

Spectrofluorimetric analysis of ticagrelor in pharmaceutical formulations and spiked human plasma using 1-dimethylaminonaphthalene-5-sulphonyl chloride reagent

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ABSTRACT: A sensitive method is presented for the determination of the ticagrelor (TCG) in human plasma and pharmaceutical preparations using 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride). Ticagrelor contains a secondary amino group which reacts with dansyl chloride in the presence of 0.5 M sodium bicarbonate (pH 10) to yield a highly fluorescent derivative that is measured at 445 nm after excitation at 340 nm. Different experimental parameters affecting the fluorescence intensities were carefully studied and optimized. The proposed method was also validated according to ICH guidelines. The fluorescence concentration plot was rectilinear over the range of 20.0–200.0 ng mL⁻¹, with a coefficient of determination of 0.9999 with a limit of detection (LOD) of 0.14 ng mL⁻¹ and the limit of quantitation (LOQ) of 0.46 ng mL⁻¹. The proposed method was applied successfully to the analysis of TCG in spiked human plasma and pharmaceutical dosage forms with good accuracy and precision.

KEYWORDS: Ticagrelor; antiplatelet; spectrofluorimetry; dansyl chloride; derivatization; validation; pharmaceutical preparations; plasma samples.

1. INTRODUCTION

Antiplatelet therapy, with P2Y₁₂ receptor antagonists, is the key of pharmacological treatment of acute coronary syndrome. Ticagrelor (1S,2S,3R,5S)-3-[7-[(1R,2S)-2-(3,4-difluorophenyl)cyclopropyl]amino]-5-(propylthio)-3H-[1,2,3]-triazolo[4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethyl)oxycyclopentane-1,2-diol (Figure 1) is a reversible P2Y₁₂ receptor antagonist intended for oral route. Ticagrelor (TCG) exerts its pharmacological activity through reversible inhibition of platelet aggregation resulting from ADP-induction. [1–3]. After oral intake, TCG is readily absorbed and displays an expected pharmacokinetic profile [4]. It is biotransformed by CYP enzymes to AR-C124910XX which is a pharmacologically active metabolite with a similar antiplatelet activity to the parent compound. [5, 6]. A steady state pharmacokinetic study showed that peak plasma concentrations of total TCG observed 1.5–3.0 h following administration, and the half-life was in the range of 6.2 and 13.1 h [6].

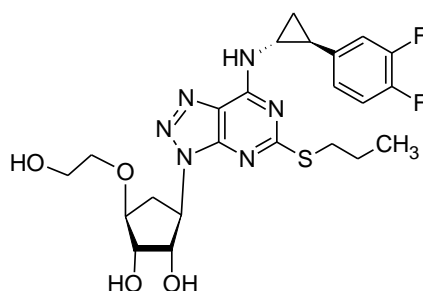


Figure 1. Chemical structure of ticagrelor.

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According to literature, UV-spectrophotometry [7], high performance liquid chromatography (HPLC) [8, 9, 10], HPLC-mass spectrometry (MS) [11], HPLC-MS/MS [12-17] analytical methods are available for quantification of TCG in biological samples and pharmaceutical dosage forms. Kelemen et al. reviewed analysis methods of TCG [18]. Sensitivity level of the above mentioned methods are not satisfactory or tiresome and necessitates advanced & dedicated equipment. Spectrofluorimetry is a preferred technique due to cost efficiency, wide availability and simplicity [19, 20]. 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl chloride) is a widely used agent for fluorescent derivatization which is firstly used by Weberl for analysis of albumin through its fluorescent derivatives. [21]. Pharmaceutical molecules containing nitrogen based functional groups like primary and secondary amines, imidazoles and also other groups like phenols were derivatized effectively with dansyl chloride and quantified accurately in biological and pharmaceutical preparations. [22-28]. The purpose of the current study is to demonstrate the efficiency of DNS-Cl as a suitable fluorescent labelling reagent for the new, selective and sensitive determination of TCG in pharmaceutical preparation and spiked human plasma. Prominent advantages of the proposed method is simplicity, rapid sampling procedure, retrieving fluorescence spectrogram with high level of sensitivity and applicability to widely used, easily accessible instruments.

