RESEARCH ARTICLE JPAR 2020

Journal of Pharmaceutical Advanced Research

(An International Multidisciplinary Peer Review Open Access monthly Journal)

Available online at: www.jparonline.com

Quantitative estimation of Clopidogrel in Rat Serum using HPLC - UV Technique: Application to Pharmacokinetics Study

Sagar Suman Panda[1]*, Ravi Kumar Venkata Varaha Bera[1], Neelakanta Rao Nagireddy[2]

[1]Department of Pharmaceutical Analysis & Quality Assurance, Roland Institute of Pharmaceutical Sciences, Khodasingi, Berhampur, India.
[2]Suraksha Pharma, Ameerpet, Hyderabad, Telangana, India.

Received: 06.04.2020 Revised: 18.04.2020 Accepted: 24.04.2020 Published: 30.04.2020

ABSTRACT: Background: Clopidogrel bisulphate is a cardiovascular drug that requires simple, economic and reliable bioanalytical method for its quantification in biological fluids. **Aim:** To develop a new and reliable HPLC method for quantitative estimation of Clopidogrel in wistar rat serum. **Method:** The analyte was extracted from Wistar albino rat serum using ethyl acetate as extraction solvent. Afterwards the analyte was separated from the serum matrix using liquid chromatography and quantified for amount of drug present. **Results:** Clopidogrel shows a retention time of 5.8 min, on a C-18 column (250 mm × 4.6 mm, 5µm) using acetonitrile: 0.01M tetrabutyl ammonium hydrogen sulfate (50:50, % v/v) flowing at 1.0 ml/min, with UV detection at 240 nm. The drug was well resoluted from serum components showing method linearity at 50 to 12,800 ng/ml with acceptable accuracy and precision. The drug was estimated from the serum samples with adequate accuracy and precision and revealed a C_{max} of 404.93 ng/ml in 1.5 h after oral administration. **Conclusions:** The newly optimized method was validated for various parameters with values in accordance to federal guidance. The current method was successfully applied for pharmacokinetic study of Clopidogrel.

Corresponding author*
Mr. Sagar Suman Panda,
Asst. Professor
Dept. of PA & QA,
Roland Institute of Pharmaceutical Sciences,
Khodasingi, Berhampur-760010, India.
Tel: +91-9438040646
Mail ID: sagarguddu2002@gmail.com

Keywords: Bioanalysis, Clopidogrel, Liquid chromatography, Liquid-liquid extraction, Pharmacokinetics.

INTRODUCTION:

Clopidogrel bisulphate, is chemically known as Methyl (+)-(S)-α-(2-chlorophenyl)-6, 7-dihydrothieno [3, 2-c] pyridine-5(4H)-acetate sulfate (1:1) (Fig 1) is an antiplatelet agent having multiple therapeutic indications [1]. Literatures revealed an HPLC and few LC-MS methods are reported to estimate Clopidogrel with its active metabolites in biological samples [2-9]. However, application of LC-MS/MS for routine bioanalysis purpose also calls for solving additional challenges of

high expenses, need of skilled analyst, few mobile phase options, variable recoveries, ion suppression and matrix effects [10]. Out of which ion suppression and matrix effects, affect the method sensitivity and ruggedness. HPLC is a better option than LC-MS/MS with regard to affordability, versatility, handling and maintenance during routine quality control and bioanalytical estimation process [11-13]. The above credentials place HPLC in the top of the different bioanalytical techniques used by small and medium research organizations.

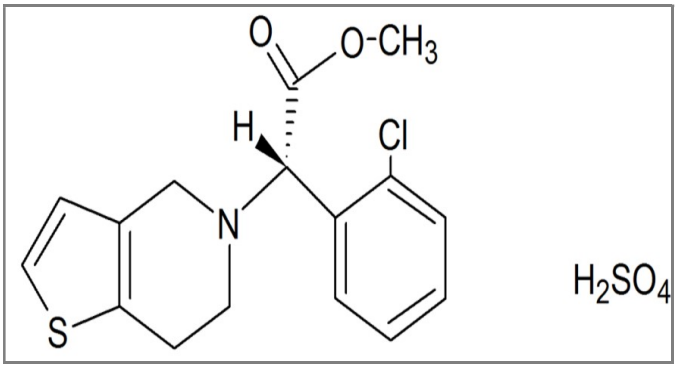

Fig 1. Chemical structure of Clopidogrel bisulphate.

