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Original Article

Simultaneous quantification of ticagrelor and its metabolite deshydroxyethoxy ticagrelor in human plasma by ultra-performance liquid chromatography electrospray ionization-tandem mass spectrometry

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ABSTRACT

A precise, highly sensitive, selective and robust liquid chromatography-mass tandem spectrometry method was developed and validated for simultaneous quantification of Ticagrelor and Deshydroxyethoxy ticagrelor in human plasma by liquid-liquid extraction. Ticagrelor d-7 and Deshydroxyethoxy ticagrelor d-7 was used as an Internal standard. The reconstituted samples were analyzed on Eclipse XDB-C8 5µm 4.6*150mm column by using Acetonitrile and 0.1% Formic acid as the mobile phase in binary mode at the flow rate of 1.0 ml/min with a chromatographic run time of 3.0 min. The calibration curve developed was linear ($r^2 \geq 0.99$) in the concentration range of 2.5 to 1000 ng/mL for Ticagrelor and 1.0 to 300 ng/mL for Deshydroxyethoxy ticagrelor. All the analytes were found to be stable in various stability studies. The mean recovery for Ticagrelor, Deshydroxyethoxy Ticagrelor, Ticagrelor d-7 and Deshydroxyethoxy Ticagrelor d-7 were found to be 99.7%, 96.6%, 105.9% and 101.1% respectively. The limit of detection of the method was 0.5 ng/mL for Ticagrelor and 0.2 ng/mL for Deshydroxyethoxy ticagrelor. The developed and validated assay method was successfully applied to a pharmacokinetic study in human volunteers.

Keywords: Ticagrelor, Deshydroxyethoxy ticagrelor, LC-MS/MS, Human plasma, Liquid-Liquid Extraction.

INTRODUCTION

Ticagrelor [(1*S*,2*S*,3*R*,5*S*)-3-[7-[[(1*R*,2*S*)-2-(3,4-difluorophenyl) cyclopropyl] amino]-5-(propylthio) -3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-5-(2-hydroxyethoxy)-1,2-cyclopentanediol); (Fig. 1)] is a platelet aggregation inhibitor used for reduction of clinical thrombotic events in patients with acute coronary syndromes. It gets readily absorbed after oral administration and reaches maximum concentration within 1 to 3 hours.

Deshydroxyethoxy Ticagrelor (AR-C124910XX, Fig. 2) is the active metabolite of Ticagrelor; which is as potent as Ticagrelor at the $P2Y_{12}$ receptor and is present in the circulation at approximately one-third of the concentration of the parent drug [1-4]. Few analytical methods have been reported for quantification of Ticagrelor and/or its metabolite Deshydroxyethoxy Ticagrelor in biological matrix. The reported methods are comparatively less sensitive and have a very broad calibration curve [5, 6] thereby making them un-suitable for clinical studies. The purpose of this study is to develop an improved, more sensitive and higher throughput method for the simultaneous quantification of Ticagrelor and its metabolite Deshydroxyethoxy Ticagrelor in human plasma to be applied to study sample analysis of clinical studies intended for regulatory submissions.

EXPERIMENTAL

Reagents and materials: The reference Ticagrelor (Drug), Ticagrelor d-7 (ISTD-1), Des hydroxy ethoxy ticagrelor (Metabolite) and Des hydroxy ethoxy ticagrelor d-7 (ISTD-2) was procured from Vivan life science and Clearsynth Labs Ltd., Mumbai. Chemical structures of same presented in Fig.1 and Fig. 2 Respectively. Water used for LC-MS/MS was prepared from Mili-Q water system. Acetonitrile, Ammonium acetate and Acetonitrile of gradient grade were procured from Merck Ltd., India and Emparta grade Formic acid was procured

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from Merck Ltd., India. The sample of human plasma was collected from in-house.

Instrumentation and chromatographic conditions: The column used was Eclipse XDB-C8 5µm 4.6*150mm. The mobile phase consists of Acetonitrile and 0.1% Formic acid solution in binary mode was delivered at 1.00mL/min into the electrospray ionization chamber into the mass spectrometer. The injection volume was 20µL and run time was 3.00 mins. The mobile phase was delivered by the ultra-performance liquid chromatography (UPLC) solvent manager and the samples were injected by a UPLC sample manager (Waters,USA). Ionization and quantification of analytes and IS(internal standard) were performed on a triple quadrupole mass spectrometer, Waters Quattro Premier XE (Waters, USA) equipped with Turbo Ion sprays, operated in the positive ion mode using multiple reaction monitoring(MRM). The compound parameters viz. the ion energy (1 & 2), cone(V), capillary(kV), extractor(V), cone Gas Flow (L/Hr), Collision energy(CE), Desolvation Gas Flow (L/Hr), were 1.0, 40, 3.5, 5.0, 40, 50 and 850 respectively. Detection of the ions was carried out in the multiple reaction monitoring (MRM) mode by monitoring the transition pairs of m/z 523.20>153.10 for Ticagrelor (Fig.3), m/z 479.20>153.10 for Deshydroxyethoxy ticagrelor (Fig.4), m/z 530.20>153.10 for Ticagrelor d-7 (Fig.5), and m/z 486.20>153.10 for Deshydroxyethoxy ticagrelor d-7 (Fig.6). Quadrupoles Q_1 and Q_3 were set on unit resolution. MassLynx software version 4.1 was used for data acquisition and evaluation of chromatographic data.

