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ORIGINAL RESEARCH ARTICLE

Development of HPLC method for isomeric impurities of key starting material of novel oral anticoagulant drug; Edoxaban Tosylate Monohydrate

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ABSTRACT

Reverse-phase high-performance liquid chromatography method has been developed for the determination of EDO-S1 stereoisomeric impurities such as isomer 1, isomer 2, isomer 3, isomer 4, isomer 5, isomer 6 and isomer 7 with good resolution using the column, Bakerbond C18 (150 × 4.6 mm; 3 µm). The separation was achieved with mobile phase-A (10 mM dipotassium hydrogen phosphate pH-7.0 with 10% orthophosphoric acid solution in Milli-Q water) and mobile phase-B (n-Propanol: Acetonitrile ratio of 20:30 % V/V), which consisted of mobile phase mixture in the combination of moilephase-A: mobile phase-B (85:15). The total run time was 30 min at 0.8 mL/min flow rate, 20 µL injection volume and 30 ℃ column oven temperature. The column eluate was monitored at 210 nm to quantify the impurities The method showed adequate specificity, sensitivity, linearity, accuracy, precision, and robustness inline to ICH tripartite guidelines. The limit of detection and quantification limits were 0.1 and 0.3 µg mL^{-1}, respectively, for all isomeric impurities and EDO-S1. The developed method was found to be linear over the concentration range of LOQ to 150% of specification range for isomeric impurities with a correlation coefficient >0.999. The method was precise (%RSD < 5.0), robust, and accurate (with 85%–115% recovery).

Keywords: edoxaban starting material; isomeric impurities; development; validation; HPLC

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1. Introduction

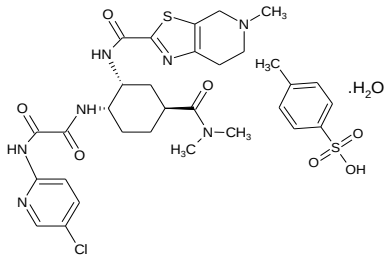
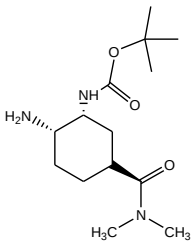
Stereoisomers are molecules that are identical in atomic bonding or constitution but distinguishable in their three-dimensional arrangement. Most of the molecules in the human body, like enzymes, proteins, amino acids, carbohydrates, nucleosides, and hormones, are chiral compounds. Further, the utmost number of drug molecules (approximately 56%) have chiral centers, whereas 88% of the molecules are marketed as racemates consisting of an equimolar mixture of two enantiomers[1–4].

Even though the enantiomers have the same chemical structure, they exhibit differences in biological activities. Hence, chiral analysis is essential in the pharmaceutical industry and in the clinic to control or eliminate the unwanted isomer from the preparation. The regulatory agencies (USFDA and EDQM) formulated the regulations for the manufacture of racemic drugs to investigate each isomer of the chiral drug individually to ensure their safety[5–8]. According to the International Conference on Harmonisation (ICH) guidelines, chiral identity and chiral assay tests are required in the specifications of the chiral products. Hence, the analytical method is indispensable for the

quantitative determination of isomers in starting materials, intermediates and drug substances and is one of the most widely used analytical tools for direct chiral analysis[9–11].

The chemical name for Edoxaban Tosylate Monohydrate (EDO) (**Table 1**) is N'-(5-chloropyridin-2-yl)-N-[(1S,2R,4S)-4-(dimethyl carbamoyl)-2-[(5-methyl-6,7-dihydro-4H-[1,3] thiazolo [5,4-c] pyridine-2-carbonyl) amino] cyclohexyl] oxamide; 4-methyl benzene sulfonic acid. EDO is generally used in the treatment of systemic embolism. Rogonic et al. EDO is a novel oral anticoagulants (NOACs) class of drugs and is a rapidly acting, oral, selective factor Xa inhibitor. It does not require antithrombin III for antithrombotic activity. Edoxaban inhibits free FXa, and prothrombinase activity and inhibits thrombin-induced platelet aggregation. Inhibition of FXa in the coagulation cascade reduces thrombin generation and reduces thrombus formation[12–15].

