Biological Forum – An International Journal **15(3): 759-770(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Synthesis, Characterization and Development of HPLC Method for Clopidogrel Metabolite: Computational Approaches for PKPD and Toxicity Predictions

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(Received: 18 January 2023; Revised: 16 February 2023; Accepted: 22 February 2023; Published: 19 March 2023)
(Published by Research Trend)

ABSTRACT: The current research work emphases on synthesis, characterization, and optimization of RP-HPLC method for determination of clopidogrel and its synthesized metabolite 2-oxo-clopidogrel as an impurity simultaneously. Also the subsequent part of the paper is, ADME and toxicity of the 2-oxo-clopidogrel were predicted through different ADME database like SWISS ADME and molesoft. The metabolite 2-oxo-clopidogrel was synthesized in the laboratory and characterized by UV, IR GC/MS, NMR spectroscopy. The method was developed using Kinetex C18, 4.6 mm x 250 mm, 5 µm as stationary phase, mobile phase comprising of acetonitrile (80%): Phosphate buffer 20mM pH 3 (20%) with flow rate 1.0 mL/min, volume 20 µL through a run time of 10 minutes.

The developed RP-HPLC method underwent validation based on ICH guidelines, demonstrating accuracy, precision, reproducibility, specificity, and robustness. Assessment of toxicity by using computational tools like Swiss tool is reported with the hope of reducing threats.

Keywords: Clopidogrel, 2-oxo-clopidogrel, RP-HPLC, SWISS ADME and molesoft.

INTRODUCTION

In the modern era, cardiovascular disease (CVD) is the main factor in death across the globe. About 17 million premature deaths (deaths occurring before the age of 70) were caused by non-communicable illnesses in 2019, with cardiovascular disease (CVD) causing approximately 38% of these deaths (Hamm *et al.*, 2011).

An antiplatelet drug called clopidogrel is frequently prescribed to those who are at susceptible to cardiovascular ailments including heart attacks and strokes to stop blood clots from forming. It is a member of the thienopyridine medication family that reduces chance of blood clots from developing by preventing platelet aggregation.

In an effort to avoid additional incidences, clopidogrel is administered to people who cannot take acetylsalicylic acid or who have already had a cardiac event or stroke while taking acetylsalicylic acid (Dipiro *et al.*, 2014).

Clopidogrel bisulphate is a prodrug. It is chemically methyl (+)-(S)(o-chlorophenyl)-6, 7-dihydrothieno-[3, 2-c] pyridine 5(4H)-acetate (O'Neil 2013).

All contaminants found in pharmaceuticals or drug products must be identified and characterized in accordance with the International Conference on Harmonisation (ICH) criteria. Any component or substance identified in a medicament which does not correspond to the therapeutic ingredient or additives specified is referred to as an impurity, accordance by the ICH.

The acceptable amounts of contaminants in pharmaceutical goods are subject to tight limits set out by the ICH. According to these criteria, contaminants that are present in amounts more than 0.1% must have been recognised, and certified. If impurities are anticipated to cause major dangers, detection and verification are still necessary despite lower quantities (ICH 2005)

It is essential to precisely detect and characterise the impurities connected to medications in order to assure their effectiveness and safety.

An important area of interest in the fields of the pharmaceutical industry and therapeutic research is the monetary and time-effective optimal synthesis and characterization of impurities and therapeutically relevant intermediates of novel drugs. Medical researchers focus on attaining correct separation, discrimination, responsive finding, and precise quantification while working on such activities.

A critical component of drug development is evaluating the administration, distribution, metabolism, and elimination (ADME) pathways. The intended emphasize participation in regards to blood-brain permeability, effectiveness, and safety might be hampered by problems with several drugs, such as off-target

interactions, important metabolic channels, and furthermore. Upfront ADME assessments throughout the course of drug creation aid in the prompt identification of possible pharmacokinetic-related problems and assist to avoid their emergence in subsequent therapeutic trials. In silico models are currently an appealing option to measurement techniques in the discipline of ADME forecasting. Anyone may utilise the SwissADME database and molesoft at http://www.swissadme.ch as examples of such resources. These technologies make it possible to predict ADMET (absorption, distribution, metabolism, elimination, and toxicity) characteristics for a variety of substances, among them the clopidogrel metabolite known as 2-oxo-clopidogrel (Daina *et al.,* 2017).

Literature survey revealed various methods as Reverse phase chromatography, UV spectrophotometric methods for determination of clopidogrel in combination with other drugs (Harahap *et al.,* 2017; Patil *et al.,* 2013; Cholke *et al.,* 2012).

The simultaneous detection of clopidogrel and associated thiol intermediate in human plasma has been shown using a variety of methods (Peer *et al.,* 2012; Delavenne *et al.,* 2010; Takahashi *et al.,* 2008; Aher and Gaikwad 2016).

The precise objective of this study is to synthesize, characterize and develop HPLC method for simultaneous estimation of Clopidogrel (CLDR) along with its metabolite that is 2-oxo-clopidogrel (CLDO). In order to quantify synthesized metabolites, this article emphasizes the value of using non-compendial reference standards with chromatographic techniques like HPLC (Mrinali, M. M. 2022).

In the subsequent part of the paper, it is emphasized how useful in in silico simulations are for predicting ADMET (absorption, distribution, metabolism, excretion, and toxicity) features and how they may be an acceptable substitute to experimental approaches. Several ADME databases, including SWISS ADMET and molesoft, were used in this investigation to estimate the toxicological effects and ADMET of the synthesized intermediate CLDO.

MATERIAL AND METHODS

Chemicals and Reagents. Dr. Reddy's Laboratories in Hyderabad, India generously provided the working standard and pure Clopidogrel samples as gift samples. The Palvix TAB tablets, manufactured by Sanofi, were procured from a local medical shop. Further analytical chemicals comprising acetonitrile (HPLC grade), Potassium dihydrogen orthophosphate, Dipotassium hydrogen orthophosphate (HPLC grade) and ortho-phosphoric acid were employed for a grade in analysis. High purity Milli-Q water was purchased from Merck (India). All other chemicals utilized in the study were of analytical reagent (AR) grade.

Synthesis of metabolite CLDO. 0.38 g of clopidogrel was placed to a 20 mL beaker, and then 10 mL of THF (tetrahydrofuran), was added gradually, one drop at a time. The outcome was then cooled to 0°C by being immersed in an ice salt combination. The reaction mixture was subsequently incorporated, and a prior 0.7 mL cold solution of lithiumdiisopropylamide in tetrahydrofuran was agitated for roughly an hour at 0°C. 1 mL of thetramethylurea was then added, and 1.65 mL of n-Butyl lithium in hexane was added after that. An extra two hours were spent stirring the resultant solution.

