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HPLC method for simultaneous analysis of ticagrelor and its organic impurities and identification of two major photodegradation products

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ABSTRACT

A simple, fast and sensitive analytical method by high-performance liquid chromatography (HPLC) was developed and validated for the simultaneous determination of ticagrelor and two synthesis impurities. The HPLC method was established using an Agilent 1200 Series equipment coupled to photodiode array detector (PDA) at 270 nm with a Zorbax Plus C_8 column (150 × 4.6 mm, 5.0 μm), injection volume of 20 μL, and a constant temperature of 25 °C. The mobile phase consisted of acetonitrile: ammonium acetate 50 mM (57:43, v/v) and pH adjusted to 8.2 with ammonium hydroxide 6 M, at a flow rate of 0.7 mL/min. No interference peaks from excipients and diluent system indicated the specificity of the method. The calibration curves showed determination coefficients (r^2) > 0.99, calculated by linear regression. The limit of quantitation (LOQ) for impurities 1 and 2 were 2.0 and 0.2 μg/mL, respectively. Intra and interday relative standard deviations (RSDs) were <2% for ticagrelor and <6% for the impurities, proving the precision of the method. Besides, two mayor degradation products formed when sample solutions of ticagrelor were exposed to UVC radiation were elucidated and the mechanisms involved in the photolytic degradation of ticagrelor were proposed.

1. Introduction

Ticagrelor (Fig. 1a) is a new drug intended for the prevention of acute coronary syndromes (ACS) characterized by the formation of atherosclerotic plaques that rupture inside the arteries. It belongs to the cyclopentyltriazolopyrimidine class, and it is the first agent that reversibly binds to P2Y12 ADP-receptor (Schneider, 2011; Stone, 2010; Widimski et al., 2012; Wijeyeratne and Heptinstall, 2011). This receptor has a central role in platelet activation and drugs that selectively target it had been widely used as antiplatelet agents (Dorsam and Kunapuli, 2004; Husted and Van Giezen, 2009). AstraZeneca produces it as coated tablets containing 90 mg of ticagrelor (Brilinta®). European Commission approved the drug product in 2010 and FDA and Agência Nacional de Vigilância Sanitária (ANVISA/BRAZIL) in 2011. To assure the quality of drugs, it is important to monitor the impurities that must be present in the final product as contaminants, directly affecting their efficacy and safety. These substances are classified as organic or inorganic impurities or residual solvents and can arise during the manufacturing process and/or storage of the drug substance as starting material, by-products, intermediates, reagents or degradation products (ICH Q3A(R2) (2006)). Several methods are available for monitoring impurities in pharmaceuticals products. The primary criterion for choosing the best method is the ability to differentiate between all compounds of interest. Furthermore, the impurities analysis requires very sensitive analytical methods, which are able to detect small amounts of these substances in samples (Luo et al., 2015). The chromatographic techniques are the most used for determination of impurities at trace levels, and the high performance liquid chromatography (HPLC) is commonly the method of choice for separation and quantification of the related substances in drugs (Ahuja, 2007; Holm and Elder, 2016). A method that offers significant advantages in terms of convenience, accuracy, speed and capacity to perform complex separations, and it can be used with different types of detectors, such as fluorescence, diode array and mass spectrometry (Ahuja, 2007). Literature data describes few studies about analytical methods for ticagrelor impurities control. Sadou Yaye and collaborators (Sadou et al., 2015) developed a stability-indicating analytical method by HPLC for the quantification of ticagrelor and determination of its impurity profile with ultraviolet detection. In the study, the authors elucidated nine related substances, which were formed after exposure ticagrelor tablets to different stress conditions (Sadou et al., 2015). In the work conducted by Kumar and collaborators (Kumar et al., 2016), five ticagrelor related process impurities were detected by HPLC. One of these substances corresponds to a reported impurity, and four were