Table 1. Product details of EDO and EDO-S1.

Compound name	**Edoxaban Tosylate Monohydrate**	**Key starting material of Edoxaban Tosylate Monohydrate**
Product code	EDO	EDO-S1
Chemical name	4-Methylbenzene-1-sulfonic acid N'-(5-chloropyridin-2-yl)-N-[(1S,2R,4S)-4-(dimethylcarbamoyl)-2-{5-methyl-4H,5H,6H,7H-[1,3]thiazolo[5,4-c]pyridine-2-amido}cyclohexyethanediamide hydrate	Tert-butyl N-[(1R,2S,5S)-2-amino-5-(dimethlycarbmoyl) cyclohexyl] carbamate
Structure		
Molecular formula	$C_{31}H_{40}ClN_7O_8S_2$	$C_{16}H_{29}N_3O_7$
Molecular weight	738.27 g/mol	375.42 g/mol
Cas number	1229194-11-9	1210348-34-7
Therapeutic category	Anticoagulant	
Packing condition	Pack the sample under inert atmosphere in double polyethylenebag followed by triple laminated aluminium bag and keep in HDPE drum	Pack the sample under inert atmosphere in double polyethylenebag and keep in HDPE drum
Storage conditions	Preserve in well closed container and store at controlled room tempararture (excursions permitted between 15 and 30 °C)	Hygroscopic material. Protect from moisture. Store below 30 °C. Keep container tightly closed in a dry and well-ventilated place
Handling precautions	Handling should be done in a ventilated area. During handling of material the person should wear all the PPEs	Handling should be done in a ventilated area. During handling of material the person should wear all the PPEs

Edoxaban has three chiral centres, and according to the chiral principle 2^n rule, it exhibits eight isomers. These eight isomers can be controlled either at the drug substance or potential source of the stage. According to the synthetic route of Edoxaban, EDO-S1 is the source of the formation of eight chiral isomers, and it can be controlled at this stage according to the ICH limits, As per the ICH, any unspecified impurity with an acceptance criterion of not more than the identification threshold, which should be not less than 0.10% for a maximum daily dose of less than 2 g/day, If the impurities are controlled in between the stages of the synthetic route (**Figure 1**), there is no need to further control the same or related impurities in API[16,17].

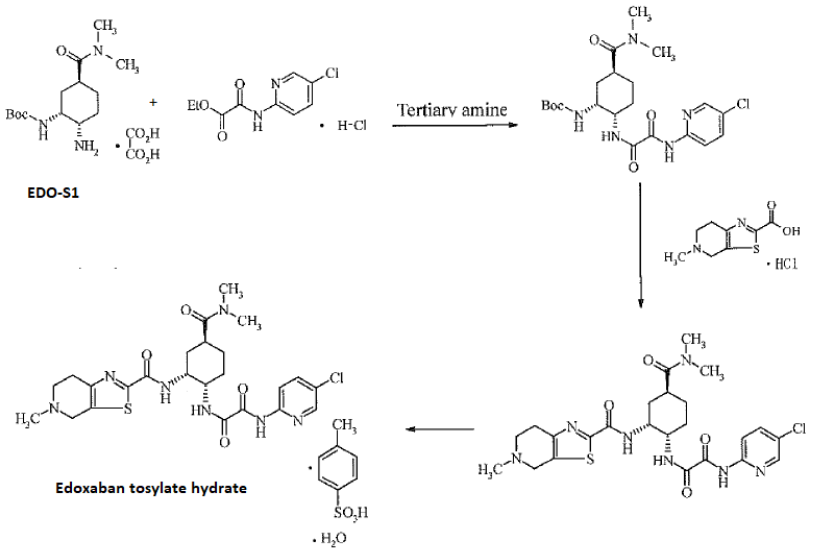


Figure 1. Edoxaban route of synthesis.

