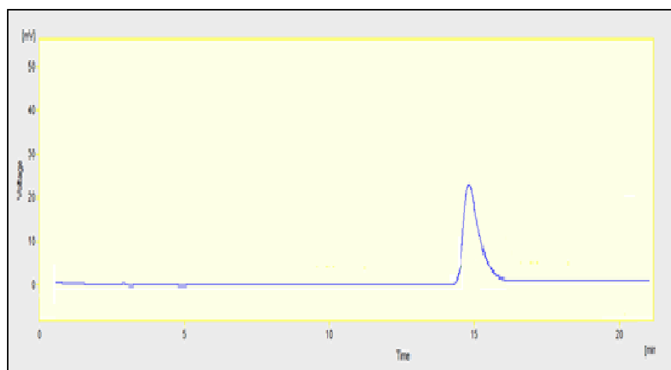


FIG. 2: RP-HPLC CHROMATOGRAM OF EDO

Stress Degradation Study: The degradation study was performed in varied stressed conditions. EDO exhibited the formation of two DPs, *i.e.* DP-I and DP-II at 3.1 and 4.3 min respectively in 1N HCl and 0.001N NaOH hydrolytic condition performed at 60 ºC **Fig. 3A** and **3B**. In both acidic and alkaline hydrolytic conditions, two major DPs were formed at the same retention time. Later on, from the LC-MS/MS study, it was proved that both DPs were identical. Furthermore, the neutral hydrolysis at room temperature and elevated temperature demonstrated no extra peak along with the peak of EDO, which concluded no degradation of EDO. This proved that EDO is highly prone to acidic and basic hydrolysis.

The peroxide degradation of EDO in the presence of 3% H_2O_2 at 60 ºC demonstrated the formation of three DPs at 3.1, 18.4, and 20.1 min as portrayed in **Fig. 3C**. The DP formed at 3.1 min was the DP-I which was formed in acid and alkaline degradation condition as confirmed from the LC-MS/MS study. The remaining two new DPs were labeled as DP-III and DP-IV respectively.

The other forced degradation conditions like thermal and photolytic condition revealed no extra peak apart from the peak of EDO. This inferred that the EDO was stable in thermal and photolytic condition for 10 days.

A mixture of all degradation condition also gave total of four DPs on the same retention time as obtained earlier as displayed in the following **Fig. 3D** and the details of degradation are summarized in **Table 2**.