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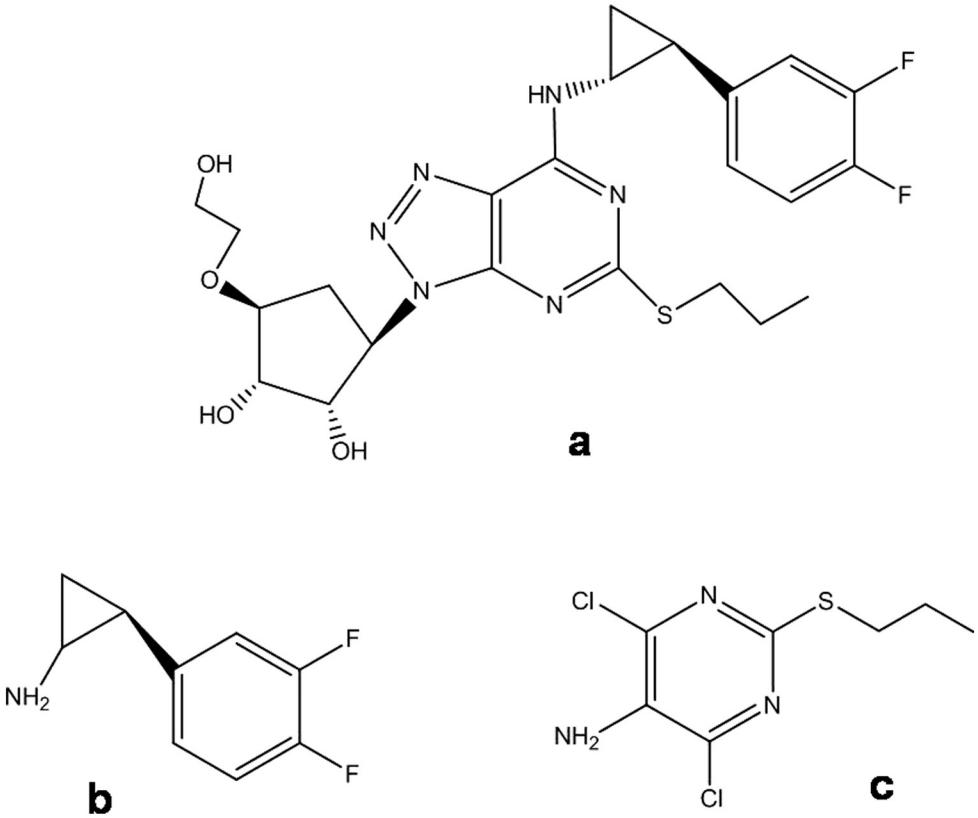

Fig. 1. Chemical structures of Ticagrelor (a), Impurity 1 (b) and Impurity 2 (c).

unknown (Kumar et al., 2016). The organic impurities are the most commonly found in pharmaceutical products, especially as intermediates that unreacted completely at synthesis steps or as degradation products formed during storage of the final product. Through a simplified retrosynthetic analysis of ticagrelor (Zhang et al., 2012), two synthesis intermediates were recognized as potential contaminants in the drug samples (Fig. 1b and c). Identify and monitor these related substances that could prejudice the quality of drugs became one of the keys to guarantee no negative interference in their clinical use. Thus, the aims of this work is to develop and validate a simple and efficient analytical method able to detect the presence of the synthesis impurities in ticagrelor, as well as elucidate the main degradation products which could be formed along the pharmaceuticals products shelf life, proposing a possible photolytic degradation route.

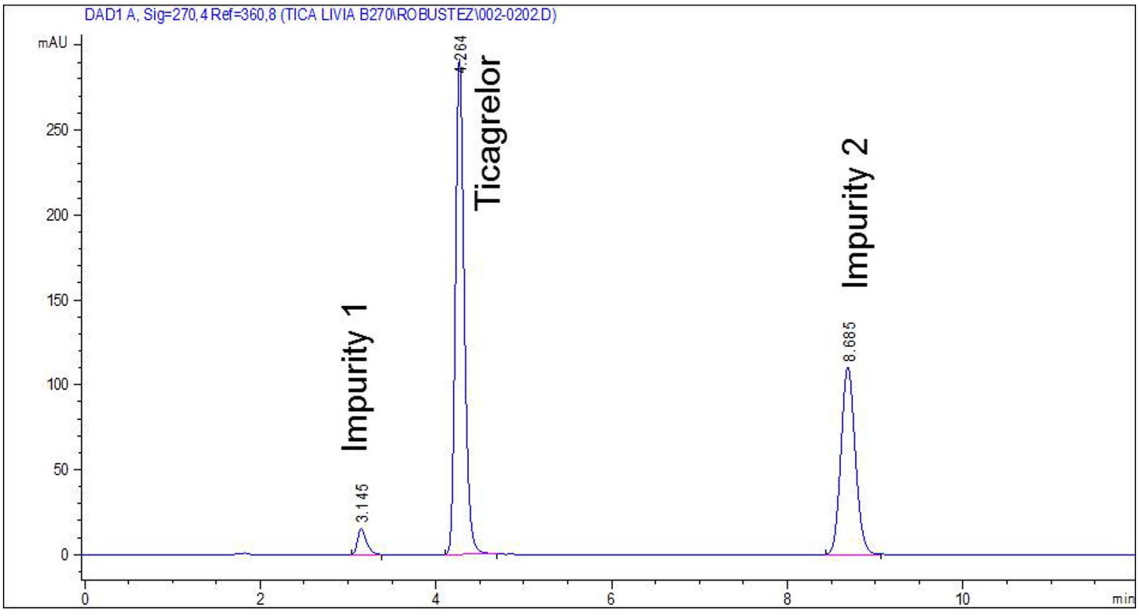

Fig. 2. Representative chromatogram of ticagrelor and their synthesis impurities. Concentrations: 20.0 μg·mL^{-1} of impurity 1, 45.0 μg·mL^{-1} for ticagrelor and 10.0 μg·mL^{-1} for impurity 2. Chromatographic conditions: C8 Zorbax Eclipse Plus column (150 × 4.6 mm, 5.0 μm) at 25 °C, with a mobile phase consisting of acetonitrile: ammonium acetate 50 mM and pH adjusted to 8.2 with ammonium acetate 6 M (57:43 v/v), at a flow rate of 0.7 mL·min^{-1} and injection volume of 20 μL. The detection was made at a wavelength of 270 nm by using PDA detector.