Preparation of stock solution, calibration curve standards and quality control samples: The stock solution of Ticagrelor, Ticagrelor d-7, Deshydroxyethoxy ticagrelor and Deshydroxyethoxy ticagrelor d-7 of 1 mg/mL were prepared by dissolving in Methanol [HPLC Grade] and stored in a refrigerator within 2 to 8°C. Calibration curve (CC) standards consisting of eight non-zero concentrations were prepared by spiking standards in 0.99 mL of blank human plasma, each with 0.1 mL working solution of Ticagrelor and Deshydroxyethoxy ticagrelor in the concentration range of 2.495-998.8 ng/mL for Ticagrelor and 1.057–300.5 ng/mL for Deshydroxyethoxy ticagrelor. Similarly, quality control (QC) standards were prepared for the lower limit of quantification (LOQQC), low quality control (LQC), medium quality control (MQC) and high quality control (HQC) levels for both the analytes within the calibration curve range. After bulk spiking, each calibration curve standards and quality control samples were stored into different pre-labeled polypropylene tubes at –65 ± 10°C until analysis.

Extraction procedure: A set of calibration curve standards (CC) and Quality control samples (QC) were retrieved from freezer and allowed them to thaw in a water bath maintained at room temperature. The thawed samples were vortexed to ensure complete mixing of the contents. From each plasma sample tube aliquot of 250µL was transferred into pre-labeled polypropylene tubes followed by addition of 50µL of ISTD dilution (about 300 ng/mL of Ticagrelor-d7 & 300 ng/mL of Deshydroxyethoxy ticagrelor-d7) and 50 µL of Methanol [HPLC Grade] to blank samples and vortexed for 1.0 minute. Then 100 µL of 100 mM ammonium acetate solution was added and vortexed for 2.0minutes to ensure complete mixing. Then 3 mL of Diethyl ether [Emparta Grade] was added and vortexed for 5minutes. After vortexing the samples were centrifuged at rcf 3345 ± 150 for 5 minutes at 10°C. The plasma layer was flash frozen and the organic layer was transferred into pre-labeled evaporation tubes. The content was evaporated at about 40°C temperature under nitrogen stream to dryness and reconstituted with 250µL reconstitution solution. The samples were vortexed for 1 minute and transferred the contents into appropriate vials for analysis.

Method validation: The validation was performed in-order to evaluate the method in-terms of selectivity, sensitivity, linearity, precision, accuracy, recovery, robustness and ruggedness, limit of detection, matrix effect, re-injection reproducibility, stabilities and dilution integrity as per the USFDA guidance on bioanalytical method validation [8]. Selectivity and specificity were determined in six different lots of normal K_2EDTA plasma and one lot each of lipemic K_2EDTA and heamolysed K_2EDTA plasma. Three precision and accuracy batches of spiked plasma calibration curve standard at eight different concentrations levels ranging from 2.495-998.8 ng/mL for Ticagrelor and 1.057–300.5 ng/mL for Deshydroxyethoxy ticagrelor were prepared and analyzed. The linear regression of $1/(\text{concentration})^2$ was used to construct the Ticagrelor and Deshydroxyethoxy ticagrelor calibration curves. Intra-day precision and accuracy were determined by analyzing six replicates at five different QC levels on three different days. Inter-day precision and accuracy were determined by analyzing six replicates at five different QC levels of five different runs. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences. Matrix effect was checked with ten different lots of K_2EDTA plasma. Recoveries of Ticagrelor, Deshydroxyethoxy

ticagrelor and IS were determined by comparing the peak area of extracted analyte standard with the peak area of unextracted standard. A recovery of Ticagrelor was determined at concentrations of 7.457 ng/mL, 502.2 ng/mL and 756.3 ng/mL whereas of Deshydroxyethoxy ticagrelor was determined at concentrations of 3.129 ng/mL, 150.0 ng/mL and 225.8ng/mL, respectively.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions at two different concentration (LQC and HQC) levels. The stock solution stability at room temperature and refrigerated conditions (2–8°C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. For Bench top stability required samples kept at room temperature for 9hrs, post processed sample stability (auto sampler stability for 114hrs, wet extract bench top stability for 2hrs), freeze-thaw stability (4cycles), long-term stability (24days), reagent stability for 8 days at room temperature and mobile phase stability for 9 days at room temperature were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable as assay values were within the acceptable limits of accuracy (85–115%) and precision (15% RSD).

