FORMULATION AND EVALUATION OF TICAGRELOR LIQUID CRYSTAL DRUG DELIVERY SYSTEMS TO ENHANCE THE SOLUBILITY

Shailaja Pashikanti[1], Aswini Lavanya Pudi[2*], Anjali Nidadavolu[3]

Abstract:
Because of the poor solubility and permeability, ticagrelor (TGL), a P2Y12 receptor antagonist, has a low oral bioavailability and is therefore classed as a Biopharmaceutics classification system (BCS) class IV drug. The bioavailability of BCS class IV drugs can be increased with the help of liquid crystals, which act as an effective delivery mechanism. Hence, ticagrelor liquid crystals are prepared to enhance the oral bioavailability and antiplatelet activity of Ticagrelor. Central composite design is followed and the optimized formulation showed a particle size of 316.5nm and encapsulation efficiency of 87.98% and zeta potential of -23.1mV. Powder X-ray diffraction (PXRD) and Differential scanning calorimetry (DSC) were performed to detect the characteristics of TGL-LC. The *invitro* dissolution and *exvivo* permeation studies are performed between pure drug and ticagrelor liquid crystal formulation in which ticagrelor showed significantly best results compared to pure drug.

Key Words: liquid crystalline drug delivery, Ticagrelor, controlled release delivery, PXRD.

[1,2*,3]Department of Pharmaceutical Technology, A.U College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India, Pin code 530003

***Corresponding Author:** Aswini Lavanya Pudi

*****Aswini Lavanya Pudi, Department of Pharmaceutical Technology, A.U College of Pharmaceutical Technology, Andhra University, Visakhapatnam, Andhra Pradesh, India – 530003
E-mail: aswini.pudi0039@gmail.com

DOI: - 10.48047/ecb/2022.12.10.564

1. INTRODUCTION

The drug Ticagrelor is used in the treatment of heart related diseases like heart attack, stroke and acute coronary syndrome, i.e., problem related to blood supply in coronary arteries. It can be used as preventive treatment in patients who are at the risk of Thromboembolism and myocardial infarction. It is a acyclopentyl-triazolo-pyrimidine, belongs to the class of non-competitive antagonist. It shows its action by inhibiting the P2Y1, P2Y12 receptors there by inhibiting the platelet aggregation [1]. Unlike the thienopyridines such as clopidogrel and prasugrel; ticagrelor is not a pro drug, not effected by CYP 450 genes and has lesser inter individual variability. The PLATO (Platelet Inhibition and patient outcomes) studies proved a significant reduction in mortality rate in case of Ticagrelor when compared to Clopidogrel [2]. The drug, Ticagrelor belongs to BCS (Biopharmaceutics classification system) class IV which implies low solubility and poor permeability. So, this trial is to improve its oral bioavailability [3].

In the recent years, the nanotechnology is growing in the fast pace as it has the advantages of avoiding the immune responses, minimization of irritant reactions and improved penetration into multiple barriers owing to their small size. Liquid crystals also comes under nanotechnology, is a fast emerging class of drug delivery systems. When they come in contact with water they reorganize into three dimensional structures called liquid crystals. Liquid crystal technology has certain advantages like ease of manufacturing, no use of organic solvents and ease to scale-up. Liquid crystal formulations contains lipids which increases the drug loading. Till date, self-micro emulsifying drug delivery systems (SEDDS), solid dispersions, nanostructured lipid carriers (NLCs) have been researched and developed to increase the bioavailability of Ticagrelor but Ticagrelor liquid crystals have not been developed yet. So, to increase the bioavailability of Ticagrelor, the development of liquid crystal drug delivery is required.

2. MATERIALS AND METHODS:

Ticagrelor was obtained as a gift sample from Lee pharma Pvt. limited. Glyceryl monostearate, polysorbate 80(Tween 80), Polaxomer 407 were obtained from Lee Pharma Pvt. Limited. Polysorbate 20, Stearic acid, palmitic acid were purchased from Loba Chemie Pvt. Ltd. Sodium Hydroxide, Hydrochloric acid, methanol, ethanol, acetone were purchased from authorized vendor.

The powder purity and authenticity were determined through pre-formulation studies that included the evaluation of a number of physiochemical characteristics including organoleptic, melting point, partition coefficient, etc. Organoleptic properties of the drug were evaluated by analyzing its colour, smell and visual appeal [4].

2.1. Determination of Melting point:

Typically, the drug is identified by its melting point. It can indicate if the drug is pure or counter felt. If the melting point of the sample drug is same as that of the standard drug, then the sample drug is an authentic and pure form of the drug. Melting-point equipment is used to ascertain the melting point. The pure drug is withdrawn into a capillary tube with a wall thickness of just 1 mm and a length of 10-15 mm. The opposite end of the tube is then sealed to prevent the drug from leaking. When measuring the melting point, the capillary tube containing the drug was heated until it melted in the melting point apparatus. That specific temperature at which the sample is melted was noted as the melting point of the drug. This temperature is compared with the temperature of the standard drug melting point and analyzed [5].