Table 1
Linear regression data in the analysis of ticagrelor and its synthetic impurities by HPLC.

	Impurity 1	Impurity 2	Ticagrelor
Concentration range (μg/mL)	2.0–60.0	0.2–60.0	15.0–75.0
Regression equation	y = 5.3853x − 0.7775	y = 139.28x − 5.6569	y = 45.31x + 1.8433
Determination coefficient (r^2)	1	0.9999	0.9998
F	138170.1232	122684.7202	14947.24021
Significance F	2.69313E-16	4.08255E-16	1.20649E-06
Lower 95%	−1.91154435	−32.81288305	−56.56328539
Upper 95%	0.298870613	21.47975187	60.76328539

2. Materials and methods

2.1. Chemicals

Ticagrelor reference substance (99.7%) and two synthesis impurities, (1R,2S)-2-(3,4-difluorophenyl)cyclopropanamine (99.3%), named Impurity 1, and 4,6-dichloro-2(propylthio)pyrimidin-5-amine (99.9%), named Impurity 2, were acquired from Sequoia Research Products (United Kingdom). Brilinta® tablets, 90 mg (AstraZeneca, United Kingdom) were purchased from local market and used for the photodegradation studies. Acetonitrile, methanol, purified water, ammonium acetate and ammonium hydroxide were used for samples preparation and mobile phase constitution. All chemicals used were of analytical grade and all solvents HPLC grade.

2.2. Chromatographic conditions for ticagrelor and impurities analysis

An Agilent 1200 series LC model, equipped with a quaternary pump, an auto sampler, compartment with thermostat and diode array detector was used for method development and validation. The system used for data acquisition and analysis of results was the ChemStation software (version B03.02). A C_8 reverse-phase column (Zorbax Plus, 150 × 4.6 mm, 5.0 μm) was used for the separation of ticagrelor and its synthesis impurities. An isocratic elution was achieved by using a mobile phase consisted of acetonitrile: ammonium acetate 50 mM (57:43, v/v) with pH adjusted to 8.2 with ammonium hydroxide 6 M. The flow rate was of 0.7 mL/min, injection volume of 20 μL, and the column temperature kept constant at 25 °C. The absorbance detection wavelength was 270 nm.

2.3. Chromatographic conditions for degradation products analysis

A Shimadzu liquid chromatography system, SCL-10 A model, equipped with manual injector and deuterium lamp detector was used for degradation products isolation. The chromatographic conditions established were: acetonitrile: water (57:43, v/v) as mobile phase, flow rate of 0.7 mL/min, UV detector set at 255 nm and temperature of 25 °C. The chromatographic column was a Phenomenex C_{18} Luna (250 × 4.6 mm, 5.0 μm) and the injection volume was 20 μL (Gobetti et al., 2014). The identification of the degradation products formed after the light exposure was performed by using a mass spectrometry AB Sciex Triple TOF 5600 model. The instrument was operated using positive electrospray ionization source, injection volume of 10 μL/min and temperature of 300 °C. The chemical structures were achieved with ACD/ChemSketch program.

2.4. Stock solutions preparation

2.4.1. Standard solution

Standard solutions of ticagrelor and its impurities were individually prepared by dissolving 5 mg of each substance in 50 mL of diluent (acetonitrile: water 57:43, v/v), obtaining stock solutions at 100 μg/mL, which were filtered in 0.45 μm membrane before injection.

2.4.2. Sample solution

Twenty Brilinta® tablets were accurately weighed and finely powdered. A quantity equivalent to 37.5 mg of ticagrelor was transferred to a volumetric flask containing methanol, which was kept in ultrasonic bath for 30 min. The volume was completed with the same solvent to reach 750.0 μg/mL and the solution was filtered using paper.

2.4.3. Placebo solution

All the excipients containing on the dosage form (mannitol, sodium amidoglicolate, calcium phosphate dibasic, hydroxypropyl cellulose, magnesium stearate, hypromellose, titanium dioxide, talc, polyethylene glycol, yellow iron oxide), except the active ingredient, were weighed in their usual concentration and prepared by the same way of sample solution.

Table 2
Results from repeatability and intermediate precision of ticagrelor and its synthetic impurities by HPLC.