RESULTS AND DISCUSSION

Method development: The method development was initiated with the tuning of compounds on mass spectrometer. Mass spectrometer parameters were optimized by infusing a solution of 200 ng/mL of analyte into the mass spectrometer using electrospray as the ionization source. The signal intensities obtained in positive mode were much higher than those in negative ion mode since the analytes and IS have the ability to accept protons. The most sensitive mass transitions were monitored from m/z 523.20>153.10 for Ticagrelor, 479.20>153.10 for Deshydroxyethoxy Ticagrelor, 530.20>153.10 for Ticagrelor-d7 (ISTD) and 486.20>153.10 for Deshydroxyethoxy Ticagrelor-d7 (ISTD). Product ion spectrum of all the analytes are represented in Fig. 3 to Fig. 6.

After optimizing the mass parameters, the chromatographic conditions were tested. Different reverse phase columns like Zorbax Eclipse C8; Phenomenex Luna C18, ACE 3 C18 were tried to achieve better chromatography, stable response, optimum retention time and desired intensity. Organic modifiers like acetonitrile and methanol and buffers like 0.1% formic acid solution, 0.1% acetic acid solution, ammonium acetate and ammonium formate buffers at different strength were also tested as a part of optimizing the analyte sensitivity, reproducibility and acceptable chromatography. Zorbax Eclipse XDB-C8 5µm 4.6*150mm as column with mobile phase composition of Acetonitrile: 0.1% formic acid solution resulted in optimum sensitivity, better resolution and acceptable chromatography. As a part of optimization of chromatography; parameters like flow rate, column oven temperature and injection volume were also optimized.

For sample clean-up different extraction techniques like protein precipitation and liquid-liquid extraction were evaluated. Protein precipitation being a simpler extraction technique was tried first. However protein precipitation resulted in low analyte recovery and ion-suppression/enhancement related issues. As it was not possible to achieve the desired LLOQ sensitivity using protein precipitation technique; it was decided to switch to liquid-liquid extraction technique. Different extraction solvents like diethyl ether, methyl tert-butyl ether, hexane, dichloromethane, mixture of solvents and others were evaluated for optimizing the recovery using liquid-liquid technique. Additionally the extraction buffers like 1% formic acid solution, 0.1% sodium hydroxide solution, 100mM ammonium acetate solution, 1% ortho-phosphoric acid solution and others were also tested to optimize the analyte recovery. Combination of 100mM ammonium acetate solution as extraction buffer and diethyl ether as extraction solvent resulted in optimum analyte recovery and better reproducibility.

Once all the parameters were optimized; its effect on method selectivity, linearity, precision and accuracy, matrix effect and stability was evaluated under pre-method validation and was found to be within the acceptable limits.

Method validation

Selectivity: Selectivity was ascertained in different lots of human plasma. No significant interference was observed in any of the screened lots at the retention time of analytes and ISTD. The chromatogram represented in fig 7 indicates the selectivity of the method.

Linearity: Calibration curve was found to be linear for eight-point concentration range of 2.495-998.8 ng/mL for Ticagrelor and 1.057–300.5 ng/mL for Deshydroxyethoxy ticagrelor. A regression equation with weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship for both the analytes in human plasma.

The mean correlation coefficient r^2 of calibration curve was found to be ≥ 0.99. Table 1 represents the results obtained as a part of linearity evaluation. Refer Fig. 8 for representative chromatogram of LLOQ.

Precision and accuracy: The inter-batch precision and accuracy were calculated after analyzing three precision and accuracy batches with spiked QC samples. The intra batch precision and accuracy were calculated after analyzing six spiked samples of Ticagrelor and Deshydroxyethoxy ticagrelor at each QC level (2.230, 9.019, 535.9, 809.5 ng/mL of Ticagrelor and 1.023, 3.242, 159.4 and 243.7 ng/mL of Deshydroxyethoxy ticagrelor). Intra-day and inter-day precision ranged from 4.3% to 10.6% and 4.4% to 9.8% for Ticagrelor, 2.0% to 9.2% and 2.1% to 10.4% for Deshydroxyethoxy ticagrelor whereas accuracy, expressed as %nominal was within 101.8 % to 114.3 % and 100.8 % to 107.7 % for Ticagrelor, 99.3 % to 109.9 % and 98.0 % to 108.6 % for Deshydroxyethoxy ticagrelor respectively, as shown in Table 2.