2.2. Screening of solid lipids

The solid lipids Palmitic acid, Stearic acid, and GMS were used for the study. In a test tube containing 10 mg of drug, 0.2 gm of solid lipid was introduced gradually while being stirred. Water was heated to 80 degrees Celsius, or 5 degrees Celsius above the melting point of the lipid being tested. Solid lipid was added in small increments until a transparent solution was obtained. Small amounts of solid lipid was added gradually till a clear solution was obtained. The quantity of lipid required to form clear solution was assessed [6].

2.3. Screening of surfactant:

The surfactants used in the experiment were Tween 20, Tween 80, and Poloxamer407. A saturated solution was achieved by adding an excess amount of the drug to a known volume of surfactant (2 ml) and then mixing the two together. The drug was dissolved using a mechanical shaker for 24 hours. After that, the mixture was centrifuged at 10,000 rpm for 15-20 minutes to remove any remaining particles. Using a UV spectrophotometer, the supernatant of

saturated surfactant systems were diluted with methanol for analysis [7].

3. Preparation of Ticagrelor liquid crystal formulations

Ticagrelor LC formulations were prepared by dissolving Ticagrelor in GMS (as the lipid phase), then adding an aqueous solution of stabilizer (tween 80) to the lipid phase. The final mixture was heated to 50°C using a bath sonicator and then sonicated. The concentration of the lipid (1, 1.5, 2 w/w %) and the concentration of the stabilizer (0.5, 5, and 10 w/w %) were the independent variables in this investigation. The drug concentration in all formulations was kept constant at 1 w/w%. Table 1 depicts the composition of different formulations [8].

3.1. Drug Identification UV/Visible Spectrophotometer ʎ max of drug: -

A UV/Visible spectrophotometer is used to find out a drug's ʎ max. An 8 g/ml solution of Ticagrelor was placed in the UV/Visible region of the spectrophotometer. It absorbs the particular wavelength of light that was passing through it and the sample was scanned between 200 and 400 nm range. Between absorbance and wavelength, an automatic curve is made that indicates the UV spectrum. The ʎ max of Ticagrelor was found to be 298 nm [9].

3.2. FTIR of pure drug and drug- excipients compatibility

The Fourier Transform Infrared spectrum (FTIR) provides information on the basic moiety present in that chemical. FTIR spectroscopy was used to study the structure of the drug and excipients in 1:1 ratio, FTIR was employed to test drug-excipient compatibility. The drug and other excipients were well mixed. FTIR was used to scan samples in the 4000-400 cm^{-1} range to determine the spectra of pure drugs and drug excipients. The spectra of a pure drug and an excipient-containing drug were compared [10].

3.3. Central composite factorial design

Optimization is a product development strategy that sets the goal for prior knowledge of trials from the design level through product stability and risk assessment while performing the experiment. The ultimate goal is to create liquid crystals with smaller particle sizes that optimize Entrapment Efficiency (EE) and drug loading (DL). Particle size reduction improves permeability and dissolution. Maximum EE and DL improves therapeutic efficiency by increasing drug bioavailability. These characteristics influence product quality as well as drug delivery effectiveness by increasing therapeutic efficiency. The liquid crystal optimization was carried out using Design Expert Software (Version 12, State ease. Inc, Minneapolis, USA). The Central Composite design was employed for liquid crystal optimization, and the polynomial equation was derived. On the basis of statistical data, the best-fitting model is choosen [11].

4. Characterization of liquid crystals

4.1.1. Particle size, polydispersity index (PDI) and zeta potential

Using a Horiba Particle Size Analyzer and Zeta Sizer, the prepared formulations' particle size, PDI, and zeta potential were assessed. The liquid crystal formulation was appropriately diluted (i.e., 10 times) with double-distilled water prior to formulation analysis. At a temperature of 25 °C and a fixed scattering angle of 173 °C, the diluted sample was assessed. The experiments were all carried out in triplicate [12].

4.1.2. Entrapment efficiency (EE):

Centrifugation was used to calculate the percentage of EE in the SLN formulation. In an eppendrof tube, 2 ml of the liquid crystal formulations were taken, and the sample was centrifuged at 10,000 RPM for 45 min in a refrigerated centrifuge. The supernatant layer was collected and methanol was used to dilute it appropriately. Utilizing a UV Vis Spectrophotometer at 298 nm, the drug content that was not encapsulated was examined. The experiments were all carried out in triplicate. The following equations were used to analyze EE.

$$\%Entrapment\ efficiency = \frac{\text{Amount of drug added in formulation}}{Amount\ of\ unencapsulated\ drug}$$

4.1.3. DSC analysis

Differential scanning calorimetric analysis (DSC) was used to characterize the thermal behavior of the drug samples. DSC study was carried out over a temperature range of 30-350°C with a heating rate of 10°C per minute in an inert environment of nitrogen gas at the rate of 20 ml per minute. Samples were sealed in aluminium pans (BO14-

3020 25 µl, 0.15 mm pans). The peak transition onset temperature of drug sample was recorded [13].

4.1.4. XRD

An analytical method known as X-ray powder diffraction (XRD) is quick and is generally used to determine the phase of crystal structures. It can also reveal information on unit cell dimensions. The material under analysis is finely powdered, homogenized and the bulk composition is determined on average. In order to perform this test procedure, an x-ray beam is pointed at the sample, and the scattered intensity is then measured as a function of the outgoing direction. After the beam has been separated, the scatter also known as a diffraction pattern shows the crystalline structure of the material.