Four of the eight chiral impurities (**Figure 2**) each have an enantiomer, and under the conditions of traditional achiral chromatography, a molecule and its enantiomeric impurity will coelute as a single peak, that is, a racemate of the impurities will be monitored as a single peak. As a result, the chromatogram will show four peaks: EDO-S1 + EDO-S1 enantiomer (isomer 1); isomer 2 + isomer 2 enantiomer (isomer 3); isomer 4 + isomer 4 enantiomer (isomer 5); and isomer 6 + isomer 6 enantiomer (isomer 7).

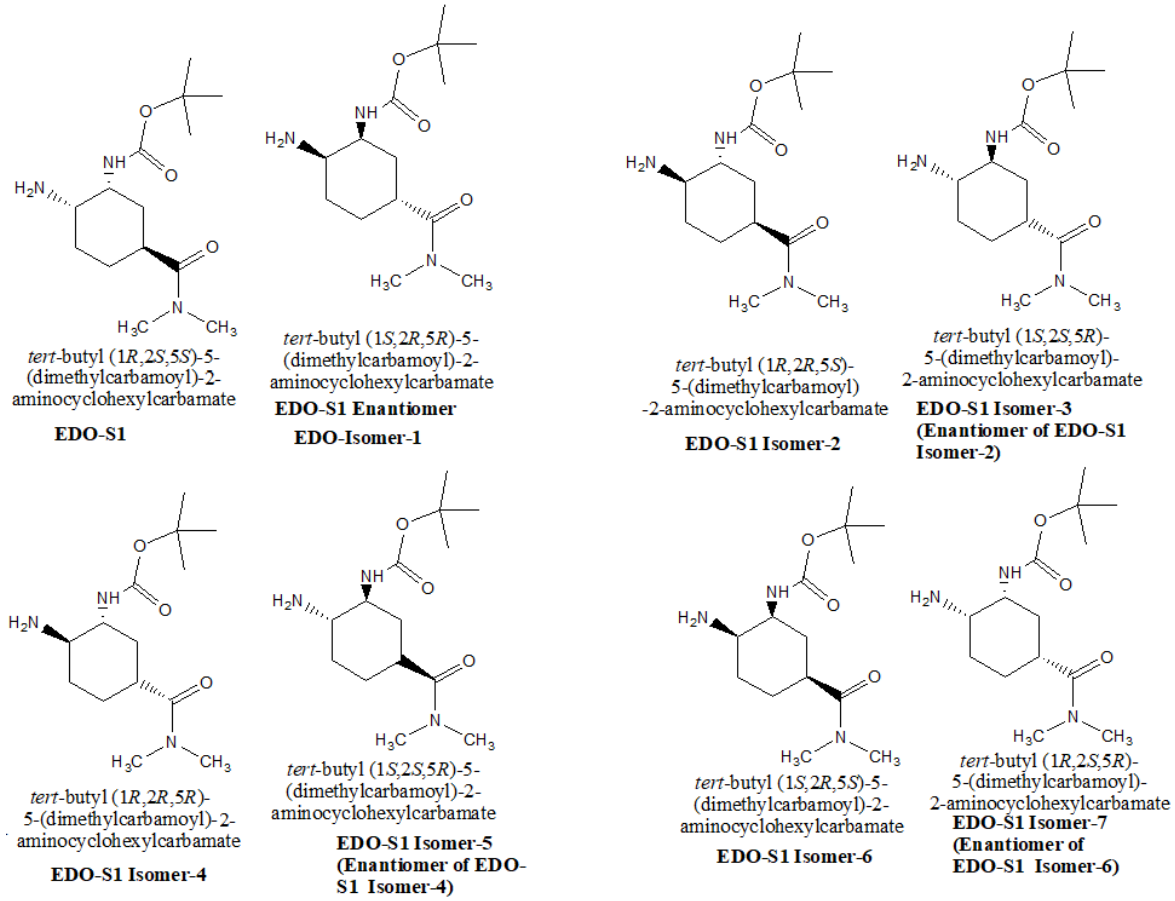


Figure 2. Impurity structures if EDO-S1 isomers.