Recovery: The absolute recoveries were estimated at low, medium and high QC samples for Ticagrelor, Deshydroxyethoxy ticagrelor, Ticagrelor d-7 and Deshydroxyethoxy ticagrelor d-7 by comparing the peak area response of the extracted samples with the recovery samples which were prepared by reconstituting the processed blank quality control samples with respective aqueous samples.. The absolute recovery determination for Ticagrelor and Deshydroxyethoxy ticagrelor was observed to be reliable, precise and reproducible. The mean recovery for Ticagrelor, Deshydroxyethoxy ticagrelor, Ticagrelor d-7 and Deshydroxyethoxy ticagrelor d-7 were found to be 99.7%, 96.6%, 105.9% and 101.1% respectively.

Matrix effect: Matrix effect was evaluated through matrix factor, which is calculated by comparing area response in presence of matrix ions with mean area response in absence of matrix ions at LQC and HQC concentration levels by using ten different plasma lots those passed selectivity criteria. The percentage coefficient of variance (% CV) for IS normalized matrix factor in ten lots was within the acceptance criteria of ≤ 15 % at each level for both Ticagrelor and Deshydroxyethoxy ticagrelor. Based on the obtained results it can be concluded that, no significant ion suppression or enhancement was observed during ionization in mass spectrometric detector for both Ticagrelor and Deshydroxyethoxy ticagrelor. Results of matrix effect are shown in Table 3.

Stability: The stability of the analytes in human plasma under different temperatures and times as well as stability of the analytes in stock solution under different conditions were evaluated. The different stability experiments carried out were Bench top stability (required samples kept at room temperature for 9hrs), processed samples stability (auto sampler stability for 114hrs, Dry extract stability 111hrs, wet extract bench top stability for 2hrs), freeze-thaw stability (4cycles), short term stability (12hrs), long-term stability (24days), reagent stability for 8 days at room temperature and mobile phase stability for 9 days at room temperature. These were performed at LQC and HQC levels using six replicates at each level. Data of various stability studies shown in Table 4.

CONCLUSION

A highly sensitive and selective method for the simultaneous determination of Ticagrelor and Deshydroxyethoxy ticagrelor in human plasma was developed using UPLC-MS/MS with turbo ion spray in positive ion mode with run time of 3.0min. The use of cost-effective, time saving and easy to handle liquid–liquid extraction method made it possible to achieve the sensitivity for both the analytes. This validated bioanalytical method is considered valid for the rapid quantification of Ticagrelor & Deshydroxyethoxy ticagrelor using Ticagrelor-d7 (ISTD-1) & Deshydroxyethoxy ticagrelor-d7 (ISTD-2) as the internal standards respectively with preferred accuracy and precision.

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Table 1: Back calculated concentrations of Ticagrelor and Deshydroxyethoxy Ticagrelor (n=3).

Analyte	Standard concentration(ng/mL)	Mean(ng/mL)	SD	CV (%)	Nominal (%)	Slope	Intercept	r^2
Ticagrelor	2.495	2.440	0.027	1.1	97.8	0.0095	0.00023	0.9978
	4.989	5.189	0.086	1.6	104.0			
	49.90	51.89	2.742	5.3	104.0			
	149.8	153.9	4.371	2.8	102.7			
	499.4	506.1	17.77	3.5	101.3			
	748.8	734.9	18.01	2.5	98.1			
	898.9	861.3	31.81	3.7	95.8			
	998.8	960.8	20.93	2.2	96.2			
Deshydroxyethoxy Ticagrelor	1.057	1.091	0.012	1.1	103.2	0.0160	-0.00033	0.9970
	2.113	1.994	0.050	2.5	94.4			
	15.00	14.12	0.099	0.7	94.2			
	45.01	43.18	0.717	1.7	95.9			
	150.0	154.7	4.749	3.1	103.1			
	225.3	230.0	1.327	0.6	102.1			
	270.4	270.6	8.796	3.3	100.1			
	300.5	321.4	4.649	1.4	107.0			

Table 2: Intraday and Interday precision and accuracy data of Ticagrelor and Deshydroxyethoxy ticagrelor

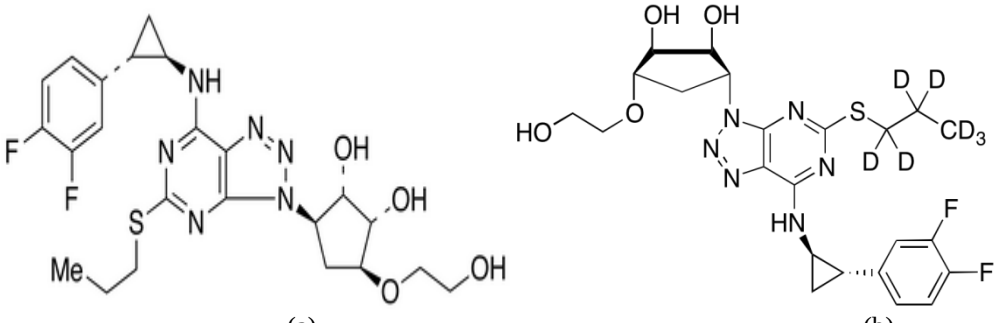
Analyte	Level	Concentration added (ng/mL)	Inter-day (n=6)			Intra-day (n=18)		
			Mean conc. (ng/mL)	Accuracy (%)	CV (%)	Mean conc. (ng/mL)	Accuracy (%)	CV (%)
Ticagrelor	LOQQC	2.536	2.581	101.8	9.8	2.589	102.1	10.6
	LQC	7.457	7.887	105.8	7.7	8.520	114.3	5.6
	MQC	502.2	540.7	107.7	6.8	560.3	111.6	5.5
	HQC	756.3	807.4	106.8	5.4	846.8	112.0	5.1
Deshydroxyethoxy Ticagrelor	LOQQC	1.064	1.042	98.0	10.4	1.146	107.7	9.2
	LQC	3.129	3.130	100.0	4.4	3.110	99.4	7.3
	MQC	150.0	159.7	106.5	2.9	159.8	106.6	2.8
	HQC	225.8	245.3	108.6	2.1	248.2	109.9	2.0