2.RESULTS AND DISCUSSION

2.1. Spectral characteristics

Derivatization with dansyl chloride and measurement of formed derivative fluorescence is preferred due to high level of sensitivity of this method. Therefore it has been chosen for analysis of TCG in pharmaceuticals and spiked plasma samples. Derivatization of TCG by dansyl chloride has been performed through its secondary amine functional group. The reaction for the derivatization between TCG and dansyl chloride is shown in Figure 2.

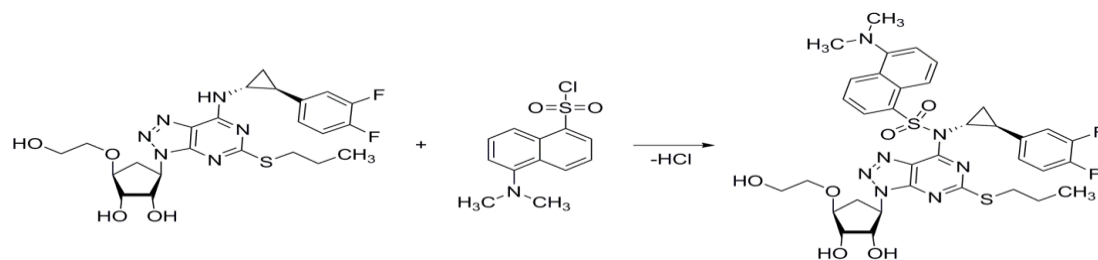


Figure 2. Proposed reaction pathway between ticagrelor and dansyl chloride reagent.

In the present study, highly fluorescent derivative of TCG was obtained through derivatization of TCG with dansyl chloride at pH 10 in bicarbonate solution. The resulting derivative exhibited an excitation maximum at 340 nm and emission maximum at 445 nm in dichloromethane (Figure 3).

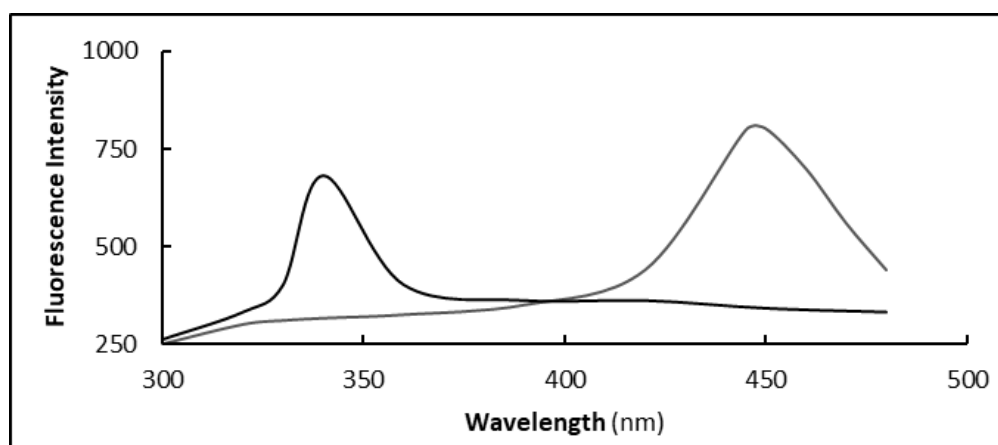


Figure 3. Excitation and emission spectra of the reaction product of TCG with dansyl chloride.

2.2. Optimization of experimental parameters

The different experimental parameters between TCG and dansyl chloride were studied and optimized. The following parameters have been individually investigated changing only one parameter at a time and keeping the other remaining parameters unchanged; pH of the medium, various concentrations of the reagent dansyl chloride, the duration of the reaction and the solvent used for dilution.