Therefore, it is possible to regulate six isomeric impurities of EDO-S1 at the stage of EDO-S1, which may aid the producers in controlling the only enantiomeric impurity of edoxaban at the API stage. In this study, we developed an analytical technique to determine the six isomeric impurities in EDO-S1 and suitable validated it in accordance with regulatory requirements.

2. Materials and methods

2.1. Materials and reagents

EDO-S1 and its isomeric impurities were obtained from Micro labs limited (Bangalore, India). Trifluoroacetic acid ACS reagent, HPLC grade acetonitrile, and methanol were purchased from Merck (Bangalore, India). Obtained Ultrapure water (Milli-Q) from Merck milli-Q direct (Bangalore, India), and analytical grade is used for all other reagents.

2.2. Instrumentation and methods

2.2.1. High-performance liquid chromatography (HPLC)

Shimadzu LC-2010 C_{HT} HPLC system (Shimadzu corp. Japan), consisting of UV, photodiode array detectors with a wavelength range of 190-800 nm, an auto sample with a 50 µL loop, and Empower-3 software (Waters chromatography division, Milford, USA) for chromatographic integration.

The chromatographic separation of EDOS-1 and its isomeric impurities was performed on a Bakerbond C18 (150 mm, 4.6 mm i.d., particle size 3 µ) procured from JTbaker (Avantor Pennsylvania, United States). The following chromatographic conditions were employed, a profile of mobile phase-A (10mM dipotassium hydrogen phosphate pH-7.0 with 10% orthophosphoric acid solution in Milli-Q water) and mobile phase-B (n-Propanol: Acetonitrile ratio of 20:30 % V/V), which consisted of mobile phase mixture in the combination of mobile phase-A: mobile phase-B (85:15). The total run time was 30 mi at 0.8 mL/min flow rate, 20 µL injection volume and 30 ℃ column oven temperature. The column eluate was monitored at 210 nm to quantify the impurities.

2.3. Solution preparation

2.3.1. Preparation of standard stock, standard, system suitability and sample solutions

The mobile phase was used as dissolvent to the preparation of samples. The stock solution of EDO-S1 and its isomeric impurities each EDO-S1isomer-1 EDO-S1isomer-2, EDO-S1isomer-3, EDO-S1isomer-4, EDO-S1isomer-5, EDO-S1isomer-6, EDO-S1isomer-7 were prepared by weighing the components appropriately and diluted with diluent to concentration of 10 µg mL^{-1}. The precise and appropriate volumes of stock solutions of the compounds were further diluted with diluent in the volumetric flask to the volume mark to prepare standards at concentration of 1.5 µg mL^{-1}. The appropriate amount of stock solutions of EDO-S1 and known impurites were transferred into the same volumetric flask and diluted with diluent to the volume mark to obtain their concentration 1000 µg mL^{-1} and 1.5 µg mL^{-1}, respectively. The obtained solution was used as the system suitability solution and also as spiked solution in the course of experiments.

The final concentration of the control sample was prepared to 1 mg mL^{-1}. All the prepared sample solutions were filtered through 0.22 µm nylon syringe filters prior to the injection in HPLC.

3. Results and discussion

3.1. Method development for the EDO-S1 isomeric impurities

HPLC method development was optimized with spiked sample, prepared by spiking with EDO-S1 isomeric impurities to EDO-S1.

3.1.1. Optimization of gradient program

The aim of the study was to develop the HPLC method for the determination EDO-S1 isomeric impurities, method development was started with Bakerbond C18 (150 mm × 4.6 mm; 3 µ) column contain mobile phase-A: 10 mm Di potassium phosphate pH-7.0 and mobile phase-B: Acetonitrile. Column flow maintained 1.0 mL/min; initially program started with Buffer: Acetonitrile (50:50). Spiked solution injected for the development and chromatogram monitored with PDA detector. Considered the above condition as a **Trial-1**. In this conditions EDO-S1 and its isomeric impurities were eluted near to the void volume. To get more retention and resolution among the same impurities, **Trail-2** has been taken by modifying the mobile phase composition to Buffer: Acetonitrile (90:10). Retention factor and resolution among three impurities were observed well, but the resolution between EDO-S1 & EDO-S1 impurity-2 + 3 and EDO-S1 impurity-4 + 5 & impurity-6 + 7 were co eluted. **Trial-3** was taken to get the more resolution between the impurities by changing the mobile phase composition to Buffer: methanol (90:10). All peaks were having broad peak shape no resolution observed among the impurities.