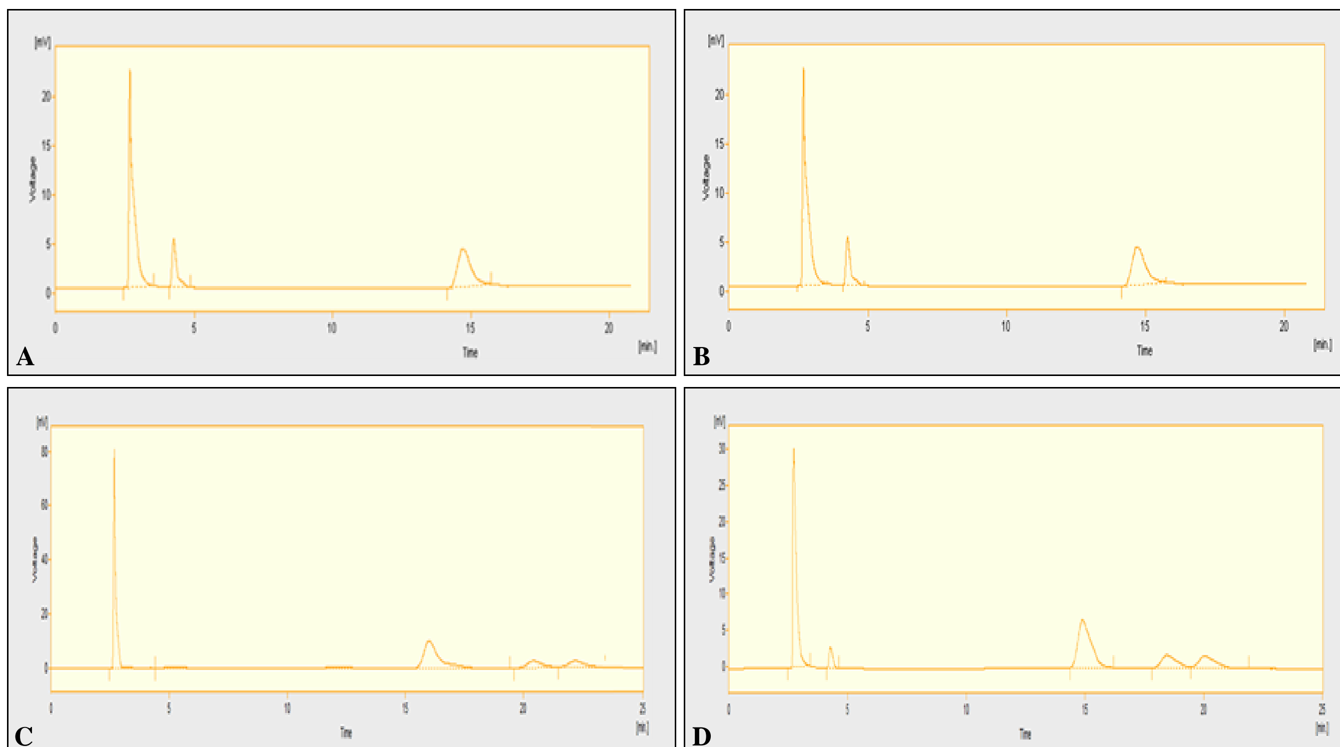


FIG. 3: RP-HPLC CHROMATOGRAM OF EDO IN (A) 1N HCl AT 60 ºC, (B) 0.001N NaOH AT 60 ºC, (C) 3% H_2O_2 AT 60 ºC AND (D) MIXTURE OF EACH STRESSED DEGRADATION CONDITIONS

TABLE 2: SUMMARY OF FORCED DEGRADATION STUDY

Condition	Parameters	EDO	DP-I	DP-II	DP-III	DP-IV
Acid (1 N HCl at 60 ºC)	Retention time (min)	15.1	3.1	4.3	-	-
	Tailing factor	1.2	1.35	0.98	-	-
	Resolution	6.78	-	3.58	-	-
	Peak purity index	1.00	0.995	0.994	-	-
	Number of theoretical plates	5982	3482	4125	-	-
	% Remaining	85.2%	9.9%	4.9%	-	-
Base (0.001 N NaOH at 60 ºC)	Retention time (min)	15.1	3.1	4.3	-	-
	Tailing factor	1.2	1.32	0.98	-	-
	Resolution	6.62	-	3.92	-	-
	Peak purity index	1.00	0.994	0.967	-	-
	Number of theoretical plates	5914	3672	4221	-	-
	% Remaining	84.5%	10.2%	5.3%	-	-
Peroxide degradation (3% H_2O_2 at 60 ºC)	Retention time (min)	15.1	-	-	18.4	20.1
	Tailing factor	1.2	-	-		
	Resolution	6.53	-	-	3.67	2.14
	Peak purity index	1.00	-	-	0.988	0.991
	Number of theoretical plates	5873	-	-	4712	3375
	% Remaining	92.8%	-	-	3.3%	3.9%
Thermal degradation	No degradation was envisaged till 10 days at 80 ºC					
Photolytic degradation	No degradation was envisaged by exposing EDO to 5382 LUX and 144UW/cm^2 for 10 days					

Validation of RP-HPLC Stability Indicating Assay Method: Validation of HPLC method was carried out for different parameters like linearity, accuracy, precision (inter-day and intra-day), LOD, and LOQ. The details of validation parameters are discussed in the following **Table 3**. Further, the chromatogram showing linearity of EDO is displayed in **Fig. 4**.

TABLE 3: RP-HPLC METHOD VALIDATION SUMMARY

Parameters		Value
Linearity (µg/mL)		1-120
Regression equation		10.58x + 2.47
Regression coefficient (R^2)		0.999
% Recovery	80%	99.25 ± 0.58
	100%	101.86 ± 0.67
	120%	101.67 ± 0.42
Intra-day precision (%RSD)		0.93
Inter-day precision (%RSD)		0.57
LOD (µg/mL)		0.98
LOQ (µg/mL)		3.35
% Assay		100.88 ± 0.79

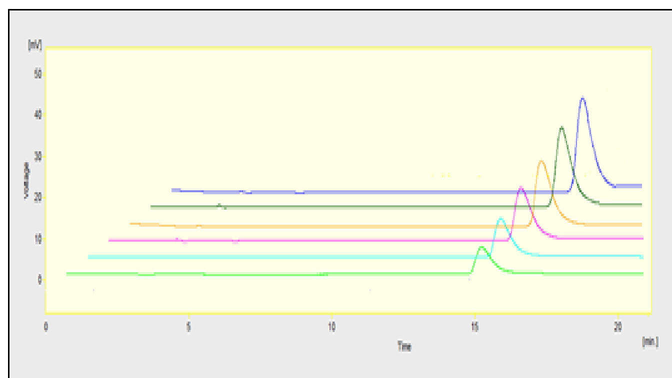


FIG. 4: CHROMATOGRAM SHOWING LINEARITY OF EDO (1-120 µg/mL)