	Day 1	Day 2	Day 3
Impurity 1			
Repeatability (μg/mL)	18.93	19.37	20.93
RSD %	4.90	0.71	2.83
Intermediate precision (μg/mL)	19.74		
RSD %	5.41		
Impurity 2			
Repeatability (μg/mL)	9.10	8.85	8.96
RSD %	1.09	0.25	0.62
Intermediate precision (μg/mL)	8.97		
RSD %	1.35		
Ticagrelor			
Repeatability (μg/mL)	45.79	45.69	45.36
RSD %	1.14	0.86	0.69
Intermediate precision (μg/mL)	45.61		
RSD %	0.95		

2.5. Method validation

The method validation was performed by evaluating the following analytical parameters: specificity, limit of detection and quantification, linearity, precision, accuracy and robustness, in accordance to current regulations (Brasil, 2003; ICH Q2B (R1), 2005).

2.5.1. Specificity

To evaluate the specificity of the method, a placebo solution was prepared at a theoretical concentration of 45 μg/mL. This solution was filtered on filter paper and then through a membrane of 0.45 μm pore size. The chromatogram obtained was compared to the standard solution.

2.5.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for impurities 1 and 2 were determined experimentally based on the signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentrations. Precision study was also carried out at LOQ level by injecting six individual preparations of each impurity and calculating the RSD of the area.

2.5.3. Linearity

The linearity of the method was verified by the construction of three calibrations curves of ticagrelor and each impurity, individually. The impurities calibration curves were prepared at nine concentration levels, from LOQ to 120% of the maximum limit specified (in the range of 2.0 to 60.0 μg/mL for impurity 1 and of 0.2 to 60.0 μg/mL for impurity 2). For ticagrelor, the calibration curve was constructed with five concentration levels, in the range of 15.0 to 75.0 μg/mL. Triplicate determinations at each concentration level were performed and after plotting concentration *versus* area values, linear equation and determination coefficient were obtained. Results were statistically evaluated by linear regression.

2.5.4. Precision

Precision was determined by repeatability and intermediate precision. Six individual solutions containing ticagrelor standard at 100% of the test concentration (45.0 μg/mL) spiked with the standard impurities 1 and 2 at the concentrations of 20.0 and 10.0 μg/mL, respectively, were analyzed in the same day and in three different days, always in triplicate. RSD values of areas were evaluated.

2.5.5. Accuracy

Accuracy was evaluated by adding known amounts of the impurities standards in the ticagrelor standard solution. Drug concentration was kept in 45.0 μg/mL and the impurities concentrations were modified at three levels, corresponding to 2.0, 20.0 and 60.0 μg/mL for impurity 1 and 0.2, 10.0 and 30.0 μg/mL from impurity 2. Each solution was prepared in triplicate and the values are expressed as percentage of the mean experimental concentration by the theoretical concentration.

2.5.6. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered, such as: flow rate, proportion of organic solvent in mobile phase and pH of mobile phase, in order to verify if the main chromatographic parameters would keep suitable for the impurities analysis. Results obtained for retention time, resolution between ticagrelor and its impurities peaks, theoretical plates and asymmetry were used to evaluate the influence of each factor in the method. The percentual variations between each condition modification and the normal established one were verified.

2.6. Photodegradation studies

An aliquot of 2 mL from sample stock solution (2.3.2) was transferred to covered disposable plastic cells, which were exposed to mirror chamber (100 × 18 × 17 cm) equipped with UVC (254 nm) lamp. After 2 h, an aliquot of 1.0 mL from the solution was diluted in a 5 mL volumetric flask using a mixture composed by acetonitrile: water (57:43, v/v). The degraded sample was filtered through a 0.45 μm membrane and then analyzed by HPLC.

3. Results and discussion

3.1. Development of reverse-phase HPLC method

The main objective of this work was to develop a single, simple, fast and suitable method for separation of two synthesis impurities, named Impurity 1 and Impurity 2, from ticagrelor. The development of analytical methods for impurities analysis in drugs products is a challenge for the quality control area, once these separations are usually hard to achieve with adequate detection at trace levels and resolution between the different analytes. Several tests and modifications in the main chromatography conditions were conducted in order to optimize the detection and separation of the samples. The work previously published for ticagrelor determination in coated tablets by Gobetti and collaborators was used as the basis for initiating the development of this method (Gobetti et al., 2014). From the chromatographic conditions established by the authors, different columns, mobile phase compositions, pH and flow rates were tested to achieve the best results. The initial tests were conducted using acetonitrile: water pH 7.0 (57:43, v/v) as mobile phase. Using a C_{18} column (250 × 4.6 mm, 5.0 μm) and wavelength of 255 nm it was observed low detection for impurity 1 and high retention time for impurity 2, leading to a very long analysis time. At this point, a change in the wavelength to 270 nm showed an increased in the impurity 1 absorption without compromising impurity 2 and ticagrelor detections. Besides, tests with a C_8 column (150 × 4.6 mm, 5.0 μm) provided faster analytical run, but was not enough to achieve a separation with adequate resolution between all the substances. Therefore, it was necessary proceed to modifications in the mobile phase composition in order to optimize it. The use of organic modifiers or different pH values in mobile phase can substantially affect the separation and retention time of the molecules under the conditions of reversed-phase chromatography (Collins et al., 2006). Testing the mobile phase in acid pH (3.66 adjusted with phosphoric acid 10%) was not able to achieve an optimal separation, probably due the poor ionization of the compounds. On the other hand, changing the pH to an alkaline range (8.2 adjusted with ammonium hydroxide 6 M) and adding 50 mM ammonium acetate (Sadou et al., 2015) to the aqueous portion of the mobile phase showed an improved in the resolution of peaks, being suitable for ticagrelor and impurities separation. Thus, acetonitrile: ammonium acetate 50 mM as mobile phase at pH 8.2 was established for the analytical method validation. Fig. 2 represents the chromatogram obtained with the optimized conditions of the method.