Trail-4 has been taken by modifying the mobile phase composition to Buffer: Acetonitrile (85:15). Retention factor and resolution among three impurities were observed well, and resolution between EDO-S1 & EDO-S1 impurity-2 + 3 and EDO-S1 impurity-4 + 5 & impurity-6 + 7 were improved. To enhance the resolution among all impurities further organic modifiers were screened by considering trail-4 as base method.

3.1.2. Different stationery phase screening

In order to understand the impurity selectivity and resolution, numerous columns comprising various stationary phases, including as C18, C8, phenyl, and phenyl hexyl columns, have been examined under trail-4 chromatographic conditions. Similar selectivity was applied to the C18 and C8 columns, however there was less resolution between EDO-S1 and EDO-S1 impurities 2 and 3 compared to the C18 column. Impurities 2 + 3 from the phenyl column EDO-S1 also eluted with EDO-S1. EDO-S1 impurities 2 + 3 were co-eluted with EDO-S1 on phenyl hexyl column, which demonstrated selectivity similar to that of phenyl column. The C18 stationary phase was chosen as an appropriate column with regard to retention and resolution when compared to all of the other stationary phases (**Table 2**).

Table 2. Method optimization trails to separate stereoisomeric impurities.

Column	**pH**	**Resolution**			
		EDO-S1 + Enantiomr	**Isomer-2 + 3**	**Isomer-4 + 5**	**Isomer-6 + 7**
Bakerbond C18 (150 mm × 4.6 mm; 3 µ)	Buffer: Organic Mixture (85:15)	-	4.4	4.1	10.6
Bakerbond C8 (150 mm × 4.6mm;3 µ)	Buffer = 10 mm Di potassium phosphate pH-7.0	-	3.2	2.9	10.1
Thermo PFP (150 mm × 4.6 mm; 2.6 µ)	Organic Mixture = Acetonitriel:	Co-eluted		2.5	6.5
Bakerbond Phenyl Hexyl (150 mm × 4.6 mm; 3 µ)	1-Propanol (60:40)	-	1.48	3.5	10.1
Bakerbond Phenyl (150 mm × 4.6 mm; 3 µ)		Co-eluted		3.0	7.2
Bakerbond C18 (150 mm × 4.6 mm; 3 µ)	pH-3.0	Co-eluted		2.5	8.5
	pH-5.0	Co-eluted		3.2	9.2
	pH-7.0	-	4.4	4.1	10.6
	pH-8.0	Broad peak shape	4.0	4.5	10.2

3.1.3. Organic modifier study in mobile phase-B

The influence of volume fraction of organic modifiers on selected chromatographic conditions (trail-4) have been investigated. Acetonitrile, methanol, ethanol, n-propanol, 2-propanol, THF modifiers composition were screened to get the impact on resolution among critical pairs. It was observed that the resolution among critical pairs has been enhanced by the addition of 1-propanol to acetonitrile in mobile phase-B (**Table 3**).

Table 3. % of different organic composition with acetonitrile study in mobile phase-B.

Mobile phase B	**Conditions**	**Resolution**			
		EDO-S1 + Enantiomr	**Isomer-2 + 3**	**Isomer-4 + 5**	**Isomer-6 + 7**
Acetonitrile	Bakerbond C18 (150 mm × 4.6 mm; 3 µ)	-	Co eluted with EDO-S1	1.6	9.3
Acetonitrile: Methanol::9:1 (v/v)	Buffer = 10 mm Di potassium phosphate pH-7.0	-	1.2	1.5	10.2
Acetonitrile: Ethanol::9:1 (v/v)		-	1.2	1.5	10.0
Acetonitrile: 2-propanol::9:1 (v/v)		-	1.5	2.1	12.5
Acetonitrile: 1-propanol::9:1 (v/v)		-	1.5	3.5	10.5
Acetonitrile: 1-propanol::8:2 (v/v)		-	2.5	4.0	10.2
Acetonitrile: 1-propanol::7:3 (v/v)		-	3.2	3.8	11.3
Acetonitrile: 1-propanol::6:4 (v/v)		-	4.4	4.1	10.6
Acetonitrile: 1-propanol::5:5 (v/v)		-	4.4	3.2	10.6