4.1.5. *In vitro* drug release:

Ticagrelor liquid crystal formulation equivalent to 10 mg of drug is used. Dissolution studies are performed in a dissolution apparatus using modified dialysis method. Liquid crystals equivalent to 10mg of drug which is suspended in 6.8 pH buffer and is filled in a dialysis bag, which is then sealed at both the ends. The dialysis bag is kept into the receptor compartment that has the dissolution medium, 250 ml of 0.2% w/v polysorbate 80 phosphate buffer of pH 1.2, and stirred continuously at 200 rpm at 37°C for initial 2h and then 1.2pH buffer is replaced with 6.8pH buffer up to 48h. At predetermined time intervals, 3ml samples are withdrawn from the dissolution media using syringes and replaced with the same amount of buffer. The above mentioned was followed for both the pure drug and Ticagrelor liquid crystal suspension and the results are compared [14].

4.1.6. *Ex vivo* permeation studies:

Ex vivo permeation of Ticagrelor from its suspension and the optimized liquid crystal formulation containing the same amount of drug (10mg) was tested using a non-everted gut sac method. The small intestine of a male Wistar rat was taken out of the animal's body after it was killed. Seven centimetres of the jejunum was cut off and washed with Krebs-Ringer phosphate buffer. A 2 ml syringe was used to fill the sac with the appropriate amount of Ticagrelor suspension or the selected Ticagrelor liquid crystal suspension, which was equal to 5 mg of Ticagrelor. Both ends were tied with cotton thread, then put in 250 ml of 0.2% polysorbate 80 phosphate buffer with a pH of 6.8 and stirred at 200rpm and 37^0C. At certain time intervals the samples are withdrawn from the release medium and replaced with the same amount of buffer. To the withdrawn sample, a few ml of methanol was added and the amount of drug was determined by using UV spectroscopy method at 298nm. The above mentioned was followed for both the pure drug and Ticagrelor liquid crystal suspension and the results are compared. The permeation flux and apparent permeability and percent of drug permeated into the receptor compartment was calculated [15].

$$\text{Papp value} = \frac{dQ}{dt} \times \frac{1}{AC0}$$

Where;

dQ/dt indicates the drug permeation rate,$\frac{dt}{AC0}$

C0 is the initial drug concentration in the mucosal compartment at $t = 0$

A is the total cross-sectional area of tissue.

5. RESULTS:

Ticagrelor in its purest form has a melting point of about 140.2 degrees Celsius. In this case, the results confirm that the drug contains pure Ticagrelor with no added impurities. To determine which materials would be used in formulation development, the drug's solubility in different solvents was examined. An ultraviolet (UV) spectrophotometer was used to examine the drug at a wavelength of 298 nm. Ticagrelor was shown to be highly soluble in both ethanol and methanol, in accordance with the results. In an investigation, the solubility of ticagrelor in water was determined to be 10ug/ml. It was found that the ticagrelor n-octanol: water partition coefficient was 1.9801± 0.001. This indicates slightly greater lipophilic nature of the medication. Ultraviolet (UV) spectra of ticagrelor were scanned at 298 nm and the results are depicted in Figures 1 and 2. The test result proves that the drug is authentic. Good linearity is shown by the ticagrelor calibration curve, with a regression equation of Y= 0.1368x + 0.093 and an R^2 value of 0.9909 in a 6.8ph buffer, and Y=0.041 x +0.0256 and an R^2 value of 0.9985 in a 1.2pH buffer. The FTIR spectra of Ticagrelor is shown in the Fig. 3 and 4

