Development and Validation of HPLC Method for Determination of Clopidogrel in Human Plasma and Its Application to Pharmacokinetic Study.

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Abstract:

Clopidogrel bisulfate, chemically *S* (+)-2-(2-chlorophenyl)-6,7-dihydrothien [3,2-C]pyridine-5 (4H)- acetic acid methyl ester sulphate is a potent antiplatelet and antithrombotic drug. Chromatography was performed with an analytical Inspire C_{18} column (250 mm x 4.5 mm, 5 µm), Shimadzu HPLC model with HPLC Pump (LC10AD) and UV-Detector SPD 10A, and using Acetonitrile: 0.1% Acetic acid in water (75:25 v/v) as the mobile phase. The average extraction recovery of Clopidogrel from healthy subjects plasma was greater than 92% at a concentration of -----, good linearity of 0.989 in plasma over a concentration range of 50 to 5000ng/ml. Interday and intraday variability was < 10% in plasma. This newly developed HPLC method was applied to the pharmacokinetic study of Clopidogrel after oral administration in cardiac patients.

Keywords: - Clopidogrel, Pharmacokinetics, HPLC.

1. INTRODUCTION:-

Clopidogrel is an inactive pro-drug that requires oxidation to its active thiol metabolite. The active metabolite inhibits platelet aggregation irreversibly by blocking platelet P2Y12 receptors, resulting in reduced Adenosine 5´-diphosphate (ADP)-mediated activation of the glycoprotein GPIIb/IIIa complex. About 85% of clopidogrel is hydrolysed via esterases to an inactive carboxylic acid derivative and only about 15% undergoes hepatic cytochrome P450 (CYP)-catalysed metabolism to a 2-oxoclopidogrel intermediate that is subsequently oxidized to the active metabolite, a thiol derivative of Clopidogrel.[1]

Clopidogrel is used for: Prevention of vascular ischemic events in patients with symptomatic atherosclerosis

For the prevention of thrombosis after placement of intracoronary stent[2] or as an alternative antiplatelet drug for patients who are intolerant to aspirin.[3] for maintainence of vascular events in heart stroke patients

Used as an antiplatlet drug for the cardiac risk patients

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1. There was no method which will of **Clopidogrel** in human plasma by using HPLC-UV instrument.
2. We developed a method and validated using

To our knowledge, no analytical method has been developed and validated to measure the concentration of Clopidogrel in biological samples. Therefore, in this study, we reported the development and validation of a sensitive HPLC assay to quantify Clopidogrel in biological samples, and the use of this assay to characterize the basic pharmacokinetic characteristics of Clopidogrel .

2. EXPERIMENTAL METHODS

2.1. HPLC method development:-

2.1.1. Chemicals and reagents

Clopidogrel and Ritonavir were kindly provided as gift sample from IPCA (Mumbai) and Aurobindo (Hyderabad) respectively. Acetonitrile, Water and Glacial acetic acid were of HPLC grade obtained from Sigma chemicals-Dombivli (Maharashtra).

2.1.2 Instruments

The Shimadzu UV-1800 model was used to determine the absorption maximum (λ_{max}) of Clopidogrel. Shimadzu HPLC model with LC10AD Pump and SPD-10A UV-Detector. The column and HPLC instrument was maintained at room temperature. The reverse phase chromatography was performed with an analytical Inspire C_{18} column (250mm x 4.6 mm, 5 µm).The HPLC detector was set at AUFS of 0.05. Column - Inspire C_{18} (250 x 4.6 mm, 6 *µm)*

Chromatographic Conditions

1. Composition of mobile phase - Acetonitrile : 0.1% Glacial acetic acid in Water
   (75:25 %v/v).
2. Flow rate - 1ml/min,
3. Wavelength- 225nm
4. Volume of injection - 20µL,
5. Retention Time (t_R) - Clopidogrel 13 min,

- Ritonavir – 5.5 min.

2.1.3. Standard solutions

Primary stock solutions of Clopidogrel and Ritonavir (Internal standard) was prepared in acetonitrile at a concentration of 1 mg/ml and stored at -80^0 C until use.

2.1.4. Standard graph procedure

1. Primary stock solution of Ritonavir was diluted with methanol to obtain the working solution of 100 µg/ml concentration.
2. Primary stock solution of Clopidogrel was diluted with methanol to obtain the working solution of 0.125, 0.250, 0.5, 1.25, 2.5, 5, 12.5, 25 and 37.5 µg/ml concentrations.
3. To 100 µL of plasma samples,20µL of 0.1N HCl, 20 µL of internal standard from 100 µg/ml of working solution was added to obtain 20 µg/ml final concentrations and 20 µL of Clopidogrel was added from each concentration to obtain 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 7.5 µg/ml concentrations of Ritonavir and 20 µL 0.1N of HCl was added
4. The resultant solution was mixed for 2 minutes on cyclomixer at room temperature, and 500 µL of methanol was added and centrifuged at 4000 rpm for 10 min.
5. The supernatant was collected and kept for evaporated to dryness on water bath, the residue was dissolved in 200 µL of methanol and after filtration through 0.2 µm syringe filter, 20 µL of the solution was spiked for the HPLC analysis.
6. The peak area of the drug and internal standard was determined and the peak area ratio was calculated.