3.2. Method validation

3.2.1. Specificity

The specificity of the current method was evaluated by injection of a placebo sample prepared at a usual concentration and verifying a possible interference of the excipients in the peaks of interest. In addition, the diluent system used to prepare the samples, composed by acetonitrile: water (57:43, v/v), was investigated by injection. Any apparent signal was observed during the analytical runs, especially at retention times near to ticagrelor or its impurities, which could prejudice the analysis.

3.2.2. Limit of detection (LOD) and limit of quantitation (LOQ)

One of the goals in the impurity analytical methods development is to achieve the higher sensitivity as possible, guaranteeing the detection and quantitation of trace levels of this contaminants in drug

Table 3
Accuracy of the method by HPLC for impurity 1 and impurity 2 obtained by the recovery test.

	Theoretical concentration (μg/mL)	Experimental concentration (μg/mL)	% Recovery
Impurity 1 (2.0 μg/mL)	2.08	2.09	100.38
Impurity 1 (20.0 μg/mL)	20.08	20.09	96.56
Impurity 1 (60.0 μg/mL)	61.2	62.56	102.21
Impurity 2 (0.2 μg/mL)	0.20	0.21	105.53
Impurity 2 (10.0 μg/mL)	10.16	8.94	88.0
Impurity 2 (30.0 μg/mL)	30.48	31.09	101.99

samples. It is important to attempt that, in this methods, the LOQ of the impurities must be lower than the specified notification limits (Cass and Degani, 2001). At this work, the limits of detection and quantitation were determined experimentally by injecting series of impurities solutions individually until stablish the signal to noise ratio of 3:1 and 10:1, respectively. The results founded for impurity 1 were 0.5 μg/mL (LOD) and 2.0 μg/mL (LOQ) and 0.07 μg/mL (LOD) and 0.2 μg/mL (LOQ) for impurity 2. The LOQ precision of each impurity was carried out by injection of six samples in triplicate and the % RSD of the areas calculated. The values obtained for both impurities were below 2%.

3.2.3. Linearity

The calibration curves analysis of ticagrelor and impurities 1 and 2 were statistically satisfactory, showing determination coefficients (r^2) higher than 0.99. The linear regression was applied to confirm the method linearity and, by residual analysis, it was verified the absence of atypical samples. The statistical parameters are presented in Table 1.

3.2.4. Precision

The experimental data obtained from the repeatability and intermediate precision are shown in Table 2. Values of intra and interday RSD were below 2% for ticagrelor and below 6% for the impurities, which are considered acceptable with respect to this kind of determination (Ribani et al., 2004).

3.2.5. Accuracy

The accuracy of the method was evaluated at three concentration levels, which were made in triplicate. The results obtained in the accuracy assay, reached by the recovery test principle, are presented in Table 3. All results must be considered acceptable, indicating the accuracy of the chromatographic method (Ribani et al., 2004).

3.2.6. Robustness

The aim of this test is to demonstrate the method is not negatively affected by small deliberate modifications in their initial condition. In all varied chromatographic conditions, all analyte peaks were adequately resolved and elution orders remained unchanged. The results observed demonstrate that main chromatography parameters were altered by modifications in the method conditions, but not enough to compromise the analysis, keeping the analytical run suitable to drug and synthesis impurities separation.

4. Photodegradation products elucidation and proposed degradation pathway of ticagrelor

Previous studies of ticagrelor stability showed that drug product was unstable under different stress conditions (Gobetti et al., 2014; Sadou et al., 2015). In the work conducted by Gobetti and collaborators (Gobetti et al., 2014), the authors exposed ticagrelor tablets to acid and alkaline hydrolysis, UVA and UVC radiation, oxidation and heat, in order to verify the behavior of the drug under these stress conditions. These results showed substantial drug degradation by UVC radiation, with three degradation products formation. It is known that photolysis is a factor that plays an important role on drugs stability, especially during the pharmaceutical storage. For this reason, it becomes relevant to elucidate the main photodegradation products formed in order to know the impurity profile of the drug when expose to radiation. With this purpose, new