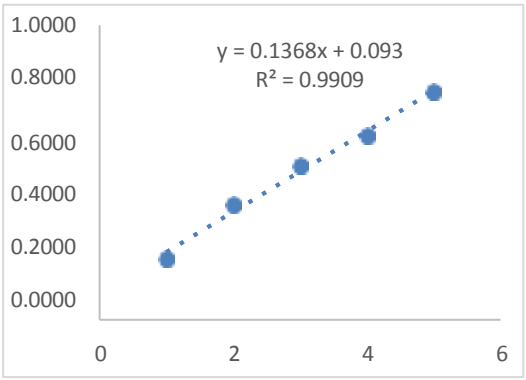


Figure 1: calibration curve of 1.2pH

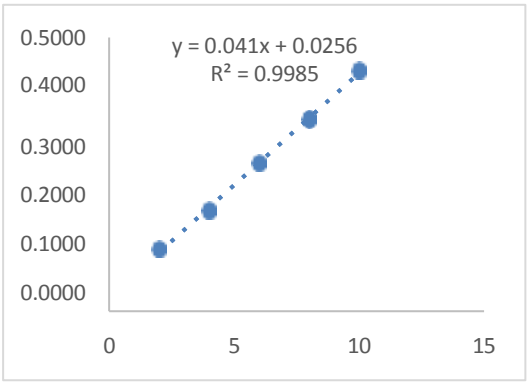
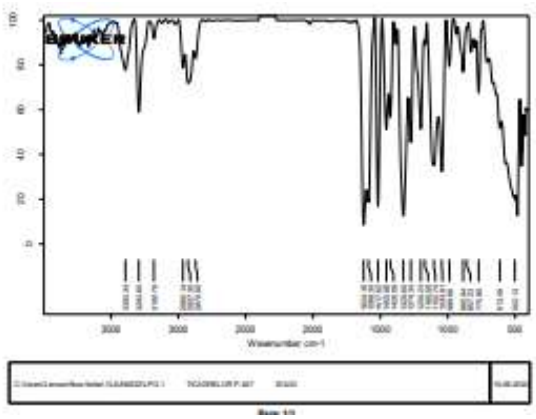
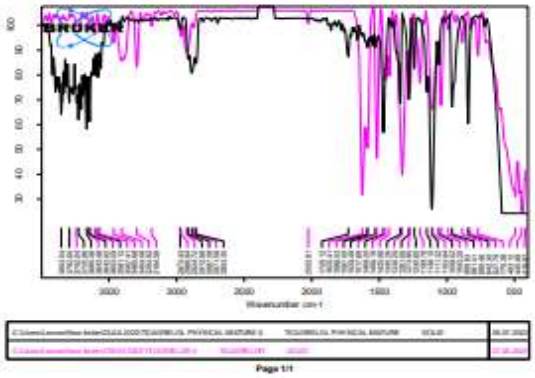


Figure 2: calibration curve of 6.8pH





The principle absorption peaks of ticagrelor of all stretches are observed in the FTIR spectra of the pure drug which indicates that the drug used is pure and authentic. The drug compatibility with excipients is also proven by the essence that the major peaks don't change. This means that the drug is perfectly compatible with excipients as shown in Fig 3

Optimization was done by central composite design by using Design Expert Software. The optimized formulations are shown in table 1

Table 1:

Run	Surfactant%	Lipid %	Particle size(nm)	Zeta potential (mV)	PDI	Entrapment efficiency %
1	8.41	1.25	448.2	-32.7	0.398	88.46
2	6	0.5	213.1	-27.8	0.25	85.36
3	7	1.25	490.2	-28.5	0.385	89.45
4	7	1.25	413.8	-29.1	0.37	88.92
5	8	0.5	205.4	-26.8	0.381	84.89
6	6	2	502.3	-27.2	0.404	94.52
7	7	0.18	195.6	-28.1	0.231	80.98
8	7	1.25	395.6	-27.9	0.454	90.23
9	5.59	1.25	380.7	-25.3	0.461	89.45
10	7	2.31	550.4	-28.5	0.413	95.61
11	8	2	510.2	-29.4	0.517	93.45

Using the data obtained by the results of particle size, zeta potential, PDI (poly dispersity index), Entrapment efficiency of runs given by central composite design, the final optimized formula is obtained using central composite design in design expert software. The 3D Surface plots of particle size, entrapment efficiency, PDI and zeta potential of the runs given by central composite design are shown in figure