3.1.4. The effect of buffer pH

To develop HPLC method, di potassium hydrogen ortho phosphate was selected as a buffer for the method development. Different pH (3.0, 5.5, 6.0, 7.0 and 8.0) were screened with trail-4 chromatographic conditions and observations were tabulated in **Table 4**. Resolution observed more than 1.5 for all impurities in pH-7.0.

Table 4. Validation summary.

Parameter	**Retention time**	**LOD**		**LOQ**		**Correlation coefficient**	**Slope**	**Intercept**	**Repeatability (% RSD, *n* = 6)**	**Repeatability (% RSD, *n* = 6)**	**% Recovery**		
									LOQ level	**Spec level (0.10%)**	**LOQ**	**0.10%**	**0.15%**
		(µg mL^{-1})	**s/n**	**(µg mL^{-1})**	**s/n**								
EDO-S1 + Enantiomer	8.59	0.1	3	0.3	12	0.9990	15,695	421.00	3.9	2.5	-	-	
EDO-S1 Isomer-2 + 3	10.09	0.1	6	0.3	20	1.0000	11,724	27.72	2.1	0.4	86.73	99.02	99.12
EDO-S1 Isomer-4 + 5	11.28	0.1	5	0.3	20	0.9990	10,416	535.82	4.3	1.0	104.90	10.90	109.90
EDO-S1 Isomer-6 + 7	14.82	0.1	4	0.3	14	1.0000	11,182	547.78	4.9	2.1	91.88	110.83	112.83

3.2. Method validation

The method was validated according to the ICH Q2 (R1) guideline to prove the suitability of the method for their intended purpose[18–21].

3.2.1. System suitability

The appropriateness of the system was assessed in order to confirm its performance. The parameters, including resolution, theoretical plates, and percent RSD for peak area, were measured after six repeated injections of the standard solution and one injection of the system suitability solution were injected in the HPLC system. The system suitability study's findings, which are presented in **Table 5 (Figure 3)**, indicated that all of the parameters fell within the predefined limits of acceptable performance.

Table 5. System suitability criteria and results.

Mobile phase B	**Acceptance criteria**	**EDO-S1 + Enantiomr**	**Isomer-2 + 3**	**Isomer-4 + 5**	**Isomer -6 + 7**
RSD (%) for peak area response (n = 6) in standard solution	Should be not more than 5.0%	1.3	2.2	1.6	3.2
S/N ratio of each peak in sensitivity solution	Should be not less than 10	15	16	18	13
USP tailing of each peak in standard solution	Should be not more than 2.0	1.6	1.2	1.5	1.3
Resolution between EDOS-S1 and Isomer-2 peak	Should be not less than 2	-	4.4	-	-
Resolution between isomer-2 and isomer-3 peak	Should be not less than 2	-	-	4.6	-