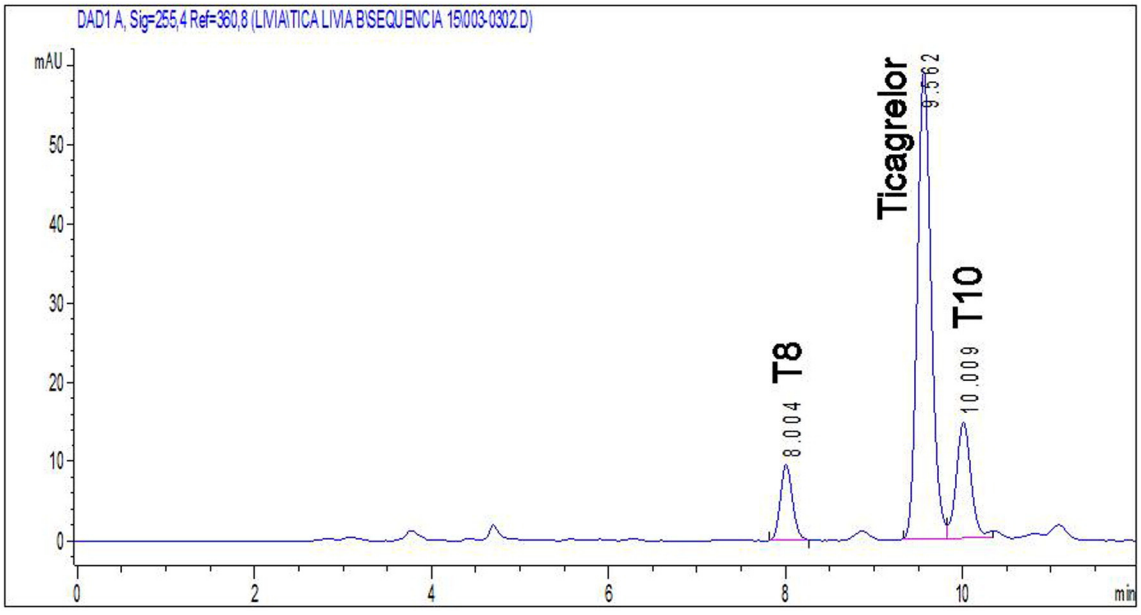

Fig. 3. Representative chromatogram of ticagrelor and two major degradation products after UVC light exposure for 2 h. Chromatographic conditions: C18 Phenomenex Luna column (250 × 4.6 mm, 5.0 μm) at 25 °C, with a mobile phase consisting of acetonitrile: water (57:43 v/v), at a flow rate of 0.7 mL·min^{-1} and injection volume of 20 μL. The detection was made at a wavelength of 255 nm by using PDA detector.