Table 3: Matrix effect data of Ticagrelor and Deshydroxyethoxy ticagrelor

	Matrix Factor	
Analyte	LQC	HQC
Ticagrelor		
Mean	0.984	0.981
SD	0.036	0.027
% CV	3.6	2.7
Deshydroxyethoxy Ticagrelor		
Mean	0.991	0.997
SD	0.032	0.015
% CV	3.2	1.5

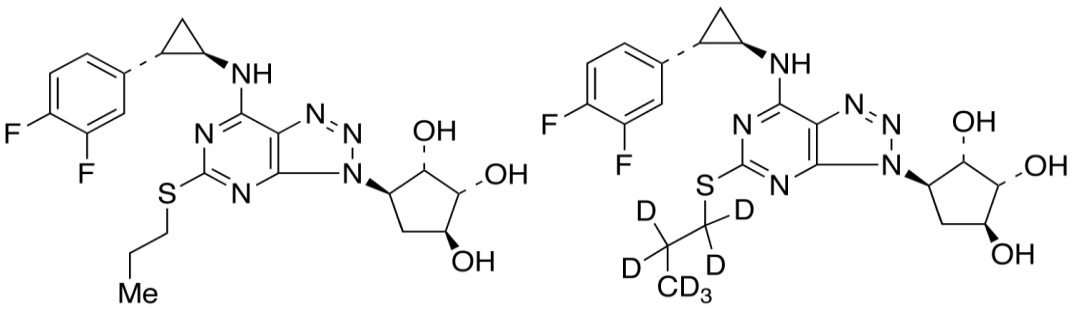
Table 4 Stability data of Ticagrelor and Deshydroxyethoxy ticagrelor of QC samples for different stability studies in different conditions (n=6).

Stability experiment	Analyte	QC (concentration spiked) (ng/mL)	Mean concentration found in fresh samples (ng/mL)	CV (%)	Mean concentration found in stability samples (ng/mL)	CV (%)	% Mean ratio
Bench top stability (9hrs)	Ticagrelor	7.457	7.189	3.2	7.161	4.2	99.6
		756.3	776.6	2.6	759.4	2.6	97.8
	Deshydroxyethoxy ticagrelor	3.129	3.268	5.3	3.312	1.2	101.3
		225.8	241.7	2.7	238.3	1.2	98.6
Auto sampler stability (114hrs)	Ticagrelor	7.457	7.744	2.0	7.836	4.4	101.2
		756.3	824.5	2.0	827.9	3.4	100.4
	Deshydroxyethoxy ticagrelor	3.129	3.200	1.5	3.268	4.3	102.2
		225.8	240.5	2.1	238.6	3.5	99.2
Dry extract stability (111hrs)	Ticagrelor	7.457	7.744	2.0	7.692	3.2	99.3
		756.3	824.5	2.2	824.6	2.9	100.0
	Deshydroxyethoxy ticagrelor	3.129	3.200	1.5	3.237	2.7	101.2
		225.8	240.5	2.1	242.9	0.9	101.0
freeze-thaw stability (4-cycles)	Ticagrelor	7.457	7.356	5.2	7.389	4.9	100.5
		756.3	807.8	1.9	809.0	3.2	100.2
	Deshydroxyethoxy ticagrelor	3.129	3.309	2.5	3.247	4.2	98.1
		225.8	242.1	1.3	246.1	1.4	101.6
			Mean Peak area of fresh samples		Mean Peak area of stability samples		%Mean ratio
long-term stock solution stability (24days)	Ticagrelor		506.7		530.8		104.8
			207773		209806		101.0
	Deshydroxyethoxy ticagrelor		330.2		337.8		103.1
			90300		91175		101.2
Short term stock solution stability (12hrs)	Ticagrelor		625.8		608.3		97.2
			230492		236208		102.5
	Deshydroxyethoxy ticagrelor		390.3		390		99.9
			107279		108483		101.1