Characterization of metabolite CLDO. CLDO has been investigated using methods of spectroscopy encompassing UV, FT-IR, MS, and NMR. A JASCO V-730 Double beam UV/Vis spectrophotometer was used to evaluate the synthesised compound's UV spectrum.

Employing a JASCO FTIR-4600 spectrophotometer, IR spectrum results were gathered and the absorption regions pertaining to the spectral oscillations of various functional groups being allocated.

NMR spectroscopy in the 1H range was used to identify the synthesised metabolite. The substance was tested for solubility in CDCl3, DMSOd6, and D2O. The substance was then submitted to SAIF, Chandigarh for further NMR testing to validate its structure.

Employing a Shimadzu QP 3010 mass spectrometer connected to gas chromatography by means of an electron impact source, the samples' mass evaluation and identification took place. The transport medium used for the specimen's examination was helium, and the fluid flow velocity were 1 mL/min. The ambient temperature of the electron impact source was maintained at 280°C. The detector chosen for this research was a gas chromatographic real analyzer. For the experiment, a cylindrical tube having an extended length of thirty-five meters and a diameter within of 0.2 mm was used.

HPLC Method Development

Chromatographic conditions and instrument. This HPLC method (Shimadzu Model LC-2030 PLUS, India) featuring an ultraviolet (UV) sensor was used for the establishment and confirmation of this experimental approach. The procedure made use of a Kinetex C18 column having 4.6 mm x 250 mm dimensions and a 5 µm size of particles. A flow velocity of 1 mL/min, an infusion amounting to 20 L, a runtime of 10 minutes, and an appropriate detection range of 223 nm were the optimised characteristics. Acetonitrile (80%) and a 20 mM pH 3 phosphate buffer (2%) were used in the phase of motion in a gradient manner. The pH meter (Mettler Toledo), ultrasonicator (Pci-Analytics), and electronic balance (Mettler Toledo) were correctly calibrated to facilitate the validation procedure.

Preparation of buffer solution. Precisely measured amounts of 1.62 g of potassium dihydrogen phosphate and 0.29 g of disodium hydrogen phosphate were added to 550 ml of distilled water. Continuous mixing resulted in the substance dissolving. A 0.45 µm Millipore membrane screen was then used to clean the buffer solution.

Preparation of mobile phase. As the moving portion, diluent, and blank solution, an 80:20 v/v combination of acetonitrile and phosphate buffer solution was made. Previous to being applied, the solution was filtered and degassed. Ortho-phosphoric acid was added to the mobile phase to bring the pH there down to 3.

Preparation of stock and standard solutions. Precisely measured amounts of CLDR and CLDO, weighing 10 mg each, were separately diluted with suitable volumes of the

mobile phase in calibrated 100 mL volumetric flasks. The medicines were completely dissolved in the prepared solutions after being exposed to sonication for 10 minutes, and the ultimate volume of 100 mL was then attained through incorporating a more mobile phase. With a concentration of 100 µg/mL, the resultant solution was given the name stock solution.

These stock solutions were used to transfer solutions ranging from 0.2 mL to 1.2 mL into 10 mL volumetric flasks for CLDR procedure evaluation, yielding ultimate concentrations varying from 2 µg/mL to 12 µg/mL. Simultaneous transfers of solutions from 2 mL to 12 mL into 10 mL volumetric flasks for CLDO analysis produced final levels that ranged from 20 µg/mL to 120 µg/mL.

Preparation of sample solution. About 20 clopidogrel-containing tablets (Palvix) were accurately weighed. The pills were broken up and well combined. The resultant powder, weighing exactly 10 mg of clopidogrel, was then put through a volumetric flask with a 100 mL capacity. After adding the proper amount of diluent, the solution was sonicated for 20 minutes. The volume was then changed using the same diluent to 100 mL. This mixture was referred to as the stock solution, and clopidogrel concentrations were adjusted by further diluting it as necessary.

Method Validation. A number of procedures have been carried out in compliance to the instructions given by the ICH in order to confirm the proposed analytical technique. The evaluation of the system's appropriateness, specificity, precision (system and method), accuracy, linearity, ruggedness, and limits of detection (LOD) and quantitation (LOQ) were all part of the validation procedure. To make sure that the testing process was reliable and robust, these extensive experiments were conducted.

System suitability study. The system compatibility characteristics of freshly made standard stock solutions of CLDR and its metabolite CLDO, were examined. Under optimal chromatographic ailments the pharmaceutical and its intermediate product have been put into the system being analysed. To assess the appropriateness of the methodology, a variety of characteristics, comprising the number of theoretical plates, resolution factor, retention duration, selectivity, as well as the LOD and LOQ, were carefully studied.

Specificity. Reference testing samples utilizing the CLDR had been presented and analysed in order to gauge the evaluation technique's accuracy. The samples' chromatograms were checked for any unwanted peaks. It was anticipated the chromatograms of the standard and test solutions ought to show comparable patterns and demonstrate comparable retention periods.

Linearity. In order to get ready the samples for examination, several 10 mL volumetric flasks were filled with CLDR stock solutions ranging from 0.2 to 1.2 mL, the mobile phase was then used to dilute these solutions to final proportions that varied from 0.2 to 12 µg/mL. To achieve final concentrations ranging from 20 to 120 µg/mL, 2 to 12 mL of the CLDO stock solution were placed into separate 10 mL volumetric flasks and diluted with the mobile phase.

Then, 20 µL of each prepared specimen were added to the chromatographic system, and the analysis was carried out for 10 minutes at a flow rate of 1.0 mL/min. 223 nm was employed as the detecting wavelength.

For both CLDR and CLDO drugs, a calibration curve was created to show the connection between concentration and peak area. The matching peak area values obtained from the chromatogram were displayed on the y-axis, and the concentration of the samples has been plotted on the x-axis.

Precision. A precision analysis was carried out to confirm the analytical method's repeatability. This study sought to evaluate the accuracy of the system and the procedure.

System Precision. The standard CLDR solution was introduced throughout the system on five times in accordance with the procedure. The peak area's relative standard deviation (RSD) was predicted to be no more than 2.0%.

Method precision. The test technique was followed, and six separate injections of the CLDR standard solution were made. It was anticipated that the CLDR test would fall between the ranges of 90.0% to 110.0%.