Reaction Kinetics Study: Reaction kinetics study performed for acid and base hydrolytic condition and in peroxide condition at different temperature *viz.*, 40, 50 and 60 °C taking different stressor concentration revealed that the degradation kinetics was following first-order reaction kinetics. % log C_o (logarithm of initial concentration) and % log C_t (logarithm of concentration remained after the time (t) for each solution at a different temperature, and concentration was calculated. The plots of the %log drug remained *vs.* time (first-order reaction kinetics) demonstrated that higher R^2 value as compared to the value obtained for the plot of %drug remained *vs.* time (zero order reaction kinetics). The summary for the same is tabulated in **Table 4** ad portrayed in **Fig. 5**. From the reaction kinetics study, it was concluded that degradation kinetics followed first-order kinetics for each degradation conditions as the r^2 value for this reaction order was nearer to unity.

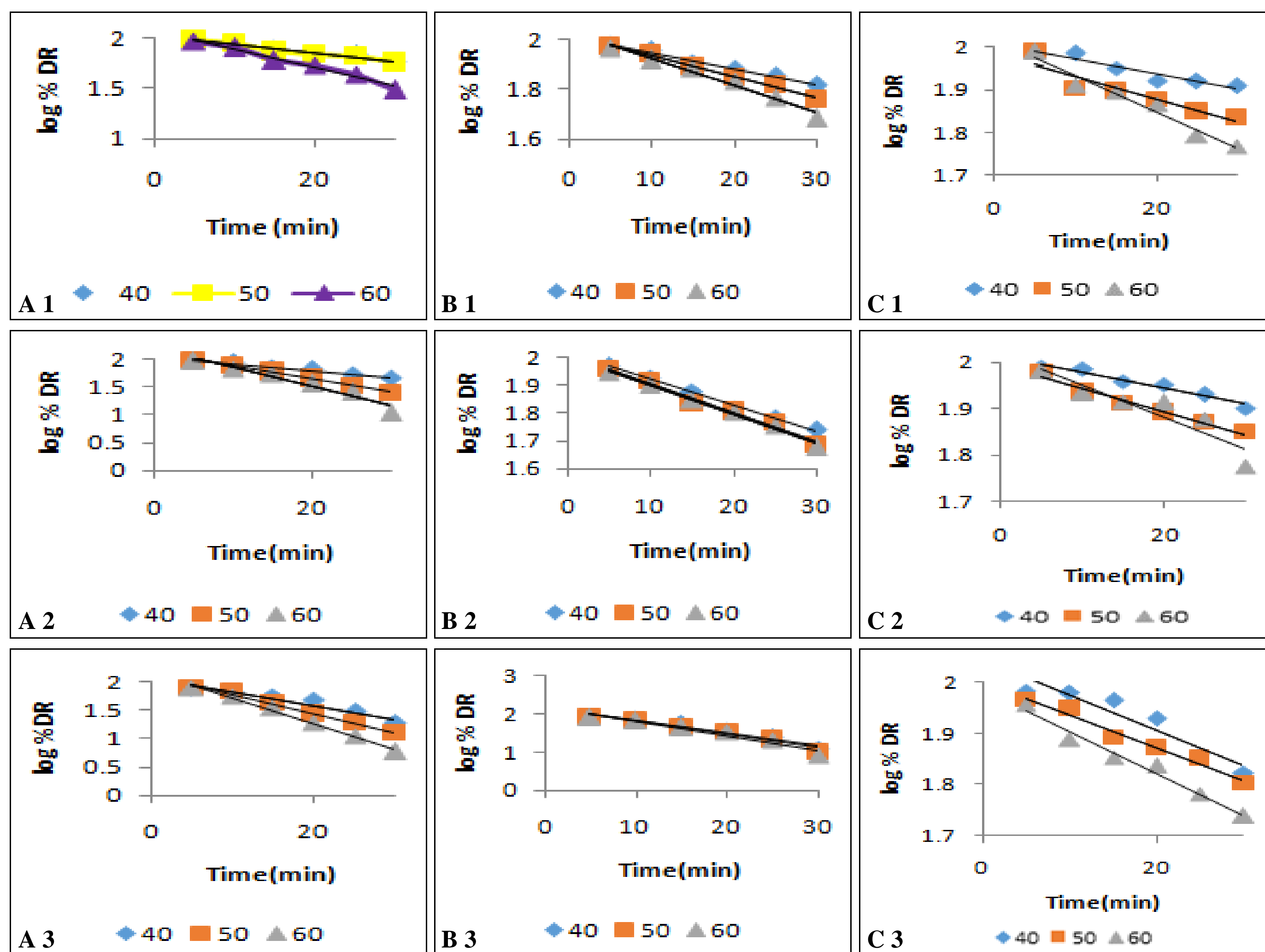


FIG. 5: DEGRADATION KINETICS STUDY OF EDO IN (A1) 1N HCl, (A2) 1.5N HCl, (A3) 2N HCl, (B1) 0.001N NaOH, (B2) 0.01N NaOH, (B3) 0.05N NaOH, (C1) 3% H_2O_2, (C2) 10% H_2O_2, AND (C3) 15% H_2O_2 AT THREE DIFFERENT TEMPERATURE

TABLE 4: R^2 VALUES FOR ZERO AND FIRST ORDER REACTION KINETICS

Condition	Concentration	R^2 at 40 ºC		R^2 at 50 ºC		R^2 at 60 ºC	
		Zero	First	Zero	First	Zero	First