In the reported HPLC method the authors have observed few critical issues like use of complex extraction solvent composition, use of a complex mobile phase utilizing gradient protocol, use of column oven, longer run time and use of internal standard which incurs additional challenges of stability, recovery and resolution with respect to analytes retention position, which calls for developing a new efficient and reliable bioanalytical chromatographic method. In view of all these aspects in this current study the authors tried to resolve the above mentioned issues and took on to develop a new bioanalytical method free of any drawbacks to estimate unchanged Clopidogrel in rat serum. Further, validation studies confirmed method aptness for the intended purpose.

MATERIALS AND METHODS:

Standard Clopidogrel (purity > 98 %) was kindly provided by Zydus Healthcare (Sikkim, India). A melting point test (Veego Instruments Corporation, Mumbai, India) ensured purity of standard drug. HPLC grade acetonitrile (ACN) (Merck Ltd. Mumbai, India) and analytical reagent grade tetrabutyl ammonium hydrogen sulfate (TBAHS) (Himedia Laboratories Pvt. Ltd., Mumbai, India), HPLC grade ethyl acetate (Merck Ltd. Mumbai, India) were used for the purpose. HPLC grade water was prepared by using TKA GenPure Ultra-Purification System, Germany.

Instrumentation:

A Shimadzu LC-10AT and LC-10AT VP series HPLC binary pumps and a SPD 10A UV-visible detector (Shimadzu, Tokyo, Japan) was used for the purpose. A reversed phase column (LiChroCART-LiChrospher 100-C18, Merck, Mumbai, India) (250×4.6 mm, 5 µm) was used for chromatography. The mobile phase contained ACN and 0.01M TBAHS in a composition of 50:50%, v/v flowing at 1.0ml/min, with UV detection at 240nm. Prior use both ACN and TBAHS solutions were ultrasonicated (Enertech Pvt. Ltd., Mumbai, India) for degassing.

Preparation of Stock Solution:

About 50 mg of Clopidogrel was dissolved in mobile phase placed in 25 ml volumetric flask (RANKEM, Mumbai, India). Working standard solutions within 50 to 12,800 ng/ml of analyte were prepared using this solution. Responses at each point (n=3) were used and calibration curve was generated.

Sample Preparation:

Fresh whole blood collection was carried out from the retro orbital area of Wistar albino rats. Afterwards, post clotting at ambient conditions, the centrifugation ((Remi Laboratories Instruments, Mumbai, India) at 2300 rpm for around 11 min helped obtaining the serum content. Until used for bioanalytical sample preparation, the collected serum was frozen (-20 °C). About 50 µl of standard Clopidogrel solution was spiked into 100 µl of serum with subsequent cyclomixing (Remi Laboratories Instruments, Mumbai, India) up to 1 min. Further, 1000 µl of ethyl acetate facilitated analyte liquid-liquid extraction (LLE).

The typical LLE process consisted of cyclomixing for 2 min, followed by 11 min of centrifugation at 2300 rpm (4 °C). The concentrations of 200 ng/ml (Low Quality Control: LQC), 800 ng/ml (Mid Quality Ccontrol: MQC) and 3200 ng/ml (High Quality Control: HQC) were analyzed as per intra and inter-day (n=3) requirements.

Validation Studies [14]:

Linearity:

By placing the responses obtained from each spiked serum sample on x-axis and concentration of Clopidogrel on y-axis, linearity of the present method was found out. The final concentrations were ranging from 50 to 12,800 ng/ml. Further, the regression analysis helped assessing suitability of linearity data.

Accuracy and Precision:

Bioanalytical method accuracy and precision was assessed at three quality control concentration with replicate analysis (n=5). The three concentration levels were 200 ng/ml (low QC: LQC), 800 ng/ml (mid QC: MQC) and 3200 ng/ml (high QC: HQC). The intra and inter-day studies were carried out on same day and different days (n=3), respectively. The method trueness (±15 %) and preciseness (% RSD = ±15 %) was calculated from results of HPLC analysis.