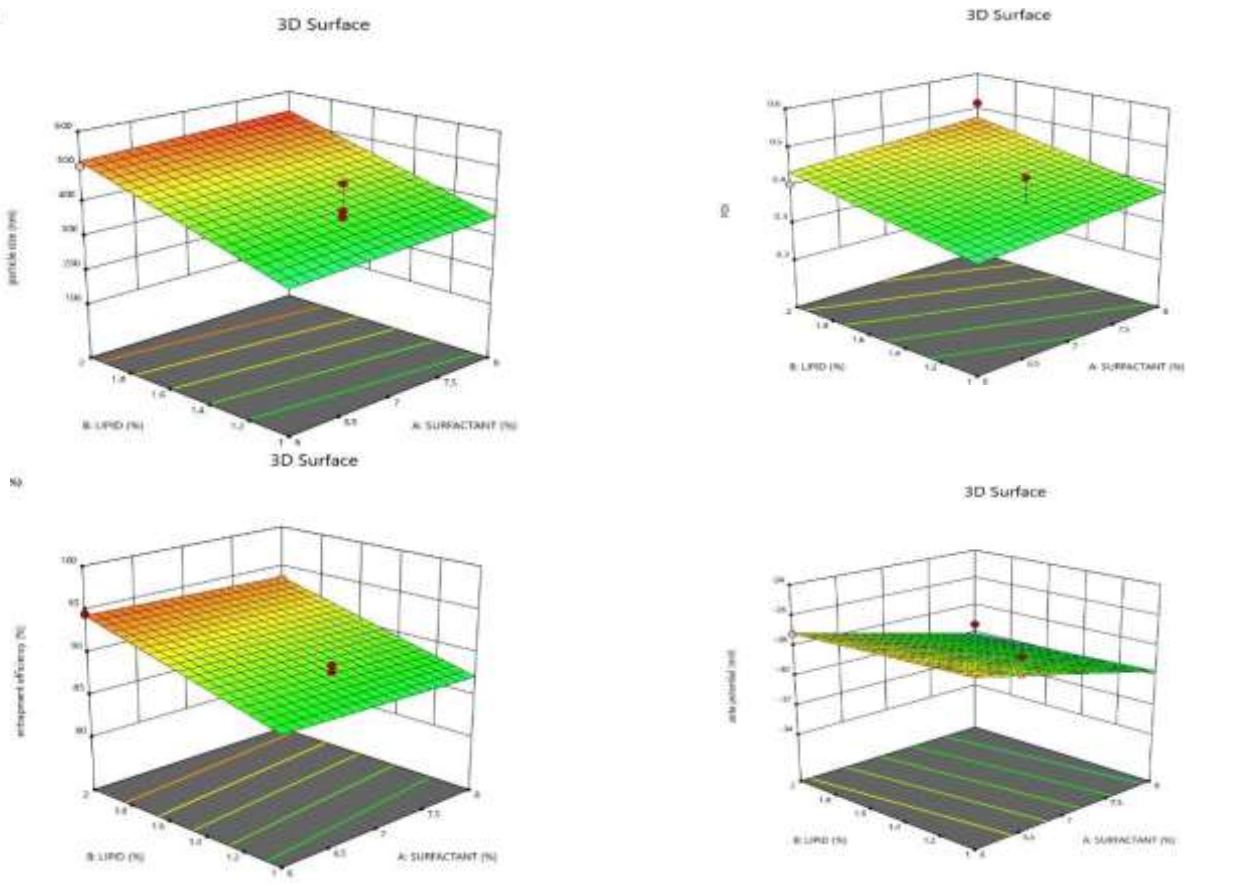


Figure 5: 3D counter plots

5.1. Characterization of Ticagrelor Liquid crystals:

Ticagrelor liquid crystals optimized formulation showed a particle size of 316.5nm which is nearer when compared to the optimized, given in central composite design which is 334.058nm. zeta potential observed as -23.1mV when compared to optimized value -26.73. The polydispersity index was observed as 0.404 aganist the observed optimized value 0.345. The instrument used to detect the particle size, zeta potential and PDI is Horiba scientific SZ 100 for windows Z type ver. 2. 40.

To evaluate the crystallinity of TGL in Ticagrelor liquid crystals, a DSC was performed. The figure depicted the DSC thermograms of raw TGL, glycerol monostearate, and TGL liquid crystal. At 140 degrees Celsius, an endothermic peak was noticed, which corresponds to the melting temperature of TGL and represents its crystalline nature. Moreover, the thermal peak of glycerol mono stearate was shown at 61^{o}C , respectively. In the Ticagrelor liquid crystal formulation, an endothermic peak at 126.3 is observed which relates to the melting point of Ticagrelor indicating the crystallinity of the formulation. However, a slight depression in the melting point of Ticagrelor is observed in the formulation.

Figure 3 depicted the powder XRD spectra of Ticagrelor liquid crystal. A number of peaks indicating crystallinity were found in Ticagrelor liquid crystal formulation. In TGL liquid crystal, the peak intensity of glycerol monostearate and poloxamer 188 exhibiting crystallinity were found. This indicates that ticagrelor is incorporated into the lipid matrix in a crystallized state.