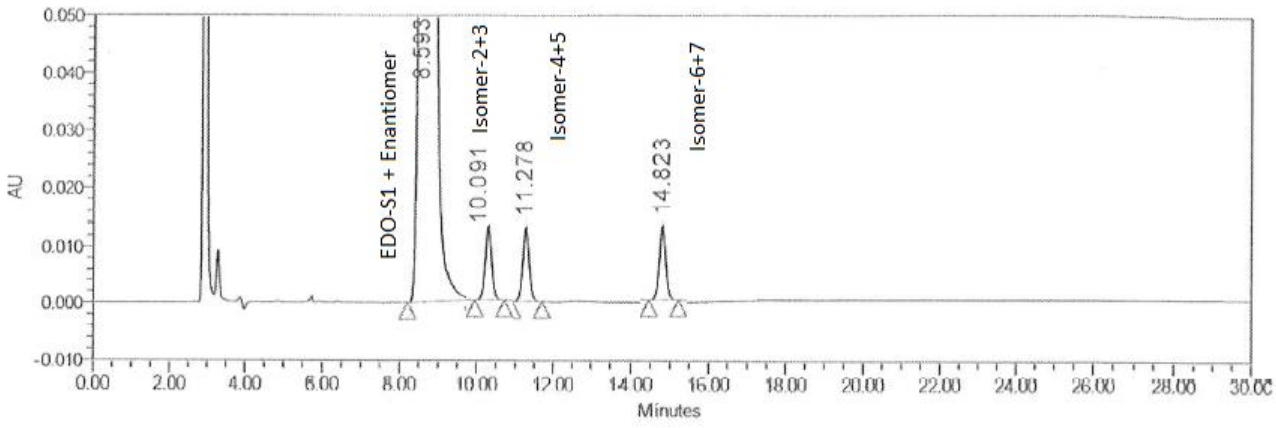


Figure 3. Spike chromatogram of EDO-S1 isomeric impurities spike chromatogram.

3.2.2. Specificity

To confirm the interference caused by impurities from degradation and the blank and sample matrices, the specificity of the devised HPLC technique was assessed. In order to make sure that there is no interference at the retention time of each target peak and resolution among all peaks, the HPLC-PDA was used to analyse the blank, test sample, and spiked samples.

The blank peaks were distinguished from all peaks pertaining to EDO-S1 and their isomeric impurities. Using a PDA detector, the peak purity of each peak was determined. The purity angles of each peak were found to be below the purity threshold, making them all pure and uniform. It implied that the approach could detect and measure the EDO-S1 isomers even in the presence of their impurities without interference from the blank.

3.2.3. Detection limit (LOD) and quantitation limit (LOQ)

The LOD and LOQ were calculated using the baseline noise technique. According to ICH-Q2 (R1), the signal-to-noise ratio is calculated by comparing the observed signals of samples with known low analyte concentrations with those of control samples. The minimal concentration at which an analyte can be consistently detected is established by LOD, and the minimum concentration at which an analyte can be reliably quantified is established by LOQ. The detection limit is estimated using a signal-to-noise ratio of 3 to 5, and the quantitation limit is estimated using a signal-to-noise ratio of 10 to 15. Precision was achieved by injecting EDO-S1 and all its isomers in six replicate samples at the QL level, and by determining the percentage relative standard deviation (RSD) of peak regions. The results are shown in **Table 4** (**Figure 4**).

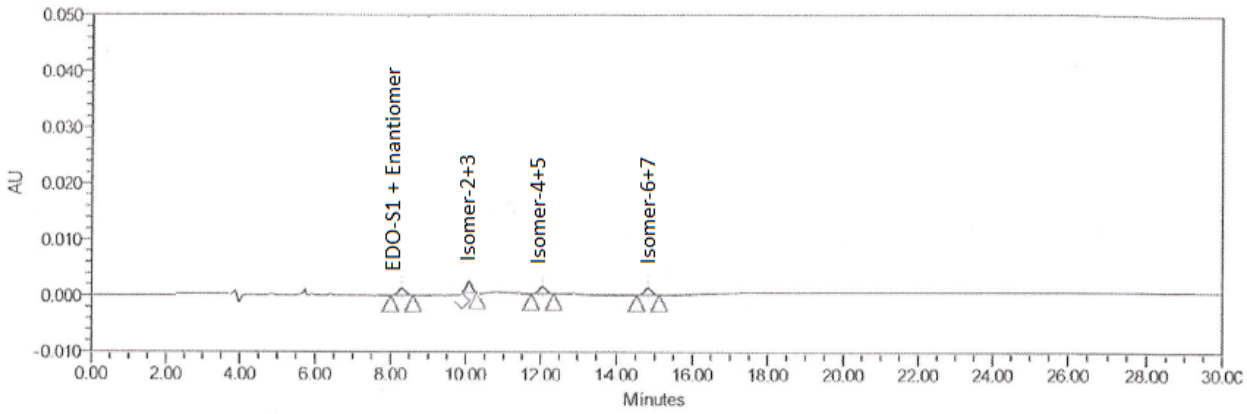


Figure 4. LOQ chromatogram of EDO-S1 isomeric impurities chromatogram.