Sensitivity and Selectivity:

Method sensitivity as LOD was determined based on S/N=3 (signal-to-noise). The lowest and highest concentrations in the linearity curve were designated as lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ), respectively. Method selectivity was established using serum samples (n=5) by evaluating possible interferences at the analytes retention time.

Recovery and Stability studies:

A comparison based assessment of amount of Clopidogrel recovered at LLOQ to that of standard Clopidogrel solution was performed. Stability studies *viz.* freeze-thaw, short-term and long-term, were performed for analyte in serum. The study concentrations consisted of LQC, MQC and HQC. The results (% recovery) obtained for stressed and recovered Clopidogrel at the above levels was comparable to initial content of analyte. The freeze thaw study included storing the samples for 24 h at -20 ºC with three subsequent thaw cycles. To examine short term stability ambient thawing of samples at pre-fixed time intervals (0, 4 and 6 h) was done. Long-term stability in serum was analyzed at long distanced time points from initial day of storage i.e. up to 7^{th} and 14^{th} day.

System Suitability:

The chromatographic suitability such as retention time, theoretical plates, resolution and tailing factor were assessed by analysing the samples at LLOQ.

Pharmacokinetics study protocol:

Two (one control and one test) groups of albino rats 8 to 10 weeks of age; were the study subjects. Each group contained 6 Wistar rats (around 200 g each). All the animals were acclimatized and were provided with rat pellet food and water for at least 15 days before experimentation. Food supply was restricted 18 h prior dose administration and supplied with the same post 4 h of dosing. A onetime oral dose of 30 mg/kg of drug was received by the test group. Around 50 ml suspension of Clopidogrel bisulphate containing acacia (0.5 %) and sorbitol (10 %) in demineralized water served the purpose of dosing. Suspension was fed to the animal by a feeding cannula. Around 0.4 ml of blood collected from the retro-orbital space of a rat into eppendorf tubes at various time points 0.5, 0.75, 1.5, 3, 6, 12 and 24 h after dose administration. The pharmacokinetic parameters that are maximum Serum concentration (C_{max}), time of maximum serum concentration (T_{max}), area under the serum concentration-time curve from 0 to the last measurable concentration (AUC_{0-t}), area under the serum-time curve from 0 to infinity ($AUC_{0-\infty}$), elimination rate constant (λ_z) and half-life of drug elimination during the elimination phase ($t_{1/2}$) were estimated using "PK Functions for Microsoft Excel" developed by Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, CA-92606, USA. The overall study protocol was approved by IAEC (Approval no. 25/Chairman IAEC, RIPS, Berhampur-10, IAEC Regd. No.: 926/ab/06/CPCSEA 22.02.2006), RIPS, Berhampur.

RESULTS AND DISCUSSION:

Chromatographic development:

Considering analyte chemistry, an HPLC method utilizing C-18 phase was found befitting for chromatography. The mobile phase contained ACN: 0.01M TBAHS. Various compositions of mobile phase and flow rates such as methanol: water (50:50), ACN: water (50:50) and ACN: 0.01M TBAHS (50:50) at 0.8 and 1.0 ml were tested to check response of Clopidogrel. A composition of ACN: 0.01M TBAHS at 50:50, v/v flowing at 1.0 ml/min, and detection at 240 nm gave optimum peak performance.

Table 1. Accuracy and precision data of the method.

Days	Conc. (ng/ml)	ACR (ng/ml)	Acc (%)	Precision (%RSD***)
Intra-day*	LQC:200	207.21	103.59	1.84
	MQC:800	781.18	98.52	3.86
	HQC:3200	3011.65	94.10	5.23
Inter-day**	LQC:200	193.6	96.74	2.67
	MQC:800	770.3	96.24	3.03
	HQC:3200	3214.3	100.44	1.64

*Average of five determinations at each QC level. **Average of fifteen determinations at each QC level. ***RSD-Relative standard deviation. ACR - Average Concentration recovered and Acc – Accuracy.

The liquid-liquid extracted and reconstituted Clopidogrel was separated at 5.8 min, with a run time of 10 min. Further, validation studies were compliant to federal guidelines.

Bioanalytical Method Validation:

Linearity:

This was proved to be linear over 50 to 12,800 ng/ml for Clopidogrel (R^2= 0.99). Results of regression studies advocated for method aptness for the purpose.