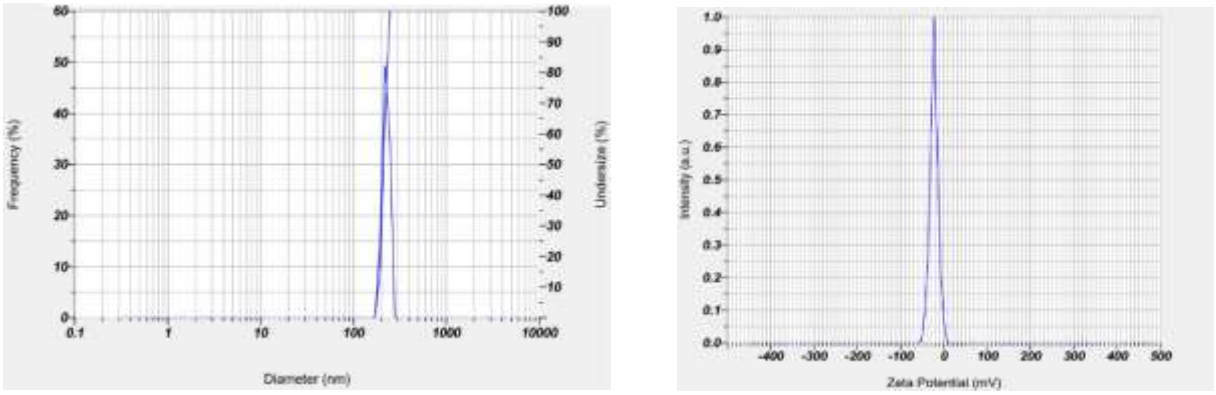


Figure 6: particle size and zeta potential of formulation

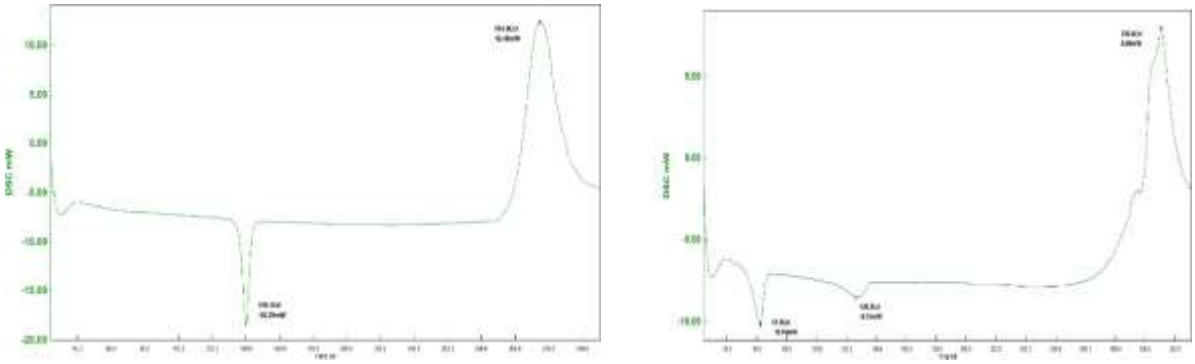


Figure 7: DSC graphs for pure drug and physical mixture

5.2. *In vitro* dissolution studies:

The table depicts the drug release pattern of the pure drug and the formulation where only 56.523 ± 0.321% of pure drug is released in 48h whereas 98.56 ± 0.521% of Ticagrelor liquid crystal was released in 48h. This indicates that Ticagrelor liquid crystal formulation shows high drug release which indicates increased solubility. It is also observed that drug release does not depend on the concentration of drug which indicates the drug release following zero order kinetics. Other kinetic studies, such as first order, Higuchi, and Korsmeyar-peppas, were also investigated in relation to the release pattern and R^2 value that they exhibited. The value of R^2 in the zero order model is 0.947, while the value of R^2 in the Higuchi model is 0.9809. This

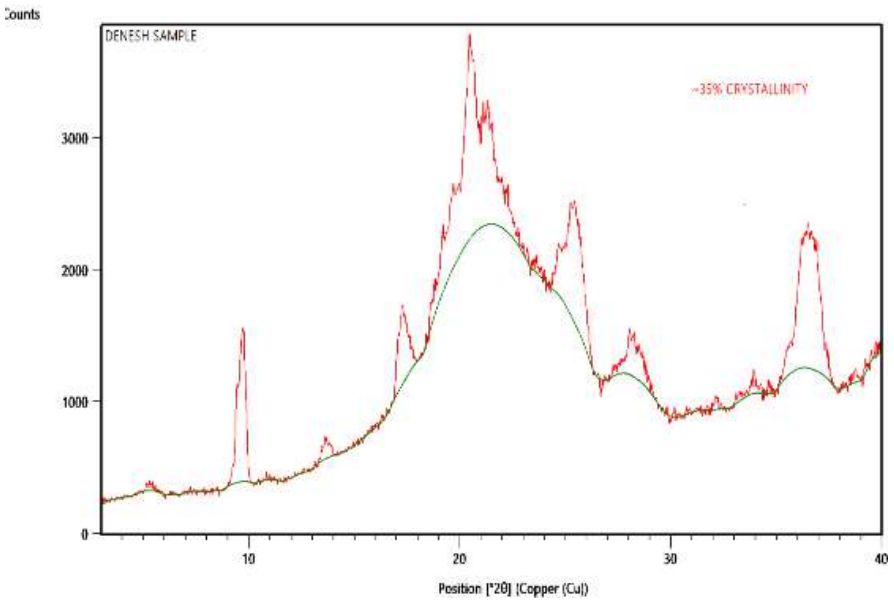


Figure 8: PXRD graph of lyophilized formulation

indicates that the drug is released in both the kinetic model and the Higuchi model in a more linear manner when compared to the first order model and the Korsmeyer-Peppas model; more specifically, the release is not dependent on the concentration of the drug, and the Higuchi model illustrates that the drug is released through diffusion mode[4]. Cumulative drug release of pure drug and formulation are shown in figure.