LOD/ LOQ. The lowest concentration that can be detected and the smallest amount that can be precisely and correctly measured were computed to define the system's LOD and LOQ. The formula below was used to get these numbers from the linearity data.

$$LOD = \frac{3.3\sigma}{S}$$

σ = standard deviation of the response

S=slope of the calibration curve of the analyte.

$$LOQ = \frac{10\sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte.

Accuracy. The CLDR assay was carried out in triplicate in accordance with the test method utilising equal quantities of CLDR concentration at 80%, 100%, and 120% of the labelled quantity. By comparing the findings to the standard solution of the same strength, accuracy was evaluated. The average CLDR recovery % was then determined.

At each spike level, the mean percentage recovery of CLDR was predicted to fall between a range of values of not less than 90.0% but not exceeding 110.0%.

Robustness. Intentional flow rate fluctuation served as proof of the proposed technique's resilience. According to the test procedure, the standard solution was prepared and introduced through an HPLC system at flow rates of 0.9, 1 and 1.1 mL/min. The requirements for system appropriateness were assessed.

PKPD predications. We used in-silico-based ADME analysis to save costs, save time, and guard against compound unsuccessful attempts in the late stages. We were able to have an early understanding of the potential for real beneficial outcomes thanks to this strategy. We used online programmes like SWISS ADME and molesoft to evaluate CLDO's toxicity, ADME (absorption, distribution, metabolism, and excretion), and drug similarity. We were able to forecast the CLDO

compound's drug-likeness and toxicity by running it via the Swiss-ADME web server.

RESULT

After conducting a comprehensive examination of the available documentation, it is possible to make the decision to utilize THF, lithiumdiisopropylamide and thetramethylurea as reagents to synthesize 2-oxo-clopidogrel shown in Fig. 1.

Characterization of metabolite. Spectroscopic techniques such as UV, IR, NMR, and GC/MS were employed to characterize CLDO. The corresponding spectra, including UV Fig. 2, FT-IR Fig. 3, NMR Fig. 4, and mass spectra Fig. 5 and Fig. 6, were obtained and analyzed. Additionally, the probable fragments of CLDO were illustrated in Fig. 7.

The analysis of the spectral data provided conclusive evidence confirming the successful synthesis of the product with the desired level of purity and quality.

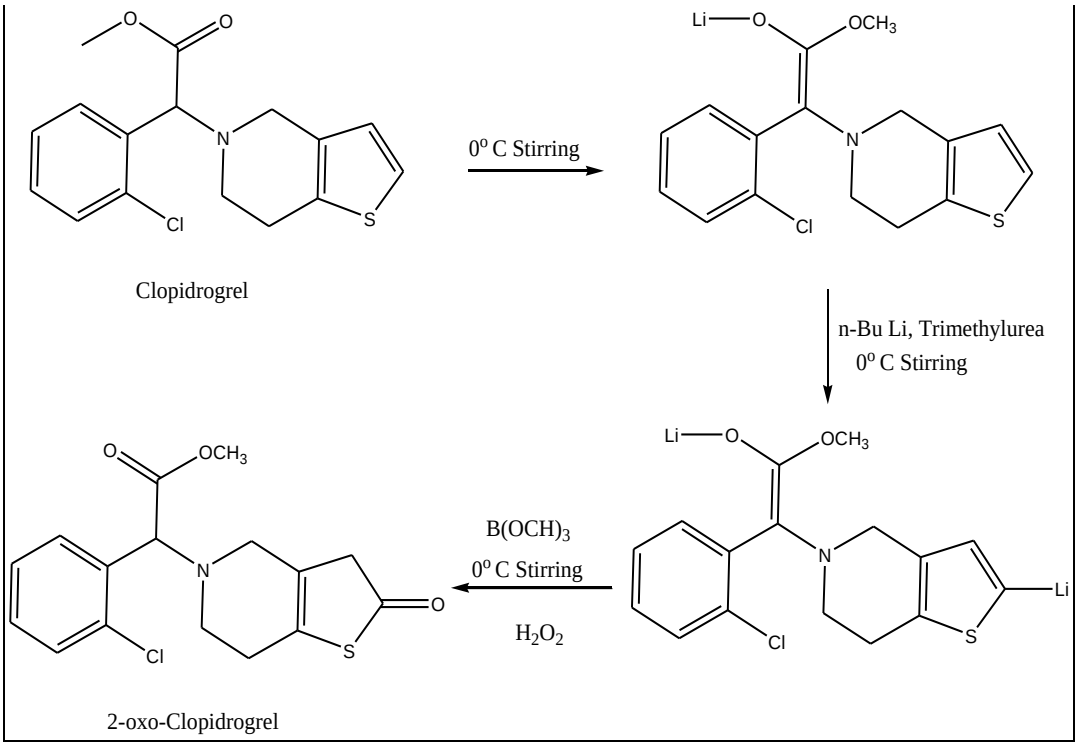

Fig. 1. Scheme of synthesis for metabolite 2-oxo-clopidogrel (CLDO).

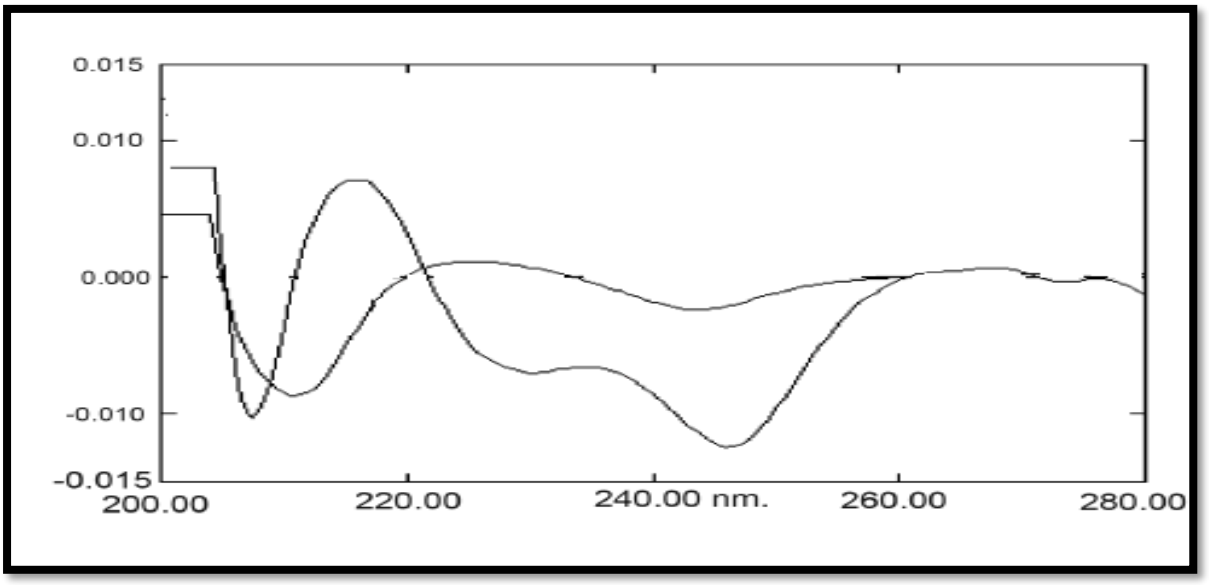

Fig. 2. Overlain UV Spectrum of CLDR and CLDO.