2.2.1. Effect of concentration of dansyl chloride

The impact of change in the concentration of derivatization agent dansyl chloride was tested changing volumes of stock solution of the reagent at 0.02% concentration, between 50 μ L and 300 μ L. It was found out that 200 μ L of dansyl chloride (0.02% solution) was sufficient for derivatization reaction as shown in Figure 4.

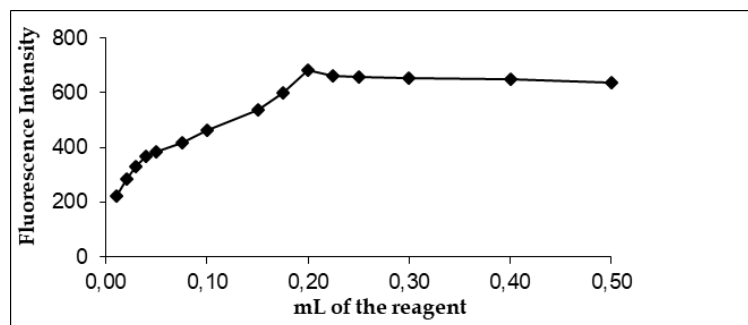


Figure 4. Effect of reagent volume on the development of the reaction product of TCG with dansyl chloride.

Job's method of continuous variation was used in order to determine the molar ratio of dansyl chloride reagent to TCG in the proposed chemical reaction [29]. Use of equivalent moles of TCG and dansyl chloride derivatization reagent in solution, the stoichiometry of the proposed reaction was determined as 1:1 ratio (drug molecule/derivatization reagent), confirming that the proposed chemical reaction involves one molecule of TCG and one molecule of derivatization reagent dansyl chloride.

2.2.2. Effect of pH

The impact of the change of pH between 9 and 11 of the reaction media on the intensity of fluorescence of the derivative compound was studied. Bicarbonate solution and borate buffer were preferred to achieve alkaline pH levels to optimize the reaction between TCG and dansyl chloride due to high yield levels of product with dansyl chloride under alkaline conditions is well described in literature. 200 μ L of bicarbonate solution at pH 10 was determined as the optimum condition to achieve peak level of fluorescence (Figure 5).

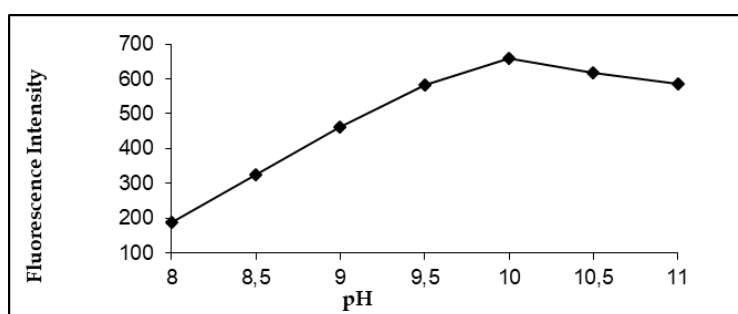


Figure 5. Effect of pH on the development of the reaction product of TCG with dansyl chloride.

2.2.3. Effect of temperature and time

The impact of temperature and different time intervals on intensity of fluorescence were investigated in the 40, 50 and 60°C. The highest fluorescence levels were achieved after 15 min using a 40°C water bath and remained stable (Figure 6).