(a) (b)

Fig. 1 Ticagrelor (a) and Ticagrelor d-7 (b)



(a) (b)

Fig. 2 Deshydroxyethoxy Ticagrelor (a) and Deshydroxyethoxy Ticagrelor d-7(b)

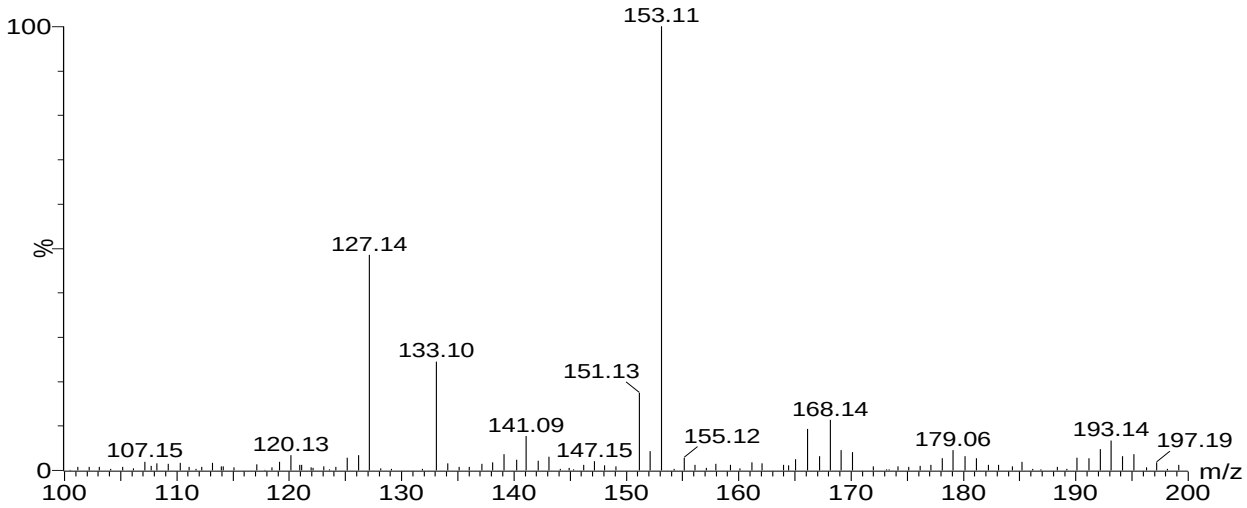


Fig. 3 Product ion spectrum of Ticagrelor

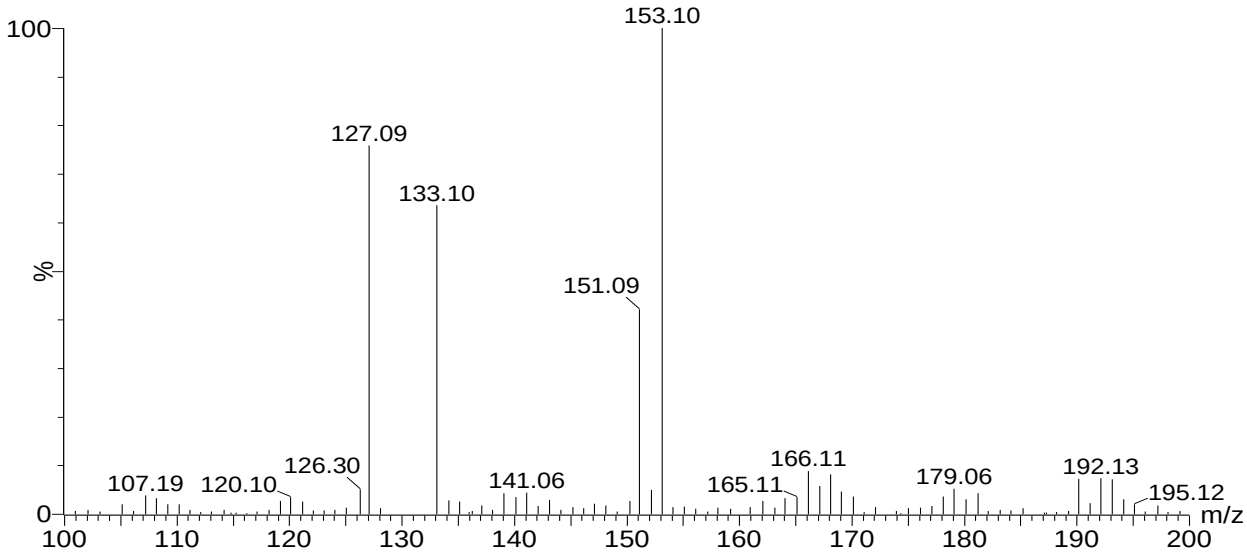


Fig. 4 Product ion spectrum of Deshydroxyethoxy ticagrelor