Acid	1	0.943	0.968	0.875	0.981	0.922	0.988
	1.5	0.936	0.954	0.917	0.958	0.917	0.932
	2	0.924	0.962	0.893	0.964	0.892	0.947
Base	0.001	0.944	0.963	0.931	0.947	0.875	0.917
	0.01	0.935	0.975	0.921	0.952	0.932	0.952
	0.05	0.924	0.941	0.894	0.928	0.921	0.942
Peroxide	3%	0.894	0.953	0.917	0.947	0.914	0.964
	10%	0.917	0.944	0.928	0.952	0.876	0.922
	15%	0.908	0.933	0.871	0.933	0.886	0.911

Applicability of Developed SIAM: The developed method was successfully applied for EDO estimation in its marketed formulation. % Assay for the same was carried out in triplicate, and it was found to be 100.88 ± 0.79 **Table 3**.

Further, the formulation was also subjected to stress degradation under identical conditions as specified for EDO API. The DPs formed for formulation were well resolved and discernible.

Isolation and Characterization of Major DPs: Two major degradation products, *i.e.* DP-I and DP-II, were isolated performing preparative TLC and characterized in detailed. The obtained degradation products were characterized by LC-MS/MS, ^{1}H-NMR, and ^{13}C-NMR study.

ESI-MS/MS and NMR Data: EDO (m/z 548.16): The ESI-MS/MS data of EDO revealed $[M+H]^+$ ion at 548.16 in positive mode. Further mass spectrum

with abundant product ions at m/z 366.12 revealed removal of the chlorinated pyridine ring. This was further confirmed from the mass spectrum, which revealed the absence of M+2 peak of chlorine **Fig. 6**.