Accuracy and Precision:

Table 1 demonstrates the method trueness and preciseness. Accuracy (intra-day and inter-day) of the method was acceptable (94.10 to 103.59 %). Overall RSD of precision studies was within 6 %.

Sensitivity and Selectivity:

Lower values of LOD (i.e., 38 ng/ml), LLOQ (50 ng/ml) and ULOQ (12,800 ng/ml), affirmed enhanced sensitivity by using HPLC. The chromatograms of blank rat serum (A), standard Clopidogrel (B), rat serum containing Clopidogrel (C) and Clopidogrel in rat serum at C_{max} (D) (Fig 2), depicted uninterferred estimation at the analytes retention time indicating method selectivity. Further, it approves the systematic LLE procedure followed for the purpose.

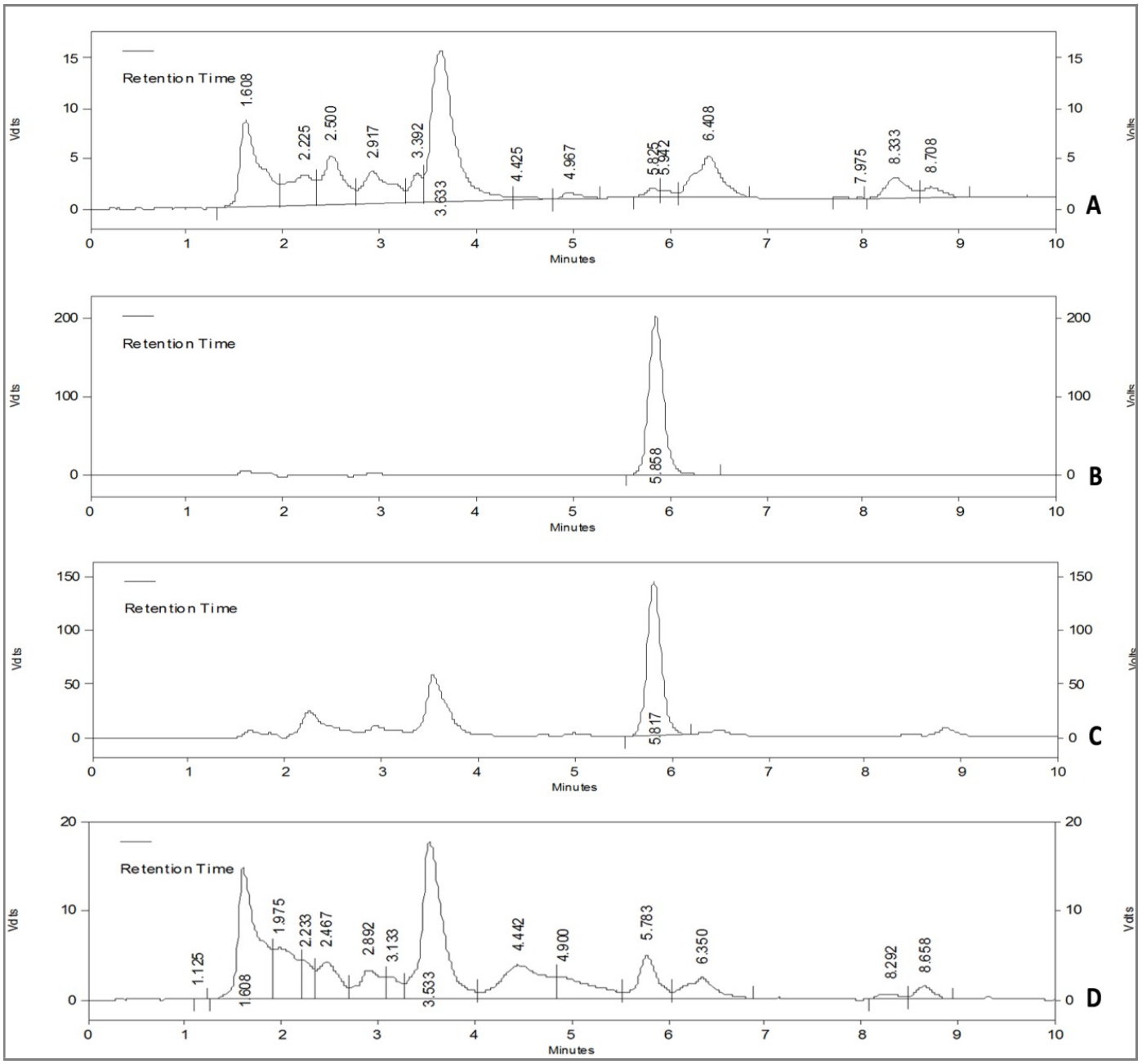

Fig 2. Typical chromatograms representing (A) blank rat serum, (B) standard clopidogrel, (C) rat serum containing clopidogrel and (D) clopidogrel in rat serum at C_{max}.