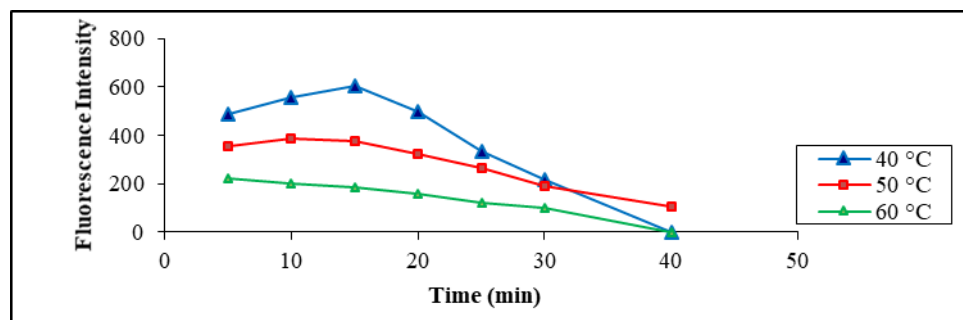


Figure 6. The effect of temperature and time of heating on the reaction between TCG with dansyl chloride (at 40 °C, 50 °C and 60 °C) at different time interval.

2.2.4. Effect of diluting solvent

Different solvent such as toluene, chloroform, dichloromethane, diethyl ether, benzen were investigated to yield the highest fluorescence intensity. The highest fluorescence intensity was achieved with dichloromethane. The samples prepared under these described conditions, remained stable for at least 2 h.

2.3. Validation of the method

The validation of the proposed method has been performed in line with the ICH guidelines [30]. The validation parameters covered linearity, specificity, repeatability, accuracy, and precision.

2.3.1. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Relative fluorescence intensity was directly proportional to the concentration over the range 20-200 ng mL⁻¹, under the optimized reaction conditions. Linear regression analysis of the results provided the following equation:

$$I_f = 4.2548C + 20.988 \quad (r^2 = 0.9999) \quad [\text{Eq. 1}]$$

Where I_f is the fluorescence intensity, C is the concentration of the drug in ng mL⁻¹ and r^2 is the coefficient of determination ($n=5$).

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOQ determines the minimum level of the analyte which can be reliably detected by analyzing the sample at a known concentration. The LOD and LOQ were determined with the following equation [26].

$$\text{LOD or LOQ} = \kappa \text{SDa} / b \quad [\text{Eq. 2}]$$

Where $\kappa = 3.3$ for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The analytical performance parameters for the proposed method is presented in the following Table 1.

2.3.2. Accuracy, specificity and precision

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The standard addition technique was implemented to check accuracy of the proposed method. A different volume of pure sample solution was added to three different concentrations of the standard drug solution and analyzed. The percent recovery of the added standard to the assay samples was calculated from the following equation:

$$\text{Recovery \%} = [(C_t - C_u) / C_a] \times 100 \quad [\text{Eq. 3}]$$

Where C_t is the total concentration of the analyte quantified; C_u is the concentration of the analyte existing in the formulation; and C_a represents the concentration of the pure analyte added to the formulation. The findings of the analysis of the commercially available dosage forms and the recovery study are presented in Table 2. Calculated average percent recoveries were quantitatively found as 100.44 % showing high level of accuracy of for the method.

Table 1. Results of analytical parameters of the proposed method.

Parameters	Found Values
Wavelength (nm)	λ_{ex} : 340, λ_{em} :445
Concentration range ^a (ng mL ⁻¹)	20-200
Regression equation ^b	$I_f=4.2548C+20.988$
Intercept \pm SD	20.988 \pm 0.685
Slope \pm SD	4.2548 \pm 0.054
Coefficient of determination (r^2)	0.9999
<i>Precision</i>	
Intra-day ^c , RSD %	0.53
Inter-day ^d , RSD %	1.02
LOD (ng mL ⁻¹)	0.14
LOQ (ng mL ⁻¹)	0.46

^a Average of five determinatons

^b $I_f=m C +b$ where C is the concentration in ng mL⁻¹ and I_f is the fluorescence intensities

^c n=5 correspond to replicate analysis for each level

^d Results of seven different days (n=3)

Table 2. Results of recovery studies by standard addition method.