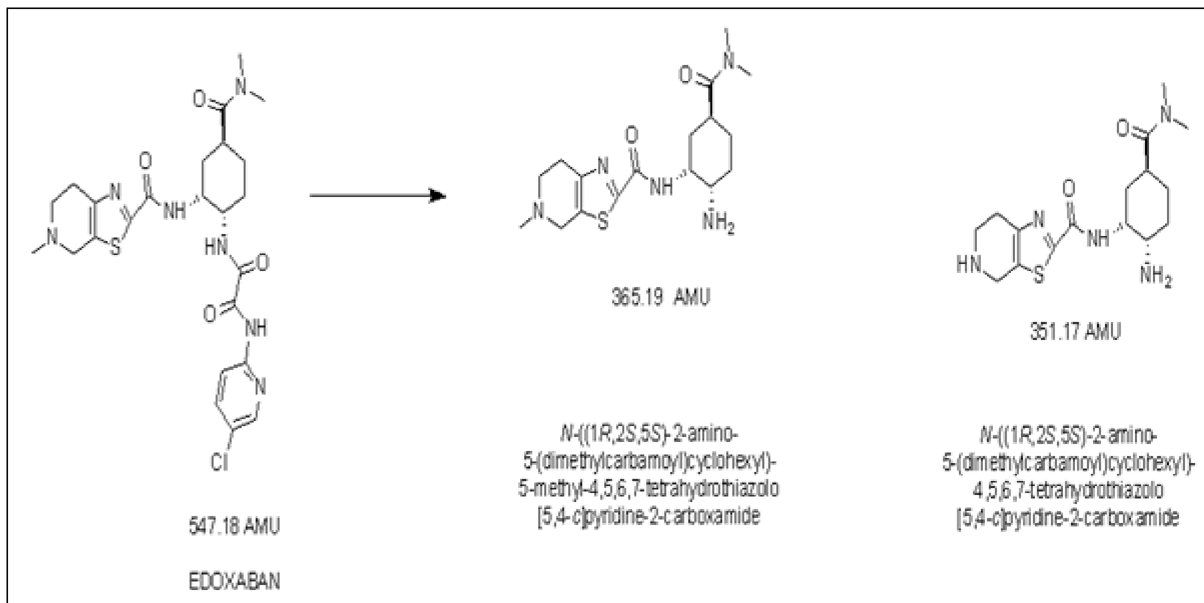
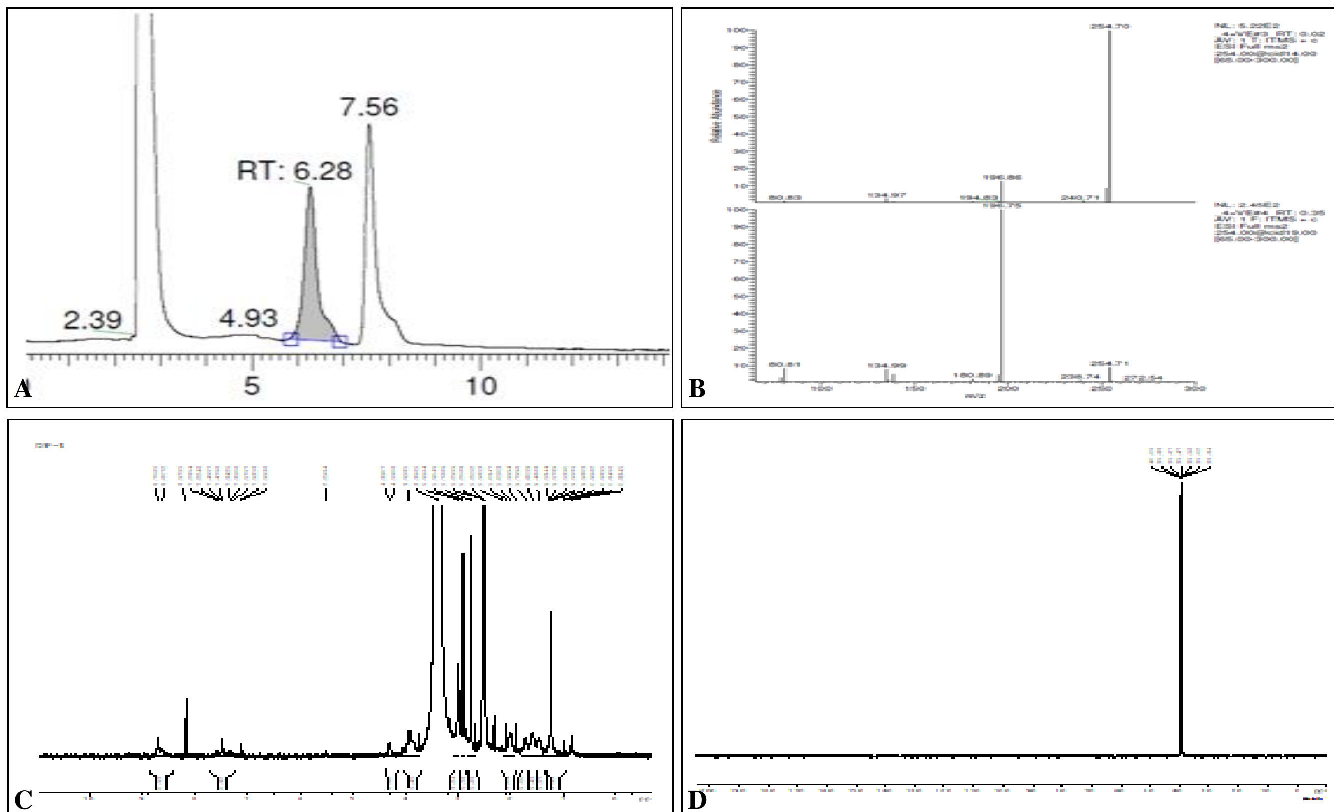