Recovery and Stability studies:

The recovery of Clopidogrel from serum was found satisfactory (>90 %) at all the four levels i.e. LLOQ, LQC, MQC, and HQC with % RSD values within 5 %. Stability results (Table 2) of Clopidogrel under studied conditions were reasonable and within 20 % deviation from nominal levels.

Table 2. Stability data of Clopidogrel in rat serum.

SS	NC (ng/ml)	AC* ng/ml)	Recovery (%)	RE** (%)
ST (6 h)	LQC:200	195.38	97.69	-2.30
	MQC:800	752.62	94.07	-5.92
	HQC:3200	3088.19	94	-5.99
LT (14^{th} Day)	LQC:200	180.71	90.35	-9.64
	MQC:800	732.09	91.51	-8.48
	HQC:3200	2862.66	89.45	-10.54
FT (3^{rd} cycle)	LQC:200	177.11	88.55	-11.44
	MQC:800	740.67	92.58	-7.41
	HQC:3200	2832.41	88.51	-11.48

***Average of 5 determinations at each QC level. **RE – Relative error. SS - Stability Studied, ST - Short Term, LT - Long Term, FT - Freeze Thaw, NC and AC - Nominal and Average Concentration.**

System Suitability:

The RSD values for retention time (5.8 min), theoretical plates (7195), resolution (2.31) and tailing factor (1.33) were within 2 % indicating system aptness for the study.

Pharmacokinetics of CLP:

The obtained results (Table 3) for various pharmacokinetics parameters concerning amount of unchanged drug present in rat serum served the study intent. The Fig 3 depicts mean serum concentration-time profile of analyte and it proposed a T_{max} and C_{max} value of 1.5 h and 404.93 ng/ml, respectively.

Table 3. Results of pharmacokinetics study.

Parameter	Observed values
C_{max}	404.93 ng/ml
T_{max}	1.5 h
AUC_{0-t}	5036.11 ng.h/ml
$AUC_{0-\infty}$	6455.23 ng.h/ml
λ_z	0.0558 h^{-1}
$t_{1/2}$	12.4 h

CONCLUSIONS:

A new and reliable bioanalytical method was established for estimation of Clopidogrel in rat serum. The new method utilized RP-HPLC technique along with a mobile phase requiring simple preparation. The issues such as use of internal standard, its stability and recovery from serum were well avoided because of optimum recovery of analyte.

Validation studies were pivotal for establishing method appropriacy. A linear and sensitive bioanalytical method free from any drawbacks was developed for estimating Clopidogrel. Method accuracy and precision in both intraday and inter-day pattern produced optimum results advocating method reliability. Further, the stability studies for Clopidogrel under vivid stress conditions indicated stability aspect of Clopidogrel during the study period. Hence, the present method is apt for estimating Clopidogrel in biological matrix. Moreover, the developed bioanalytical method projects it's applicability for routine therapeutic drug monitoring purpose.

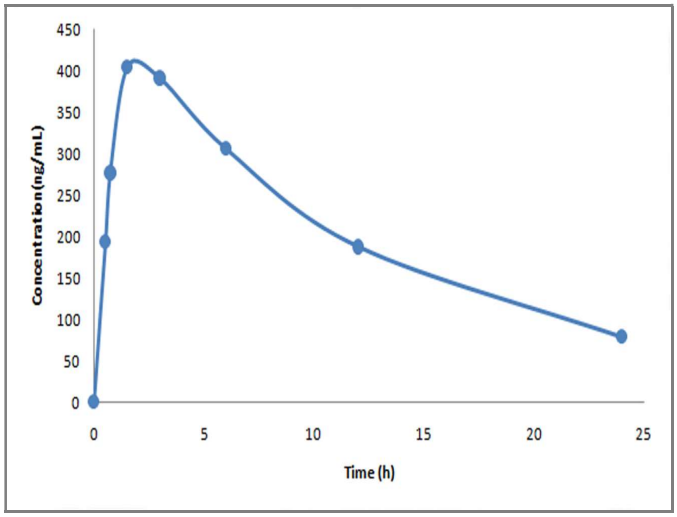

Fig 3. Mean serum concentration-time profile of clopidogrel in wistar rat serum.