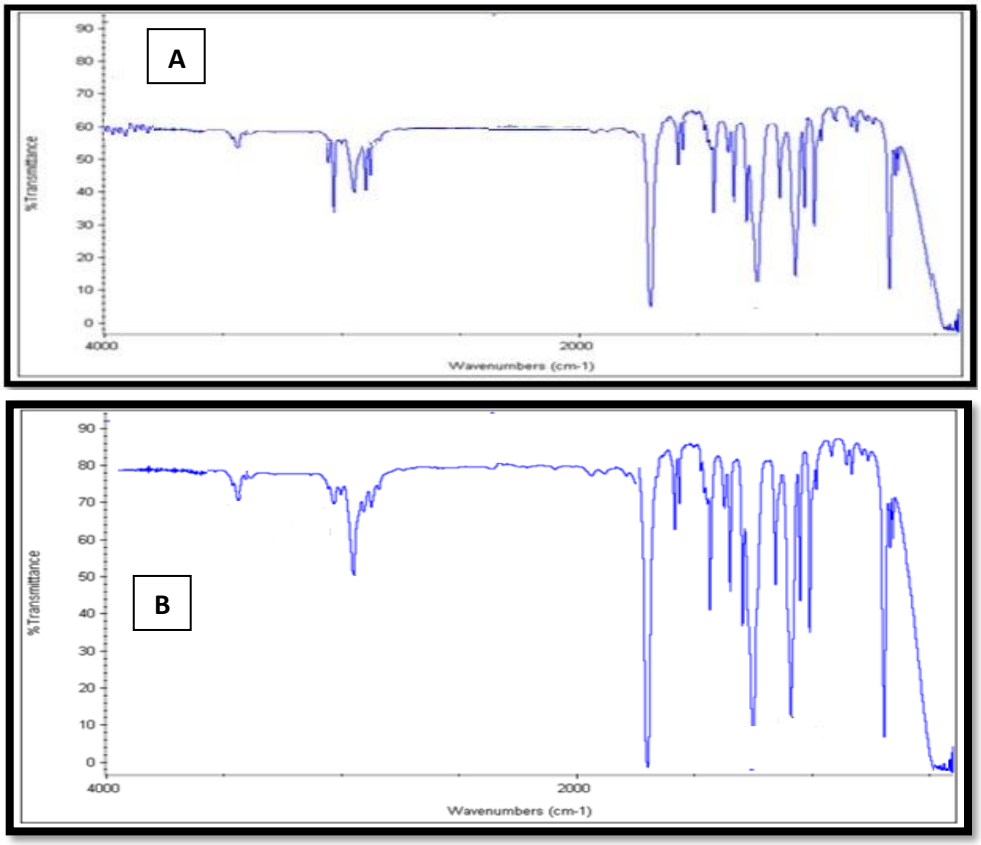

Fig. 3. Infrared Spectrum of CLDR (A) and CLDO (B).

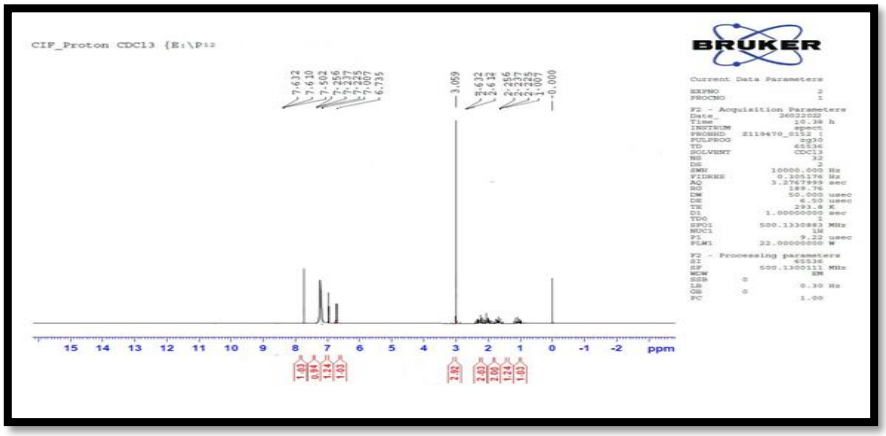

Fig. 4. NMR of CLDO.

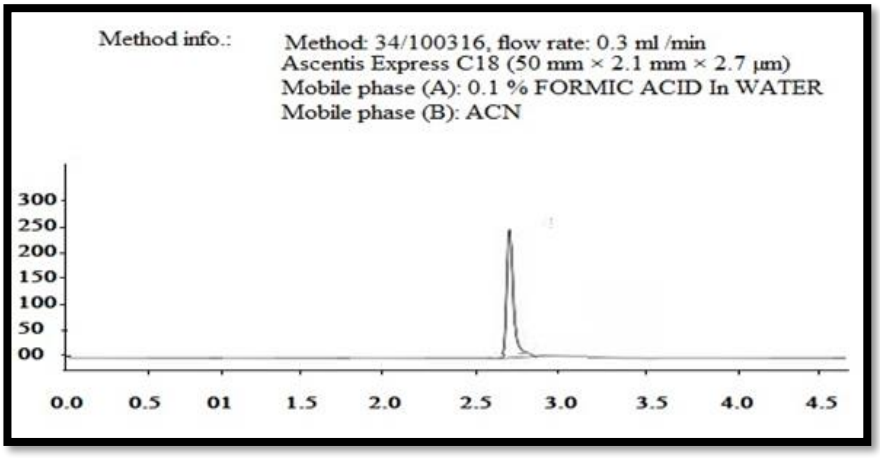

Fig. 5. Mass spectra of CLDO.