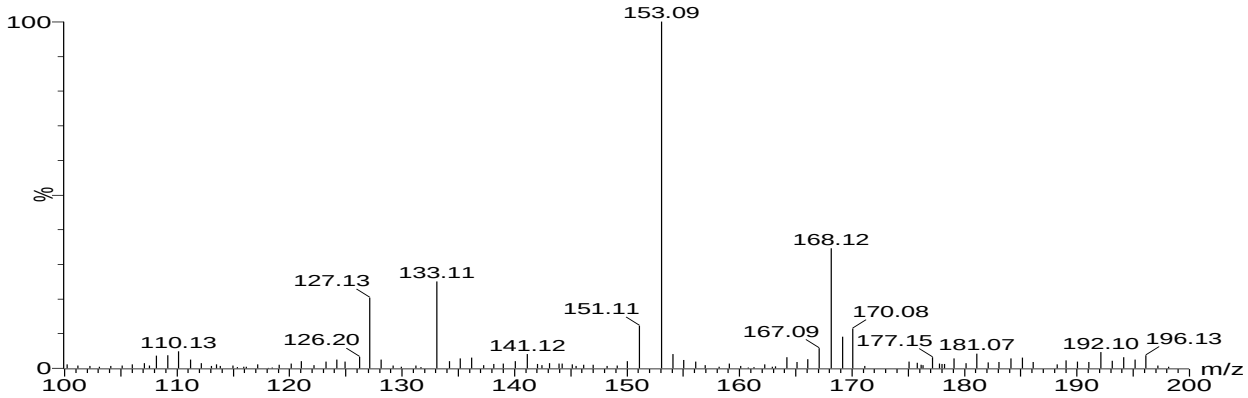


Fig. 5 Product ion spectrum of Ticagrelor-d7

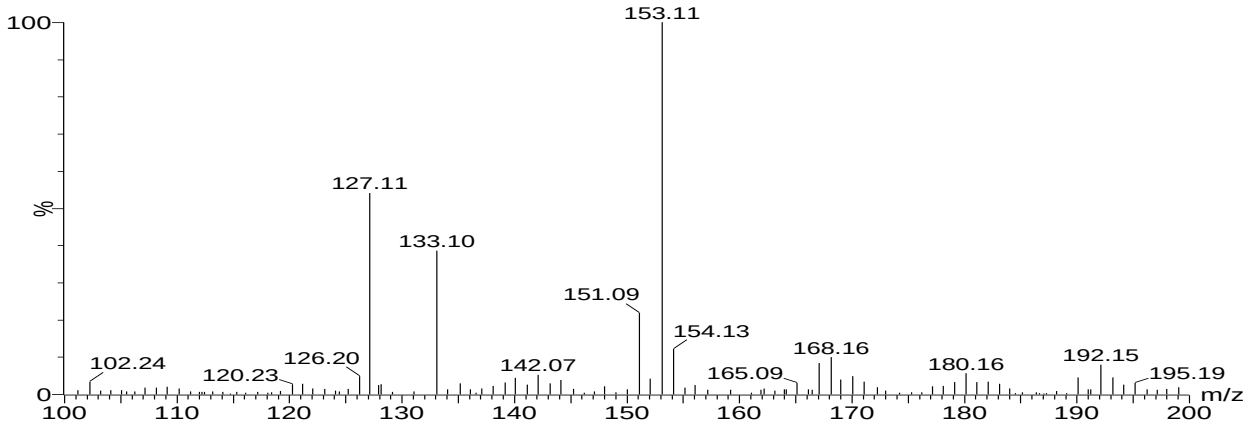


Fig. 6 Product ion spectrum of Deshydroxyethoxy ticagrelor-d7

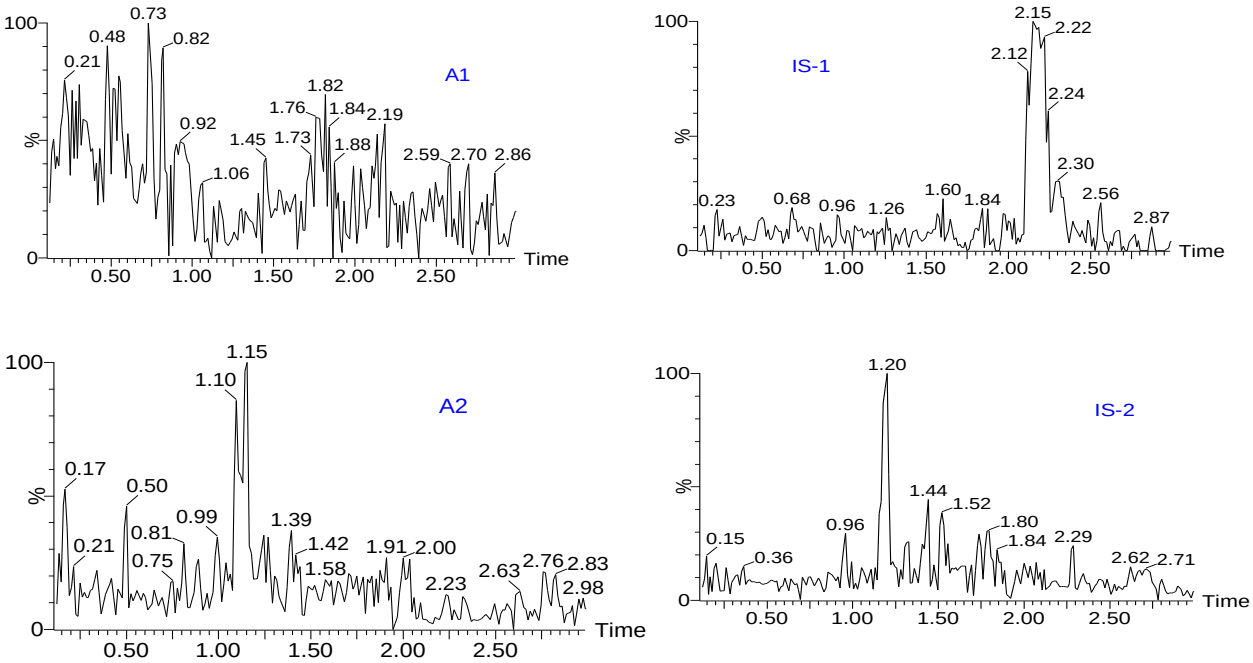


Fig. 7 Representative chromatograms of extracted blank plasma of Ticagrelor (A1), Ticagrelor –d7 (IS-1), Deshydroxyethoxy ticagrelor (A2) and Deshydroxyethoxy ticagrelor-d7 (IS-2).

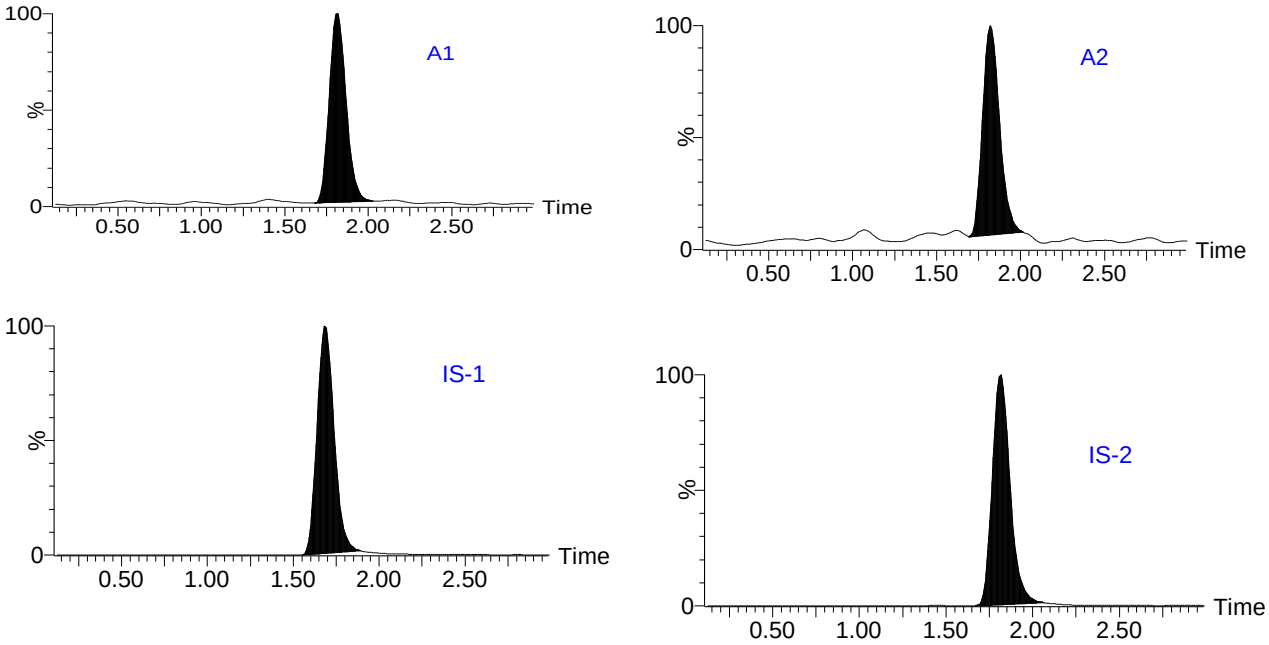


Fig. 8 Representative chromatograms of Ticagrelor (A1) and Deshydroxyethoxy ticagrelor (A2) at concentration of 2.5ng/mL and 1.0ng/mL with internal standard.

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