Proposed Method	Amount taken (ng mL ⁻¹) ^a	Amount added (ng mL ⁻¹)	Total amount found ^b (ng mL ⁻¹) (Mean \pm S.D. ^c)	Recovery (%)	RSD (%)
		10	20.11 \pm 0.14	101.10	0.69
	10	50	60.08 \pm 0.37	100.16	0.62
		100	110.05 \pm 0.614	100.05	0.56

^a BRILINTA Film Tablet ® (90 mg)

^b Five independent analyses.

^c Standard error.

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the method was tested by taking into account the possibility of interference coming from the widely used excipients in tablet formulation, such as talc, lactose, starch, mannitol and magnesium stearate. According to the results, there is no interference detected from these mentioned excipients.

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The inter- and intra-day precision level was assessed through analysis of TCG at the three different drug concentrations (n=5) for seven consecutive days. The RSD values calculated for intra-day precisions and inter-day precisions were 0.53%, and 1.02%, respectively and were showed an acceptable. The results of the tests are presented in Table 1.

2.3.3. Robustness of the method

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was evaluated for 100 ng mL⁻¹ concentration (n=3) by testing the susceptibility of measurements to planned variation of the analytical conditions for dansy chloride concentration and reaction temperature and time.

Variation in the dansyl chloride concentrations (% w/v ± 0.5), temperature (optimum ± 2 °C), and time (optimum ± 1.0 min) did not have any significant impact on the procedures; recovery and the RSD values were within 2%. As the pH had a great effect on the fluorescence of the product pH is kept within the range of 10.0 \pm 0.2.

2.4. Applications of the method

2.4.1. Determination of TCG in tablets

The proposed method was successfully implemented to the analysis of commercially available drug product (BRILINTA Film Tablet®). The results are summarized in Table 3. According to the test results there is no interference coming from the excipients, e.g. lactose, glucose, fructose, magnesium stearate and starch. The achieved results for accuracy and precision are satisfactory as proven by the high level of percentile recovery and RSD less than 2%.

Table 3. Determination of TCG in tablets by the proposed methods (n=5).

Proposed method	Label claim ^a (mg/per tablet)	Mean ^b \pm S.D	Recovery (%)	RSD (%)
	90	90.24 \pm 0.12	100.27	0.13

^a BRILINTA Film Tablet® (90 mg)

^b Five independent analyses.

2.4.2. Determination of TCG in plasma samples

After oral intake of 120 mg ticagrelor as single oral dose (at 12 subjects), a mean C_{max} of 1013.1 (\pm 277.2) ng mL⁻¹ was reached in 2.0 (1.0–4.0) hours [31]. With reference to study, the proposed methods could be successfully applied for the quantification of TCG in spiked plasma.

The extraction procedures and implementation of the proposed methods to plasma samples were described in detail at Assay Procedures for Spiked Plasma Samples. The achieved results presented in Table 4 are satisfactorily accurate and precise.

Table 4. The recoveries of TCG from plasma (n=5).

Proposed method	Added (ng mL ⁻¹)	Determined \pm S.D (ng mL ⁻¹)	Recovery (%)	RSD (%) ^a
	20.0	14.01 \pm 0.12	70.05	0.86
	100.0	69.06 \pm 0.36	69.06	0.52
	200.0	144.28 \pm 0.41	72.14	0.28

^a Five independent analyses.

3. CONCLUSION

The current study proposes a new highly specific new spectrofluometric method for the determination of TCG in film tablets and spiked human plasma. There is no need to complicated handling like as chromatographic techniques. Furthermore, the developed method offers better sensitivity and less experimental steps than the previously reported HPLC methods [8-13]. When the developed method compared with UV spectroscopic method [7], it has more advantages of increased sensitivity. The developed method is suitable for routine analysis of ticagrelor in biological fluids and can be used for drug monitoring purposes and for regular analytical applications of TCG in pharmaceutical industrial procedures, and research laboratories

4. MATERIAL AND METHODS

4.1. Materials

4.1.1. Reagents and solutions

Ticagrelor was kindly supplied by Zhejiang Ausun Pharmaceutical Co., Ltd and its commercially available pharmaceutical product (BRILINTA Film Tablet®) containing 90 mg of ticagrelor per film tablet was procured from local pharmacy. 5-dimethylaminonaphthalene-1-sulphonyl chloride (DNS-Cl) was procured from Sigma-Aldrich (Germany). All chemicals, reagents, solvents used were of analytical-reagent grade.