ACKNOWLEDGEMENTS:

The authors are thankful to Zydus Healthcare Ltd, India for the gift sample of standard Clopidogrel bisulfate and Principal, Roland Institute of Pharmaceutical Sciences, Khodasingi, Berhampur, India for providing the necessary research facilities.

REFERENCES:

1. O'Neil MJ, editor. The Merck Index. New Jersy (NJ): Merck & Co. Inc; 2006.
2. Singh SS, Sharma K, Barot D, Ram Mohan P, Lohary VB. Estimation of carboxylic acid metabolite of Clopidogrel in wistar rat plasma by HPLC and its application to a pharmacokinetic study. J Chromatogr B, 2005; 821: 173-180.
3. Nirogi RV, Kandikere VN, Shukla M, Mudigonda K, Maurya S, Boosi R. Quantification of

Clopidogrel in human plasma by sensitive liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom, 2006; 20: 1695-1700.
4. Shin BS, Yoo SD. Determination of Clopidogrel in human plasma by liquid chromatography/tandem mass spectrometry: application to a clinical pharmacokinetic study. Biomed Chromatogr, 2007; 21: 883-889.
5. Takahashi M, Pang H, Kawabata K, Farid NA, Kurihara A. Quantitative determination of Clopidogrel active metabolite in human plasma by LC-MS/MS. J Pharm Biomed Anal, 2008; 48: 1219-1224.
6. Elsinghorst PW. Quantitative determination of Clopidogrel and its metabolites in biological samples: a mini-review. J Chromatogr B Analyt Technol Biomed Life Sci, 2013; 917-918: 48-52.
7. Liu G, Dong C, Shen W, Lu X, Zhang M, Gui Y, *et al*. Development and validation of an HPLC-MS/MS method to determinate Clopidogrel in human plasma. Acta Pharm SinB, 2016; 6: 55-63.
8. Robinson A, Hillis J, Neal C, Leary AC. The validation of a bioanalytical method for the determination of Clopidogrel in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci, 2007; 848: 344-354.
9. Javed MK, Iqbal Z, Khan A, Khan A, Shah Y, Ahmad L. Development and validation of HPLC-UV method for the determination of Clopidogrel in pharmaceutical dosage form and human plasma. J Liq Chromatogr Relat Technol, 2011; 34: 2118-2129.
10. Taillon MP, Furtado M, Garofolo F. Challenges of developing a bioanalytical method for a macrolide immunosuppressant compound by LC-MS/MS. Bioanalysis, 2011; 3: 1201-1215.
11. Van de Merbel NC. Is HPLC becoming obsolete for bioanalysis? Chromatographia, 2002; 55: S53-S57.
12. Begum A, Sowjanya Ch, Kumar KS, Ravindra N. Development and validation of RP-HPLC method for determination of citicoline and methylcobalamin in bulk and its pharmaceutical dosage form. J Pharm Adv Res, 2019; 2: 717-722.
13. Pappula N, Padmini LN, Jahnavi M, Navya N, Vasanth P, Ravi Chandra S. Analytical method development and validation of a RP-HPLC method for determination of sofosbuvir in pharmaceutical dosage from. J Pharm Adv Res, 2019; 2: 746-753.
14. Bioanalytical Method Validation Guidance for Industry, US Food and Drug Administration, Rockville, MD 20857. http://academy.gmp-compliance.org/guidemgr/files/4252FNL.Pdf (Accessed April 21, 2020).

Conflict of Interest: None
Source of Funding: Nil
Paper Citation: Panda SS*, Bera RKVV[1], Nagireddy NR[2]. Quantitative estimation of Clopidogrel in Rat Serum using HPLC - UV Techniques: Application to Pharmacokinetics Study. J Pharm Adv Res, 2020; 3(4): 837-842.