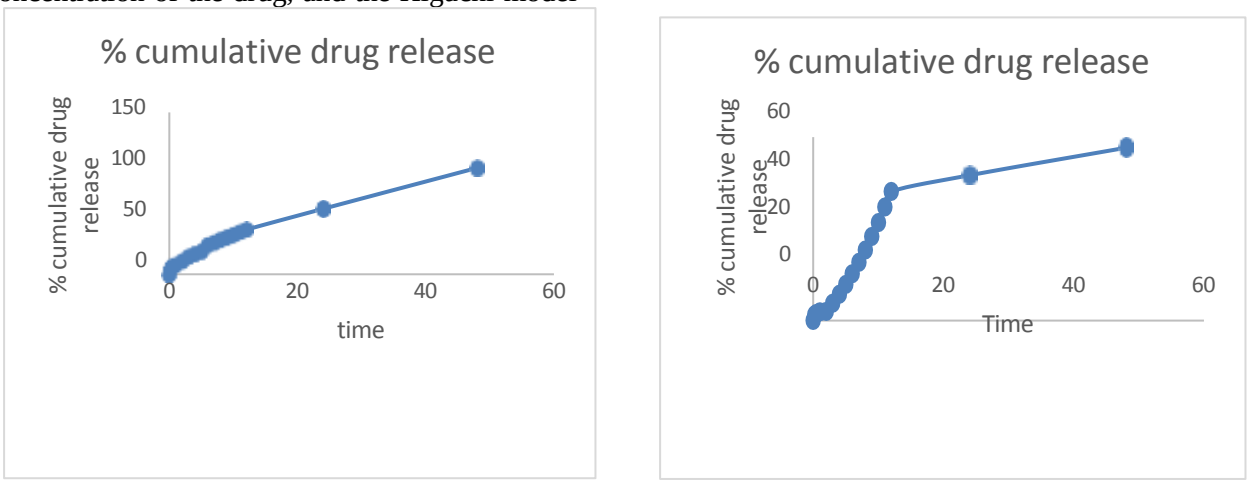


Figure 9: In vitro drug release of pure drug and formulation

Table 2:

Time	Pure drug	Formulation
0	0	0
0.25	1.87±0.117	5.272±0.047
0.5	2.264±0.223	7.81±0.431
1	2.814±0.114	8.99±0.048
2	2.93±0.332	12.638±0.221
3	5.610±0.125	16.727±0.213
4	8.55±0.112	19.24±0.414
5	11.87±0.138	21.203±0.076
6	15.299±0.335	27.356±0.054
7	19.057±0.115	29.592±0.115
8	23.043±0.338	32.156±0.117
9	27.46±0.327	34.505±0.265
10	32.086±0.342	36.65±0.274
11	37.08±0.115	39.163±0.119
12	42.184±0.174	41.337±0.323
24	47.53±0.114	60.667±0.424
48	56.523±0.321	98.564±0.521

5.3 *Ex vivo* permeation studies:

The permeation profile of Ticagrelor from optimized Ticagrelor liquid crystals and drug suspension were studied by using non everted gut sac model. It clearly indicated that liquid crystals increased the permeation of Ticagrelor compared with the drug suspension. After 4 h, a cumulative amount of Ticagrelor was permeated to receiver compartment increased. The graph plotted indicates that at the end of the 4 hours, 0.50674 mg/cm^2 of pure drug and 1.695247 mg/cm^2 liquid crystal formulation was permeated per unit area of intestine. The steady state flux was found to be 0.0037 for pure drug and 0.0096 for liquid crystal formulation.

Table 3:

Time	Pure drug	Formulation
0	0	0
30	0.006284	0.11351
60	0.018243	0.248404
90	0.048242	0.416439
120	0.10074	0.604034
150	0.177764	0.831053
180	0.266444	1.086243
210	0.379751	1.374785
240	0.50674	1.695247