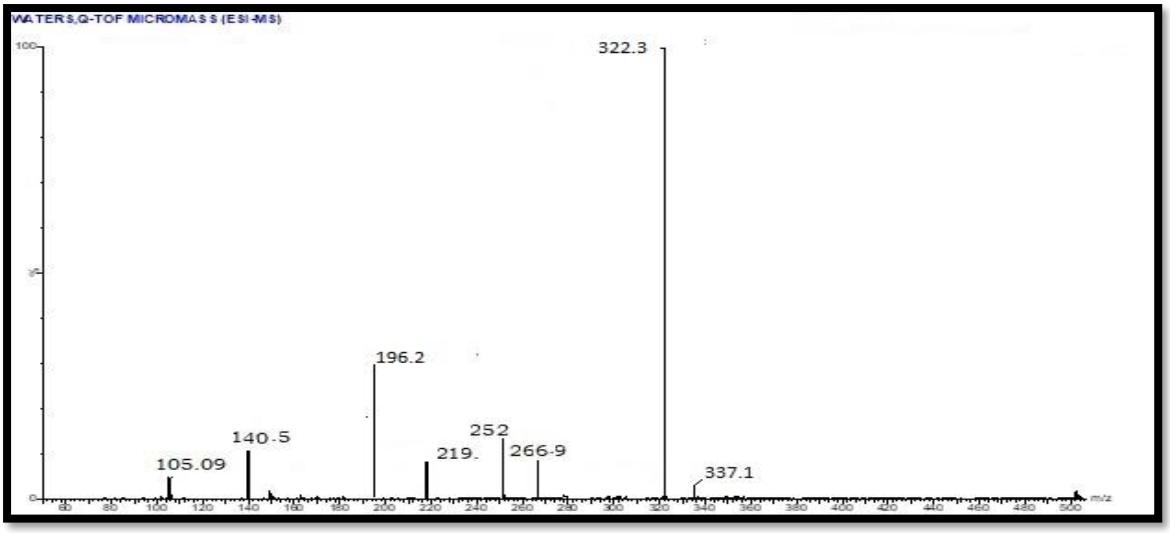

Fig. 6. Mass fragment of CLDO.

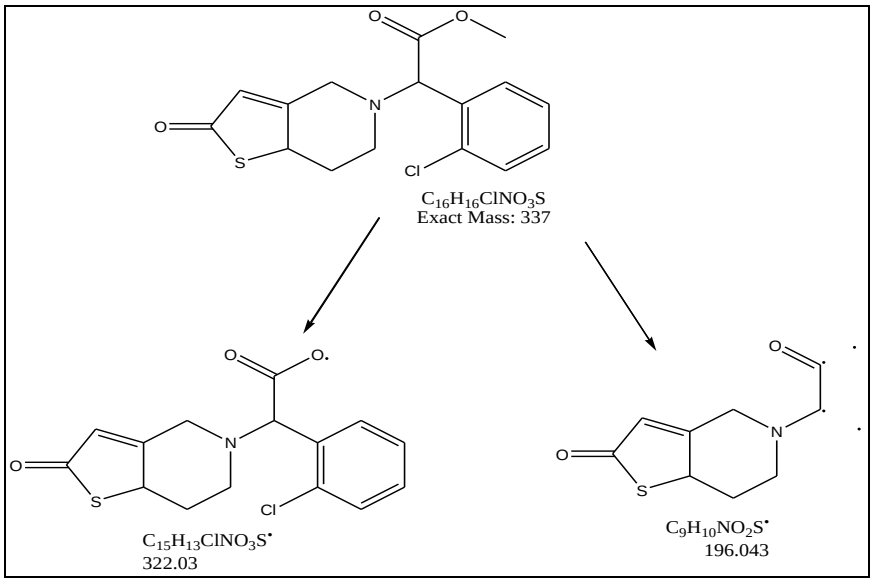

Fig. 7. Mass fragment of CLDO.

System Suitability. The selection of the stationary phase, which mostly depends on the molecule's molecular weight and solubility, is a crucial step in the creation of an approach to analysis. The C18 column was used for the evaluation in the present investigation based on prior research that suggested CLDO is best examined using RP-HPLC.

Different acetonitrile and buffer concentrations were tested and optimised in order to provide the best results as trial shown in Fig. 8. The objective was to generate a symmetric peak for the drug while reducing the analysis's total run duration.

Proper separation with well-resolved and symmetrical peaks was accomplished when employing a mobile phase made up of acetonitrile (80%) and a 20mM phosphate buffer at pH 3 (20%). CLDR and CLDO had retention durations of 4.24 and 6.05 minutes, respectively. Table 1 provides a summary of the observations for the various mobile phases that were attempted during the optimisation process. Table 2 provides the ideal chromatographic conditions.

The chromatograms from the standard and test solutions are shown in Fig. 9. The approach was validated in accordance with the recommendations in ICH Q2B (R1).

Linearity. For CLDR and CLDO, calibration curves were created in order to evaluate the method's linearity. For CLDR and CLDO, the concentration ranges for the calibration curves were established as 2 to 12 µg/mL and 20 to 120 µg/mL, respectively. The linearity graphs for CLDR and CLDO are shown in Fig 10 and Fig 11, respectively.

Plotting the mean peak area measurements against the respective concentrations yielded the least square line, as shown in Table 3. A strong linear connection between the concentrations and the mean peak areas is shown by the high correlation coefficients of 0.9992 for CLDR and 0.9994 for CLDO. This suggests that the procedure used was linear and appropriate for both CLDR and CLDO quantitative analysis.

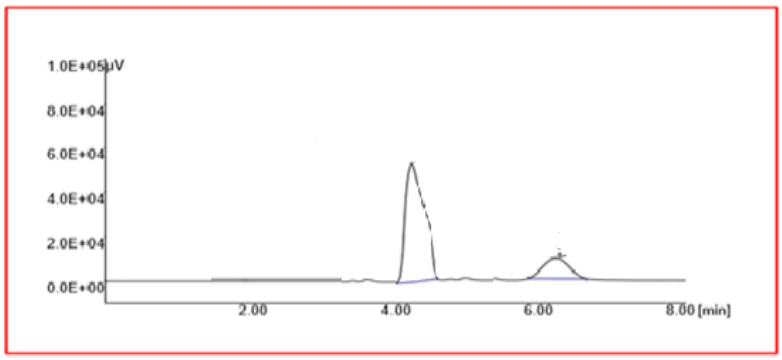

Fig. 8. Chromatogram representing CLDR and CLDO with composition of mobile phase Acetonitrile: Phosphate buffer (75:25).