FIG. 6: FRAGMENTATION PATTERN OF EDO IN ESI-MS/MS

DP-I (m/z 254): The ESI-MS/MS data of DP-I of acidic and alkaline conditions revealed $[M+H]^+$ peak at 254.70 in positive mode. This could be due to the removal of tetrahydro-thiazolopyridine ring and chlorinated pyridine ring. The absence of M+2 peak at 256.70 m/z supports the formation of DP-I having structure devoid of chlorine. Further, the mass spectrum displayed an abundant product ion at m/z 196.75, which could be due to loss of methylated tertiary amine and a primary amine group. The proposed fragmentation pathway is portrayed in **Fig. 7**.



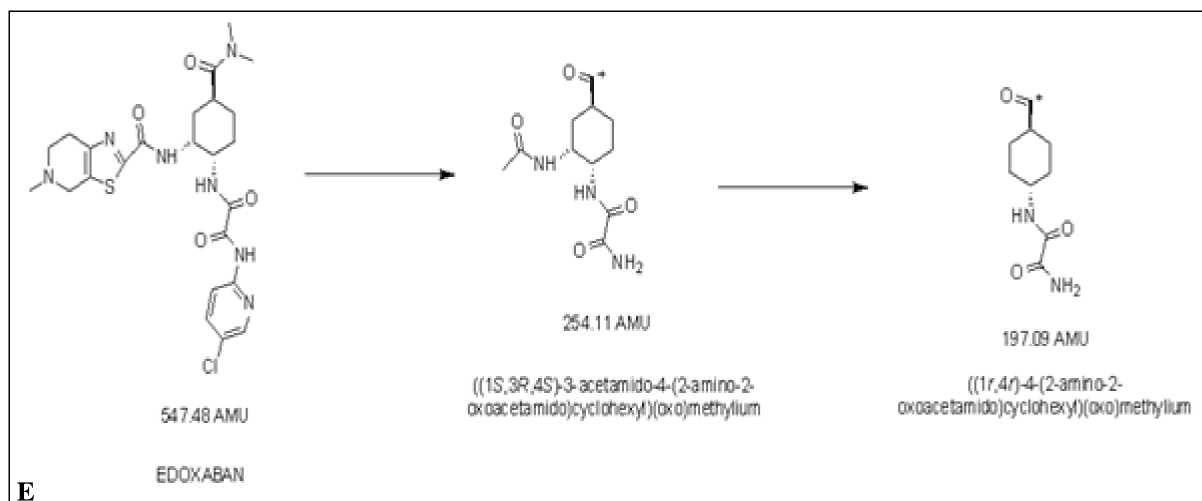


FIG. 7: CHARACTERIZATION OF DP-I SHOWING (A) LC-MS/MS CHROMATOGRAM, (B) LC-MS/MS SPECTRA, (C) ^{1}H-NMR, (D) ^{13}C-NMR AND (E) FRAGMENTATION PATTERN FOR DP-I

The peak in the ^{1}H-NMR at beyond 8.0 and 7.2 δ value indicated the presence of secondary amide group and primary amide group, respectively. Further, the two-methyl group attached to nitrogen showed a distinct peak at 3.43 δ value. The bunch of peaks in the region of 1-3 δ value is corresponded to –CH and –CH_2 of cyclohexane. The ^{13}C-NMR exhibited numerous peaks near 39.85 to 40.10 δ, which is corresponded to the carbon of cyclohexane and methyl group **Fig. 7**.

DP-II (m/z 128.95): The ESI-MS/MS data for DP-II showed $[M+H]^+$ peak at m/z 128.95 along with isotopic peak due to chlorine at 130.95 ([M+2] peak). This revealed the removal of bulky groups and the presence of chlorinated pyridine ring. This was further fragmented and gave abundant product ions at m/z 111.03, which could be due to the removal of the primary amine group. Further, the peak in the ^{1}H-NMR in the region of 6-8 δ is due to the presence of aromatic protons **Fig. 8**.