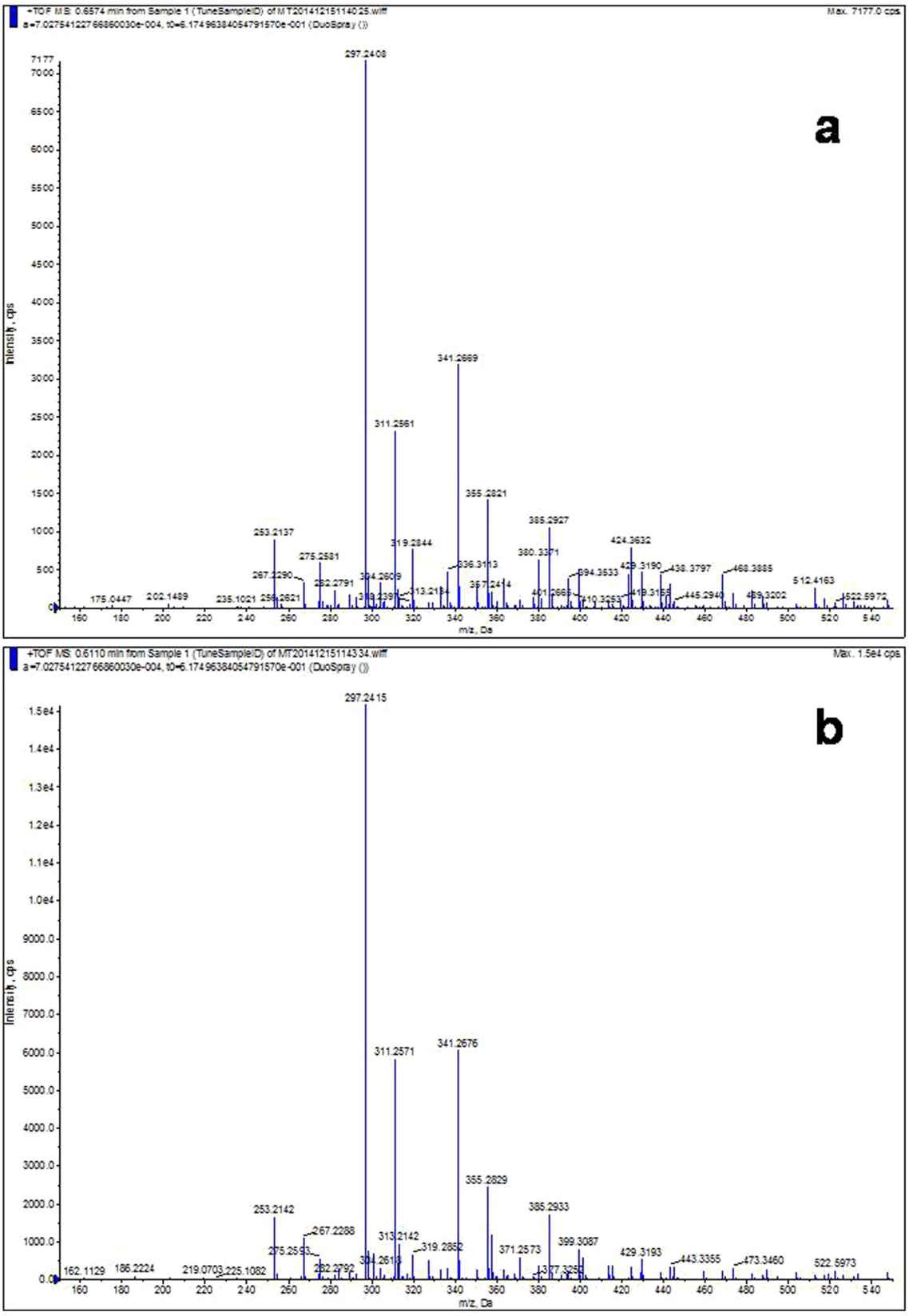

Fig. 4. Representative mass spectra of degradation product T8 (a) and T10 (b) after individual injection in mass spectrometer operating by positive electrospray ionization. Injection volume of 10 μL and temperature of 300 °C.

samples were submitted to degradation by UVC radiation (Fig. 3). The chromatogram indicates two major peaks formed from ticagrelor, which were named according to their retention time as T8 and T10. These products were individually collected from the chromatographic system and then injected into the mass spectrometry. Fig. 4 represents the spectra of T8 (Fig. 4a) and T10 (Fig. 4b), respectively, with their

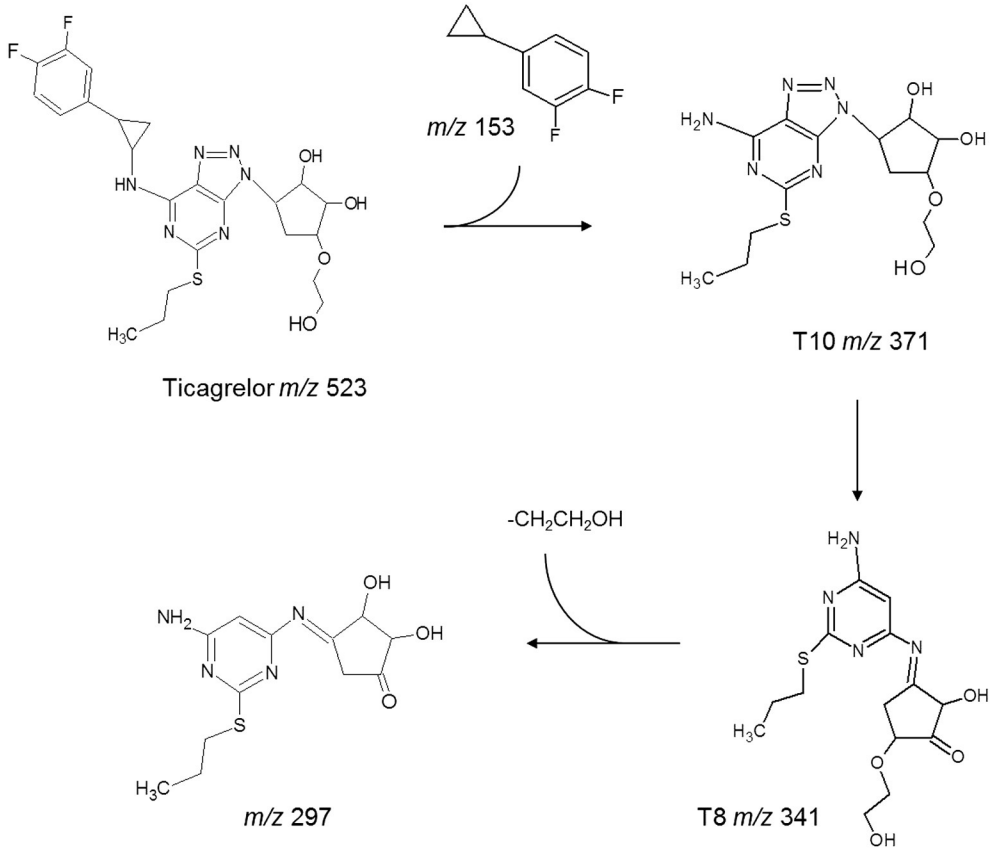

Fig. 5. Proposed photolytic degradation routes with the chemical structures of the T10 and T8 degradation products.