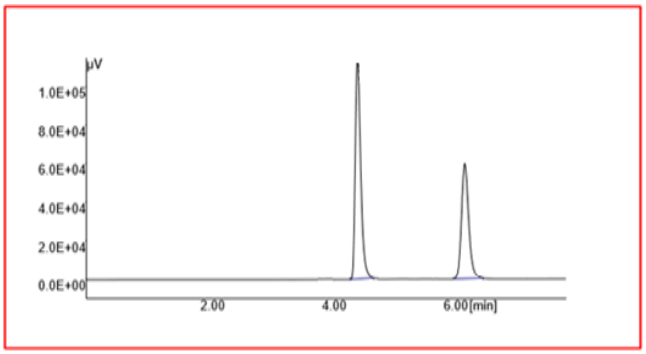

Fig. 9. Chromatogram representing CLDR and CLDO with retention time 4.243 and 6.055 respectively.

Table 1: Chromatographic behavior of CLDR and CLDO in mobile phase of various compositions.

Sr. No.	Mobile Phase	Concentration (% *v/v*)	Retention Time (min)	
			CLDR	CLDO
1	Acetonitrile: Water	80:20	4.02	Peak not obtained up to 10 min
2	Acetonitrile: Water	90:10	4.56	Peak not obtained up to 10 min
3	**Acetonitrile: Phosphate Buffer 20 mM pH 3**	**80:20**	**4.24**	**6.055**

Table 2: Optimized chromatographic conditions.

Sr. No.	Parameters	Details
1	Flow rate	1.0 mL/min
2	Column	Kinetex C18, 4.6 mm x 250 mm, 5µm column
3	Detector wavelength	223 nm
4	Injection volume	20 µl
5	Run time	10 min
6	Retention time	4.24 and 6.05 min

Table 3: Linearity studies on CLDR and CLDO.

Sr. No.	Sample			
	CLDR		CLDO	
	Conc^{n} (µgmL^{-1})	Area (N=3)	Conc^{n} (µgmL^{-1})	Area (N=3)
1	2	54467.28	20	42928.9
2	4	98222.92	40	89282.1
3	6	152929.1	60	132887.7
4	8	199892.92	80	169892.2
5	10	248922.13	100	208967.9
6	12	268736.26	120	247861.2
Straight line equation	y = 22489x + 14020		y = 2029.6x + 6564.3	
R^2	0.9992		0.9994	
SD	4573.3		67382.1	
Slope	22489		2029	

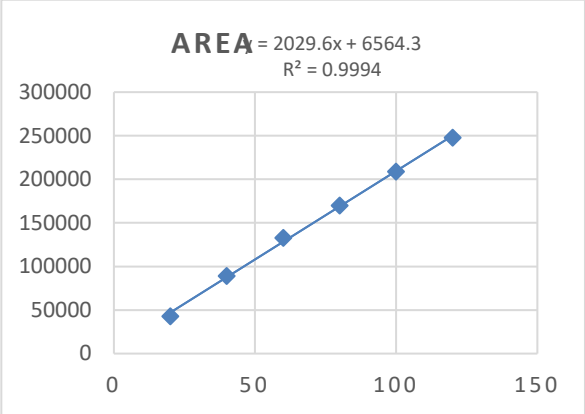

Fig. 10. Linearity graph of CLDR.

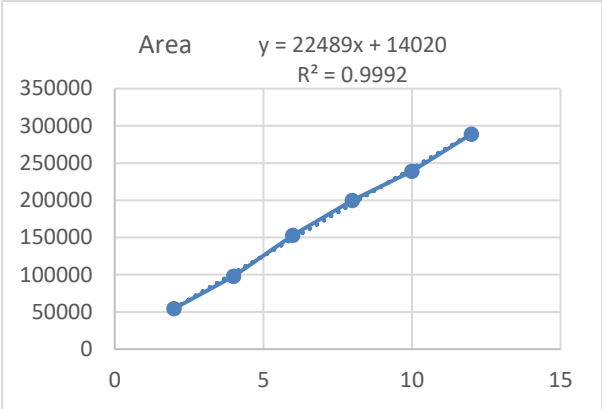

Fig. 11. Linearity graph of CLDO.

Accuracy. The recovery studies conducted on CLDO yielded results within the range of 99.25% to 100.75%, with a %RSD of ≥1.44%. These findings indicate the accuracy of the developed method, as presented in Table 4.

Precision. The intra-day and inter-day precision studies were carried out to assess the repeatability and reproducibility of the method. The results obtained demonstrate the consistency of the method both within the same day and across different days. The precision study results are summarized in Table 5.

Table 4: Accuracy studies on CLDR and CLDO.

Sample	Drug in formulation (mg)	Spiked level	Conc^{n} added (mg)	Area (N=3)	Conc^{n} found (mg)	% recovery	SD	%RSD
CLDR	75	80%	60	1921102	79.64	99.55	27445.5	0.98
	75	100%	75	2147861	99.81	99.81	24754.6	0.87
	75	120%	90	2399386	120.19	100.15	17141.1	1.19
CLDO	7.5	80%	6	200915	8.06	100.75	2853.3	1.32
	7.5	100%	7.5	239312	09.72	97.2	2042.1	1.44
	7.5	120%	9.0	257719	11.88	99.25	1467.501	0.99

*Acceptance criteria < 2.0.

Table 5: Intra and inter day variations of CLDR and CLDO.

Intra and inter day variations		Sample	
		CLDR	CLDO
Day 1	Mean (N=3)	1886265	734961.3
	SD	21859.08	2119.007
	%RSD	1.43	1.21
Day 2	Mean (N=3)	1859803	736340.7
	SD	21823.63	2398.382
	%RSD	1.09	1.21
Day 3	Mean (N=3)	1879365.26	726418.87
	SD	209998.04	2597.492
	%RSD	1.82	1.08

Specificity. In both the standard and test solutions, there was no interference seen at the CLDR and CLDO retention times. The retention times for the standard and test solutions were the same, meeting the specificity test's acceptance requirements. Fig. 12 gives this observation a graphic depiction.

LOD/ LOQ. The LOD for CLDR was determined to be 0.14 µg/mL, while for CLDO it was found to be 0.19 µg/mL. The LOQ was observed to be 0.52 µg/mL for CLDR and 0.46 µg/mL for CLDO. These results indicate that the method exhibits excellent sensitivity in terms of LOD and LOQ.

Robustness. Variations in the flow rate between 0.9 mL and 1.1 mL were added to test the method's adaptability. It was noted that these modifications had no appreciable effect on the proportion of drugs recovered. The created approach is resilient, according to the robustness study's findings, and is unaffected by little changes in the chromatographic settings. An overview of the results obtained as a result of parameter modifications made during the robustness investigation is shown in Table 6.