A stock solution of TCG base at a concentration of 1 mg/mL was prepared in methanol and working solutions were prepared through dilution of the stock solution.

DNS-Cl solution was newly prepared at a concentration of 2.0 mg/mL (0.02%) in acetone. The solution of sodium bicarbonate at 0.5 M concentration was prepared in water and its pH was adjusted to 10 with 0.5 M sodium hydroxide, using a pH meter. Prepared solution was stored in refrigerator and used approximately for 1 week.

4.1.2. Apparatus

Fluorescence measurements were performed by using the Hitachi spectrofluorometer (Model U-2900). The system used Xenon lamps and 1 cm light path cells. The excitation and emission wavelengths were identified as 340 and 445 nm, respectively. The pH was measured by WTW pH 526 digital pH meter (Mettler Toledo, Germany).

4.2. Method

4.2.1. General procedures

Aliquots of 0.1-1.0 mL TCG standard solution ($1 \mu\text{g mL}^{-1}$) were transferred into a series of tubes and then the volume was completed to 0.2 mL with methanol. A 200 μL volume of bicarbonate solution at pH 10 and 200 μL of DNS-Cl solution were transferred to each one of the tubes. The reaction mixture was left at 40°C for 15 min. It was left to cool down at ambient room temperature in air. Then, the derivative was extracted with 5 mL of dichloromethane for 1 min. Blank experiment was carried out simultaneously. Fluorescence intensity of the reaction product was evaluated at 445 nm following excitation performed at 340 nm against a blank assay which was measured separately. The corrected fluorescence intensity was plotted versus final drug concentrations to get the calibration curve. The calibration curve was plotted with corrected fluorescence intensity against the final TCG concentrations

4.2.2. Assay procedure for tablets

Tablets were powdered and powder weight having theoretically 10 mg of TCG was accurately weighed and put into a 50 mL calibrated flask. About 15 mL of methanol solution was transferred and the extraction procedure was implemented mechanically for 20 minutes and sonicated for another 20 more minutes. The total volume was completed to 50 mL with addition of methanol and final solution was filtered using quantitative filter paper. This filtrate was further diluted using methanol to get working solutions then processed as detailed under the preparation of calibration curve. The nominal content of each tablet was calculated through the calibration graph or the corresponding regression equation.

4.2.3. Assay procedures for spiked plasma samples

5.0 mL of medication free human blood sample was drawn from healthy volunteers into a heparinized tube prevent coagulation and centrifuged at 3000 rpm for 30 min to separate aliquot. (Ethics committee approval was provided for this study by BVU Ethics Clinical Research Committee, number: 71306642-050.01.04).

An aliquot of plasma (100 μL) was transferred in a centrifuge tube and was spiked with various concentrations of TCG and the resulting sample was vortexed for 10 s. 1.3 mL ethyl acetate was transferred to the sample and the extraction mixture was vortexed for 5 min and centrifuged at 4000 rpm for 10 min [31]. An aliquot (1.0 mL) of the supernatant from the sample, was evaporated through evaporated to dryness under nitrogen at ambient room temperature. The organic phases were removed through evaporation to dryness using a water bath at 40°C. The resulting residual mass was dissolved with 0.2 mL methanol. The analyses were executed as the General Assay Procedure. All the procedures for blank assay were carried out in the

same way. The percentage recoveries were estimated by using the corresponding calibration graphs for plasma.

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Conflict of interest statement: The authors declared no conflict of interest in the manuscript.

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