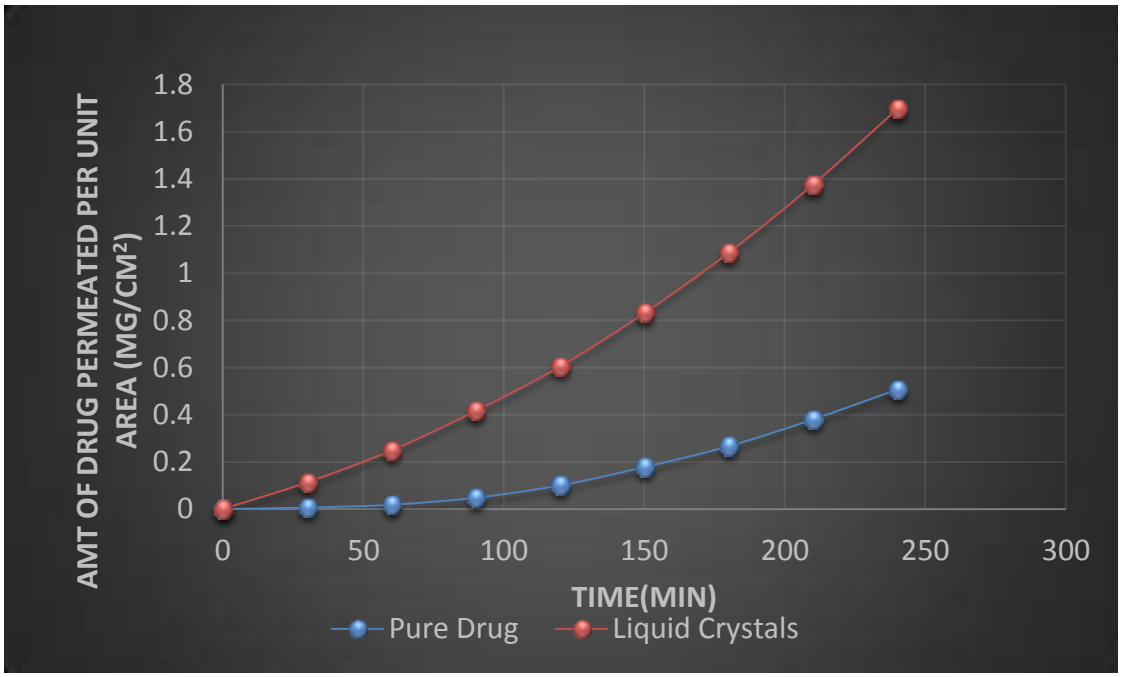


Figure 10 : *Ex vivo* permeation studies

6. Conclusion:

The research work demonstrates that the preformulation parameters, such as solubility, λ max, FTIR investigations, and compatibility studies were found to be a standard parameters. The preparation technique and the formulations increased quality, novel ingredients are ideal for liquid crystallization. The observation that the maximum absorbance of Ticagrelor was measured at 298 nm, which is extremely near to its official value, confirms the drug's credibility. Studies on particle size, zeta potential, differential scanning calorimetry, and powder X-ray diffraction all confirmed the formulations crystallinity. The release study of drug has also shown the best result for the optimized formulation of concentration 1%w/w drug, 1% Glyceryl mono stearate, 6% of polysorbate 80 exhibited the best release with 98.564±0.521 in 48h. The R^2 values of zero order and Higuchi model were found to be 0.947 and 0.9809 respectively, which indicates the formulation follows zero order and follows diffusion mechanism.

Acknowledgements:

The authors sincerely acknowledge Lee pharma pvt. ltd for providing gift sample of the drug, Dr. K. Suresh Babu; Senior principal scientist, CSIR-IICT and Dr. K. T. Sunil kumar, Shri Vishnu college of pharmacy for providing lab facilities for research work.

REFERENCES:

1. Husted S, Van Giezen JJ. Ticagrelor: the first reversibly binding oral P2Y12 receptor antagonist. Cardiovascular therapeutics. 2009 Dec;27(4):259-74. [Cross Ref]
2. Kubisa MJ, Jezewski MP, Gasecka A, Siller-Matula JM, Postuła M. Ticagrelor–toward more efficient platelet inhibition and beyond. Therapeutics and clinical risk management. 2018 Jan 17:129-40.

[CrossRef]
3. Norgard NB, Abu-Fadel M. Comparison of prasugrel and clopidogrel in patients with acute coronary syndrome undergoing percutaneous coronary intervention. Vascular Health and Risk Management. 2009 Nov 2:873-82. [CrossRef]
4. SHARMA RB, KUMARI C, KAPILA A, BORA KS, SHARMA A. Formulation and in-vitro evaluation of emulsion loaded topical gel for the enhancement of diffusion through the skin for the treatment of skin irritation. Journal of Research in Pharmacy. 2022 Sep 1;26(5).
5. Sharma M, Dhiman N, Singh P, Sharma R, Sharma RB, Arora V, Arora S. Gel incorporated lipid nanoparticles for the treatment of psoriasis. Materials Today: Proceedings. 2022 Jan 1;48:1690-701. [CrossRef]
6. [6]. Mekonnen Y, Bekele A, Suleiman S, Chali BU. Physicochemical Quality of Metformin Hydrochloride tablet Brands available in Jimma Town, South west, Ethiopia. [CrossRef]
7. Lala RR, Shinde AS. Development, optimization, and in vitro evaluation of atorvastatin calcium and vinpocetine codelivery by solid lipid nanoparticles for cancer therapy. Future Journal of Pharmaceutical Sciences. 2021 Dec;7(1):1-4. [CrossRef]
8. Kazemi M, Varshosaz J, Tabbakhian M. Preparation and evaluation of lipid-based liquid crystalline formulation of fenofibrate. Advanced biomedical research. 2018;7. [CrossRef]
9. SHARMA RB, KUMARI C, KAPILA A, BORA KS, SHARMA A. Formulation and in-vitro evaluation of emulsion loaded topical gel for the enhancement of diffusion through the skin for the treatment of skin irritation. Journal 0f Research in Pharmacy. 2022 Sep 1;26(5).
10. Xia XR, Baynes RE, Monteiro-Riviere NA, Riviere JE. Determination of the partition coefficients and absorption kinetic parameters of chemicals in a lipophilic membrane/water system by using a membrane-coated fiber technique. European journal of pharmaceutical sciences. 2005 Jan 1;24(1):15-23. [CrossRef]
11. KAUSHIK K, SHARMA RB, SHARMA A, AGARWAL S. Formulation and evaluation of antifungal activity of gel of crude methanolic extract of leaves of Ipomoea carnea Jacq. Journal of Research in Pharmacy. 2020 May 1;24(3).
12. Kim SJ, Lee HK, Na YG, Bang KH, Lee HJ, Wang M, Huh HW, Cho CW. A novel composition of ticagrelor by solid dispersion technique for increasing solubility and intestinal permeability. International journal of pharmaceutics. 2019 Jan 30;555:11-8. [CrossRef]
13. Khan AW, Kotta S, Ansari SH, Sharma RK, Kumar A, Ali J. Formulation development, optimization and evaluation of aloe vera gel for wound healing. Pharmacognosy magazine. 2013 Oct;9 (Suppl 1) :S6. [CrossRef]
14. Varshosaz J, Taymouri S, Jahanian‐Najafabadi A, Alizadeh A. Efavirenz oral delivery via lipid nanocapsules: formulation, optimisation, and ex‐vivo gut permeation study. IET nanobiotechnology. 2018 Sep;12(6):795-806. [CrossRef]