3.2.4. Linearity and range

The capacity of an analytical process to produce test results that are directly proportional to the concentration (quantity) of analyte in the sample is known as linearity.

By carrying out the necessary dilutions to achieve the desired concentrations, a series of linearity solutions containing EDO-S1, EDO-S1isomer-1, EDO-S1isomer-2, EDO-S1isomer-3, EDO-S1isomer-4, EDO-S1isomer-5, EDO-S1isomer-6, and EDO-S1isomer-7 solution were prepared. The above-prepared EDO-S1 and impurity solutions are LOQ to 150% of the known impurity specification limit, or 0.15%. The concentration of each component was plotted against the peak area of the corresponding known component after each solution was injected once. The regression line's y-intercept, slope, square correlation coefficient, correlation coefficient, and correlation coefficient were tabulated in **Table 4**. By determining the percentage RSD for six replicates of lower and higher values, the analytical method's range was assessed. RSD percentage was found to be under 5%.

3.2.5. Accuracy (recovery) and repeatability

With respect to the sample concentration of 1.0 mg mL^{-1}, spiked samples were prepared at QL, 50%, 100%, and 150% of the prescribed limit (i.e., 0.15%). By dividing the content measured in the spiked sample by the content spiked to the sample, the accuracy was determined. For each of the required contaminants at all levels, the percentage recovery was computed, and the results are shown in table. Six spike sample preparations were injected at 100% level to complete the repeatability test, and the findings were summarised in **Table 4** along with the percent RSD for each impurity's content.

The accuracy of all impurities was determined to be between 85 and 115% at all levels, including QL, 50, 100, and 150%. The reproducibility was determined to be 5% for all contaminants for six preparations of spiked sample solution at 100% level.

3.2.6. Robustness

Applying slight changes to the chromatographic conditions had little impact on the developed procedure. The resolution between the closest pairs was used to assess the procedure after slight variations in the flow rate (0.1), column oven temperature (2 °C), and pH (7.0 ± 0.2) were made (**Table 6**). The observed results showed that the created method was unaffected when the chromatographic conditions were changed slightly, proving that the method is robust to deliberate, slight modifications.

Table 6. Robustness study.

Robustness parameter	**Resolution**		
	Isomer-2 + 3	**Isomer-4 + 5**	**Isomer-6 + 7**
Ideal (0.8 mL/min; 30 °C; pH-7.0)	4.1	4.4	10.6
Flow (0.7 mL/min)	4.0	4.2	10.4
Flow (0.9 mL/min)	3.9	4.2	10.3
Temperature (25 °C)	3.8	4.4	10.8
Temperature (35 °C)	4.2	3.8	11.2
Buffer pH-6.8	4.0	4.0	10.2
Buffer pH-7.2	4.1	3.8	10.9

4. Conclusions

To the best of the literature's knowledge, the first RP-HPLC report method for the determination of potentially plausible isomeric impurities of edoxaban was created for the purpose of determining EDO-S1 isomeric impurities from edoxaban. According to ICH criteria, the devised method was verified for specific parameters that fall within the permissible limits. The new approach, on the other hand, indicated exceptional values in linearity, accuracy, and precision with decreased LOD and LOQ. As a result, the proposed RP-HPLC method in the current study was suitable for the bulk-scale quantification of the isomeric impurities of EDO-S1.

Author contributions

Conceptualization, VKB and SN; methodology, VKB; software, VKB; validation, VKB and SN; formal analysis, VKB; investigation, VKB; resources, VKB; data curation, VKB; writing—original draft preparation, VKB; writing—review and editing, SN; visualization, SN; supervision, SN; project administration, VKB; funding acquisition, VKB. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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