PKPD predications. For the assessment of ADME, toxicity and drug likeness of CLDO using online server SWISS ADME and molesoft has been used. Assessment

of drug-likeness of the compound CLDO is carried out using ***molsoft*** online server package Fig. 13.

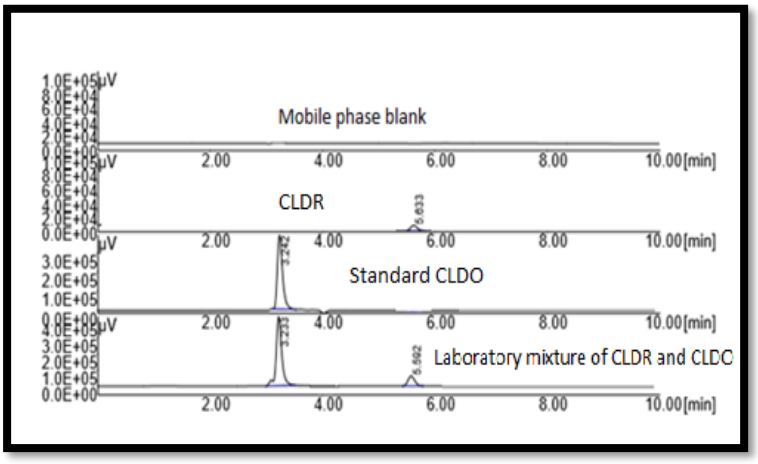

Fig. 12. Specificity of the method.

Table 6: Variations in flow rate.

Sr. No.	Flow rate (mL/min)	CLDR		CLDO	
		RT (min)	Peak area	RT (min)	Peak area
1	0.9	4.45	2222245.22	6.09	44209.10
2	1.0	4.41	2234764.22	6.04	45845.25
3	1.1	4.21	2265922.12	5.96	97123.96

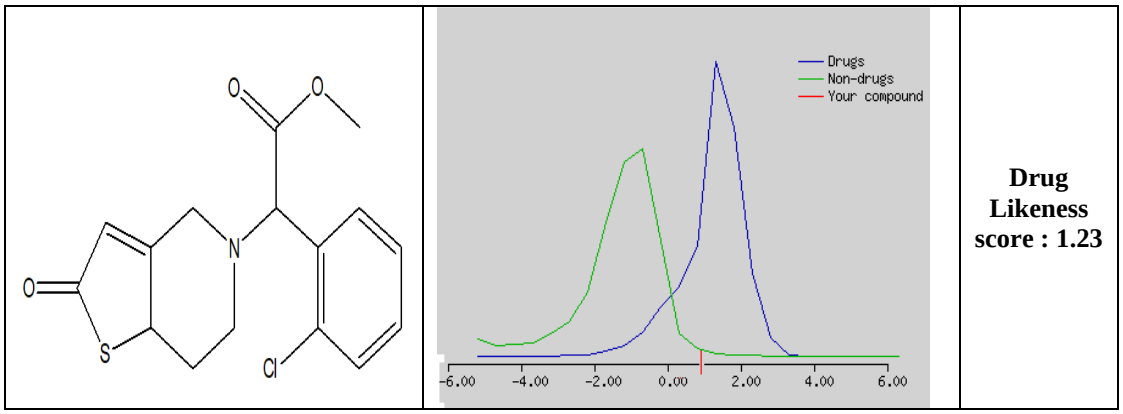

Fig. 13. Graphical data drug-likeness score of CLDO.

Smile:
Clc1ccccc1C(N2CC3=CC(=O)SC3CC2)C(=O)OC

The graphical data clearly indicates that the drug-likeness score of the compound CLDO is 1.23, suggesting that it can be considered a promising drug candidate from a pharmacokinetic perspective.

Compound CLDO was subjected to Swiss-ADME online server for their drug likeness and toxicity prediction. The compound PP02 revealed a (XlogP3) lipophilicity of 2.56; Molecular weight 337.82; Total polar surface area (TPSA) 71.91; ESOL (Log S) value -3.49. Considering the flexibility of structure the compound exhibited a 4 rotatable bonds followed by maximum 4 h-bonds acceptors and 0.0 hydrogen bond donors exhibits that the designed compounds could possess a good oral bioavailability score of 0.55 with no violations in the drug likeness activity (Lipinski rule of five). GI-absorption is high, permeable to Blood Brain Barrier hence high chance of crossing the CNS system. Compound PP02 is predicted to be a non-substrate to (P-glyco protein) Pgp, hence chance of efflux out is minimum or negligible which are shown in Table 7.

Further assessing the metabolic factor here we identified that compound CLDO is predicted as substrate only to CYP2D6 and predicted inhibitor to the CYP250 (CYP1A2, CYP2C19, CYP2C9 and CYP3A4) class of enzymes, hence the selected compound could not be easily metabolized and can be delayed in excreted out of the system.

Due to inhibition of the selective CYP450 class of enzyme following pathways and metabolism can be affected they are: Linoleic acid metabolism, Drug metabolism, Chemical carcinogenesis, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Arachidonic acid metabolism, Steroid hormone biosynthesis, Serotonergic synapse, Caffeine metabolism and Tryptophan metabolism are shown in Fig. 14. The toxicity of compound CLDO was further analyzed using ADMET SAR version 2, employing the following methodology in Table 8.

Table 7: PKPD analysis of CLDO from Swiss-ADME server.

Sr. No.	Parameter	Predication
1	GI absorption	High
2	BBB permeant	No
3	Pgp substrate	Yes
4	CYP1A2 inhibitor	No
5	CYP2C19 inhibitor	No
6	CYP2C9 inhibitor	No
7	CYP2D6 inhibitor	No
8	CYP3A4 inhibitor	Yes
9	Lipinski #violations	0
10	Ghose #violations	0

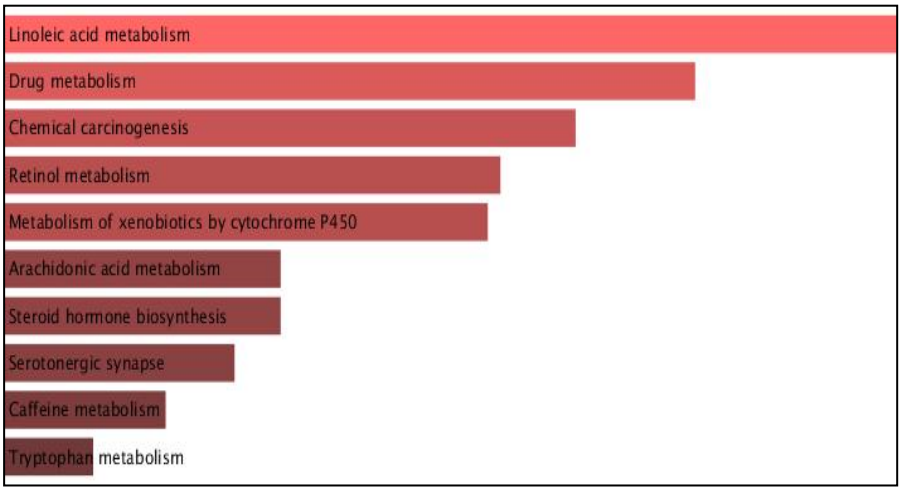

Fig. 14. Due to CYP450 (CYP1A2, CYP2C19, CYP2C9 and CYP3A4) class of enzyme inhibition following pathway may affect.