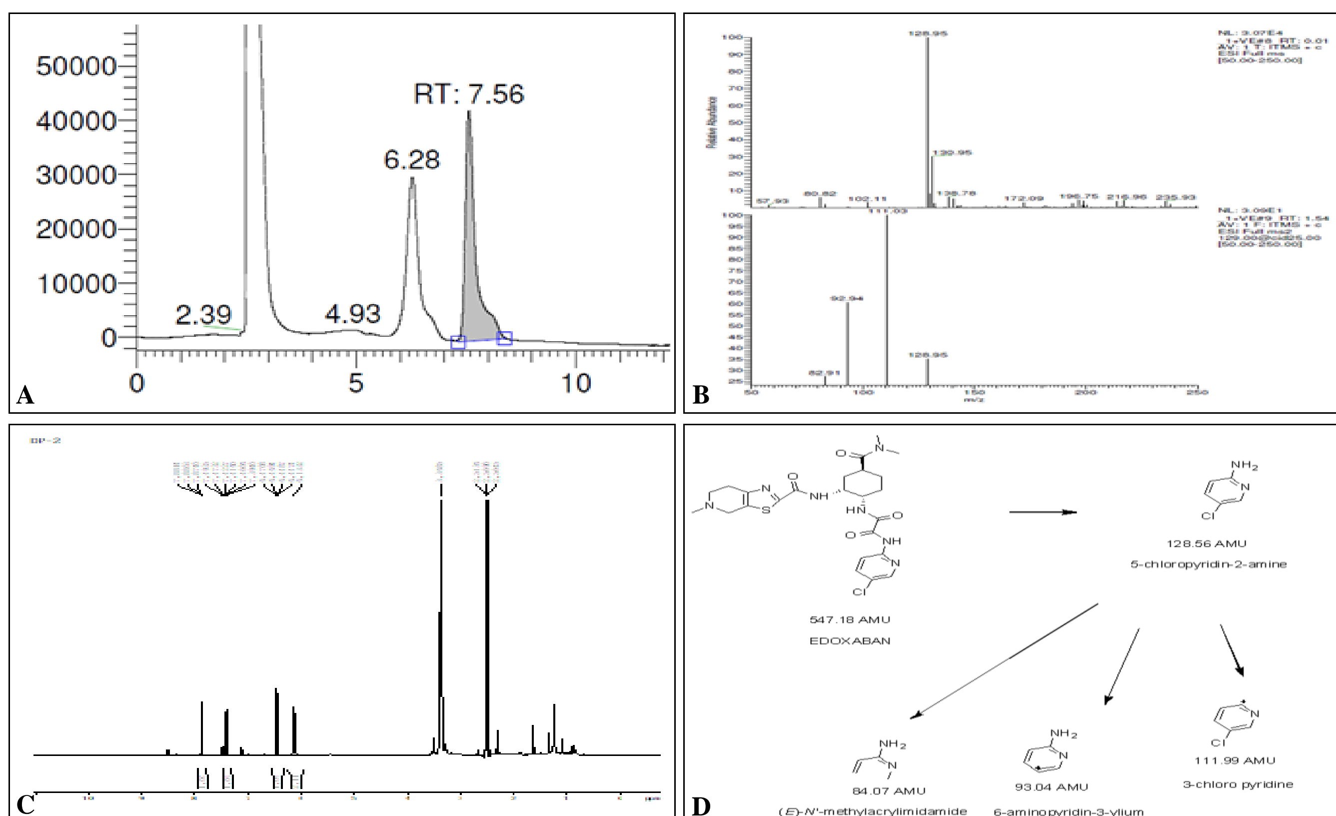


FIG. 8: CHARACTERIZATION OF DP-II SHOWING (A) LC-MS/MS CHROMATOGRAM, (B) LC-MS/MS SPECTRA, (C) ^{13}C-NMR AND (D) FRAGMENTATION PATTERN FOR DP-II

DP-IV (m/z 364.10): The ESI-MS/MS data for DP-III showed [M+H] + peak at 564.10 which was higher than the m/z of the parent moiety, *i.e.* 548.16. This observation gave evidence of formation adduct. The oxygen adduct could be formed at the 5th nitrogen position of the pyridine ring. This was further fragmented and gave abundant product ions at m/z 382.12, which could be due to the removal of thiazolo-piperidine ring **Fig. 9**.