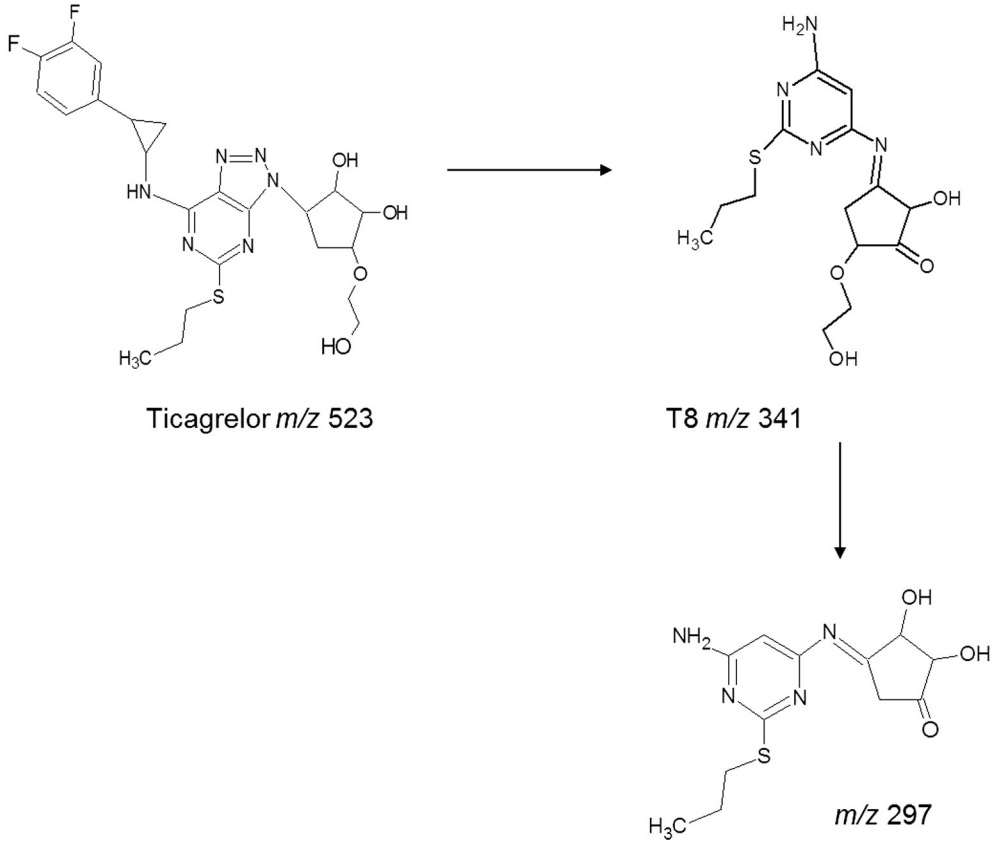

Fig. 6. Alternative degradation pathway suggested to T8 degradation product directly from ticagrelor molecule.

positive ion fragmentation. Starting from the molecule of ticagrelor (*m/z* 523.1946), it is suggested a N-dealkylation with direct N—C bond cleavage at the 3,4-difluorophenylcyclopropyl group ($C_9H_7F_2^+$; *m/z* 153.0509) leading to a structure with *m/z* 371.2573 (Fig. 5). This fragmentation is present in the T10 mass spectrum (Fig. 4b) and corresponds to the major ticagrelor metabolite, AR-C133913XX, which was already described in literature as (1S,2S,3R,5S)-3-[7-Amino-5-(propylthio)-3H-1,2,3-triazolo[4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxy)-1,2-cyclopentanediol. (Sadou et al., 2015; Kumar et al., 2016; Teng et al., 2010). After analyzing T8 mass spectrum, it was possible to propose a chemical structure, following two degradation routes. The first one was through the cleavage of the T10 molecule in its triazolopyrimidine ring with loss of N_2 and autoxidation (Sadou et al., 2015; Tonessen, 1996), resulting in a structure with *m/z* 341.2674 ($C_{14}H_{20}N_4O_4S+$) (Fig. 5), chemically 3-{[6-amino-2-(propylsulfanyl)pyrimidin-4-yl]imino}-5-hydroxy-2-(2-hydroxyethoxy)cyclopentan-1-one. In the second suggested route, T8 product could be formed directly from ticagrelor molecule by the same mechanisms already described, followed by the loss of its ethyl alcohol chain (CH_2CH_2OH) (Kumar et al., 2016) resulting in the *m/z* 297.2408 fragment, which presents significant intensity in both mass spectra analyzed (Fig. 4). Fig. 6 represents this degradation route. The structure proposed for T8 degradation product is not cited in literature yet, representing a contribution for impurities profile study of this drug.

5. Conclusions

The HPLC method developed for the simultaneous analysis of ticagrelor and its synthesis impurities demonstrated to be suitable for the purpose. This simple, fast, robust and sensitive technique can be used with reliability to monitor the presence of these contaminants in drug samples during the quality control routine. Besides, two photodegradation products were chemically identified with degradation pathways suggested, contributing to the knowledge about the organic impurity profile of ticagrelor.

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