Table 8: ADMET TOX Prediction using ADMETSAR tool box.

Sr. No.	Parameter	Prediction	Probability
1	EYE corrosion	-	0.5617
2	Eye irritation	-	0.043
3	Carcinogenicity	-	0.6733
4	Hepatotoxicity	-	0.1283
5	Acute oral toxicity	+	1.34
6	Ames Mutagenicity	-	0.8019
7	Respiratory toxicity	-	0.623
8	Nephrotoxicity	-	0.4872

The ADMET property table includes classification and regression results. For classification, the columns of the table are endpoint, value and probability, respectively. The value is the predict labels. For example, for toxicity endpoints, the value "+" mean s Positive/Toxic while "-"means Negative/Nontoxic. The probability is related to the value, and it is generally higher than 50% because if the probability was less than 50%, it should have been predicted as the other result. For the regression models, the columns are endpoint, value and unit.

DISCUSSION

This study set out to synthesize and characterise the clopidogrel metabolite 2-oxo-clopidogrel as well as to develop a novel validated method for precisely measuring both the synthesised metabolite and the parent drug using the RP-HPLC method. Following a thorough

review of the documentation, one can decided to use THF, lithiumdiisopropylamide along with thetramethylurea to create a 2-oxo-clopidrogrel.

According to published works, there are no documented analytical methods for the evaluation of synthesized metabolite 2-oxo-clopidogrel along with parent clopidogrel by RP-HPLC method.

The cost- and time-efficient optimum synthesis and characterization of impurities and therapeutically essential intermediates of pharmaceuticals is a significant topic of interest in the realms of pharmaceutical industry and therapeutic research. Due to their critical significance in guaranteeing the quality control of drugs, analytical techniques for the determination of new ligands employing RP-HPLC are now being developed with a great deal of effort. Given its extreme relevance, this has attracted a lot of attention.

The absorption bands observed in the IR spectrum of the compound were characterized by 1082 (C-O str), 1117 (C-O str), 1726 for ester (C=O stretching), 2966 for aliphatic (CH), 3078 for aromatic (C-H stretching) respectively.

In the proton of NMR revealed following of 2-oxo-clopidrogrel interpretation found to be (δ ppm): 7-7.6 9, (4H, m aromatic), 6.7 (1H, s, olefinic H-C=C), 3.059 (3H, s, -CH3), 2.6 (2H, t, ring –CH2), 2.56 (2H, d, ring –CH2), 2.2 (1H, d, ring –CH). The mass spectrum of the compound showed a molecular ion peak at M/z = 322.03 of $C_{15}H_{12}ClNO_3S$ and 196.04 for $C_9H_{10}NO_2S$.

In accordance with the guiding principles outlined by the ICH, the developed method underwent validation to assess its accuracy, precision, reproducibility, specificity, and robustness. The UV method was optimized for the analysis of clopidogrel and 2-oxo-clopidogrel, with the optimized wavelengths found to be 218 nm and 228 nm, respectively. However, during HPLC analysis, it was observed that both compounds exhibited a prominent peak at a wavelength of 223 nm when overlain. Therefore, this specific wavelength was chosen for analysis. An acceptable symmetric peak was attained using the Kinetex C18 column (250 mm × 4.6 mm id, 5 µm particle size).

The most optimum ratio for the mobile phase containing acetonitrile: phosphate buffer 20mM pH 3 at a ratio of 80:20% v/v yielded satisfactory retention time and parting of CLDR and CLDO with retention times of 4.24 min and 6.05 min, correspondingly. The run time of the method was fixed at 10 min.

In the subsequent part of the paper, it is emphasized how useful in in silico simulations are for predicting ADMET (absorption, distribution, metabolism, excretion, and toxicity) features and how they may be an acceptable substitute to experimental approaches. To achieve cost-effectiveness, save period, and prevent late-stage disappointments of compounds, we conducted an in-silico ADME investigation of the synthesized metabolite CLDO, allowing for early identification of true positive results. Compound CLDO was subjected to Swiss-ADME and molesoft online server for their drug likeness and toxicity prediction.

CONCLUSION

The developed HPLC method is characterized by its simplicity, accuracy, precision, sensitivity, and selectivity for the estimation of clopidogrel and its metabolite, 2-oxo-clopidogrel, which was synthesized and characterized in the laboratory. This method can also be applied to quantify 2-oxo-clopidogrel as an impurity often found in bulk drugs and formulations of clopidogrel.

Moreover, there is a possibility to further optimize the method to enable the accurate quantification of 2-oxo-clopidogrel in biological fluids. This optimization would make the method highly valuable for conducting clinical and bioequivalence studies. To ensure safety, computational tools such as Swiss tool have been utilized to assess the compound's toxicity. The objective is to minimize any potential risks associated with the compound through thorough evaluation.

FUTURE SCOPE

Overall the future scope of this research entails broadening the research to investigate different facets of the metabolite, including identification as an impurity if present in formulation, optimization of synthesis, validation of analytical methods, assessments of the pharmacokinetic and pharmacodynamics effects, safety profiling, and further development towards clinical applications.

Acknowledgment. The authors are grateful to thank for the JJTU University for providing facilities to complete this research work. We are also thankful to Dr. Jat, Dr. Ashish, Mr. Yogesh, Mr. Bhaskar for kind support with constant motivation towards my research work.

Conflict of interest. None.

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How to cite this article: Pathade P.A., Kaushik A. and Pawar A.Y. (2023). Synthesis, Characterization and Development of HPLC Method for Clopidogrel Metabolite: Computational Approaches for PKPD and Toxicity Predictions. *Biological Forum – An International Journal*, *15*(3): 759-770.