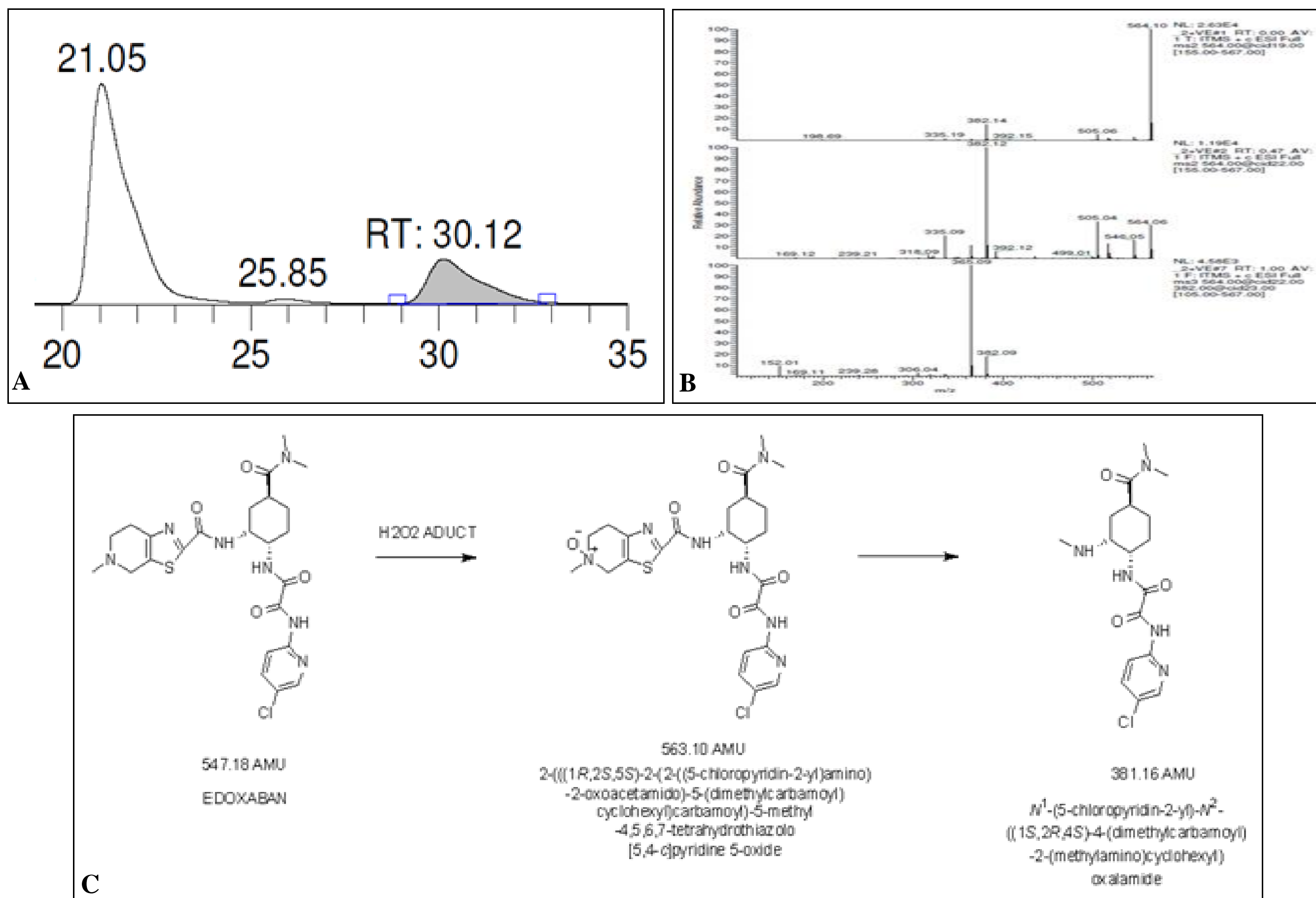


FIG. 9: CHARACTERIZATION OF DP-IV SHOWING (A) LC-MS/MS CHROMATOGRAM, (B) LC-MS/MS SPECTRA AND (C) FRAGMENTATION PATTERN FOR DP-IV

CONCLUSION: The simple, robust, selective, and isocratic RP-HPLC SIAM was developed for EDO estimation, which was able to easily and effectively discriminate the EDO specifically in the presence of numerous degradation impurities. The degradation study was carried out in different stressed conditions. The experiment revealed that EDO was extremely labile for alkaline hydrolytic condition and labile for the acidic condition.

In both the hydrolytic condition it formed two major identical DPs, *i.e.* DP-I and DP-II. This was isolated and characterized thoroughly employing ESI-MS/MS and NMR study. Further, the peroxide degradation also gave two DPs, *i.e.* DP-III and DP-IV along with DP-I. The developed SIAM was selective enough to estimate EDO in the presence of all DPs with the accepted resolution. Further, the validation of developed SIAM met all the criteria as specified in ICH Q2(R1). The detailed degradation kinetics study at different temperature and different stressor conditions exhibited first-order reaction kinetics of degradation.

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