   Peak area ratio = peak area of drug / peak area of internal standard
7. Graph was plotted by using concentration on X-axis and peak area ratio on Y-axis.
8. The standard graph was considered to be significant when the r^2 value is ≥ 0.99.

2.1.5 Samples

To 100 µL of plasma sample, 20 µL of internal standard from 2 µg/ml of working solution and 20 µL 0.1N of HCl was added. and the proteins are precipitated by adding 400 µL of acetonitrile, the resultant solution was mixed for 2 minutes on vertex shaker at room temperature, and

centrifuged at 4000 rpm for 15min and the supernatant was evaporated to dryness, the residue was dissolved in 200 µL of mobile phase and after filtration through 0.2 µ syringe filter, 20 µL of the solution was used for the HPLC analysis.

2.2 HPLC method validation.

2.2.1. Specificity and selectivity

The chromatographic interference from endogenous compounds was assessed by comparing chromatograms of blank plasma, with that of the samples spiked with Clopidogrel and IS.

2.2.2. Sensitivity

The lowest limit of quantification (LLOQ) was determined as the minimum concentration that could be accurately and precisely quantified with the relative standard deviation of < ± 10%. The lowest limit of detection (LLOD) was defined as the amount that could be detected with a signal-to-noise ratio of 4.

2.2.3 Linearity

Calibration curves of ten concentrations of Clopidogrel ranging from 10 to 10000 ng/ml were used. Blank samples were analyzed to confirm the absence of interferences. Calibration curves were plotted by taking Peak Area Ratio of Clopidogrel/ Ritonavir on Y-axis and Concentration of corresponding values on X-axis. The minimally acceptable correlation coefficient (r^2) for the calibration curve was 0.989 or greater.

2.2.4 Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy for the assay, Clopidogrel samples at low, medium and high concentrations (10,500,10000 ng/ml,) were prepared as described above. The intra-day precision of the assay was assessed by calculating the coefficient of variation (CV) for the analysis of samples in three replicates. And inter-day precision was determined by the analysis of samples on three consecutive days. Accuracy was calculated by comparing the measured values to the true values and was expressed in percent. The precision was accepted when the coefficient of variance for each concentration doesn't exceed ± 10, and

accuracy was accepted when the averaged values are > 95% of true concentration except for the LLOQ where the limit was > 92%.

2.2.5 Recovery

After determination of LLOQ from the standard samples, plasma samples (0.1ml/sample) (n=3) were spiked with known amounts of Clopidogrel to yield a final concentration of 50, 500 and 5000 ng/ml, and 100 µL of Internal standard from 2 µg/ml was added to obtain a resultant solution of 10 µg/ml. The spiked samples were extracted following the above described extraction procedures.

The extraction recovery (ER) was calculated using the formula :

ER%= (Peak area of extracted samples/peak area unextracted samples)×100%.

2.2.6 Stability

Stability of Clopidogrel in Plasma was analyzed at room temperature for 4h. Three freeze-thaw cycles (-80^{0}C/room temperature). Freezing stability of Clopidogrel was assessed by analyzing QC samples stored at -80^{0}C for one month. The peak area of Clopidogrel in plasma at initial condition was used as reference to determine the relative stability of Clopidogrel in experiments described above.

2.3 Pharmacokinetics of Clopidogrel in human subjects

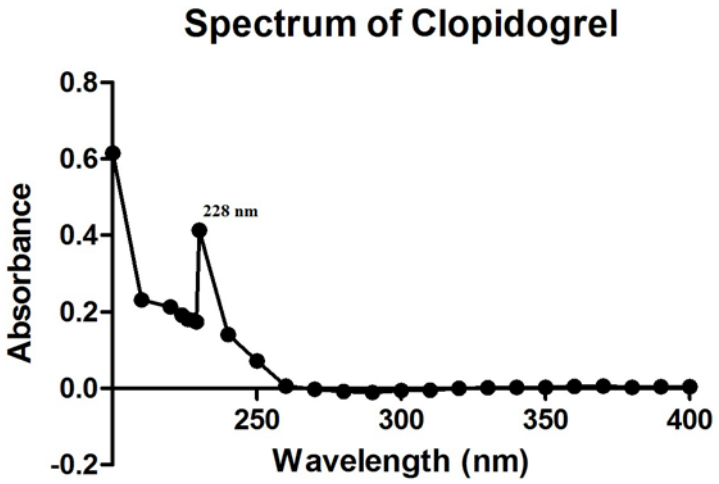
Human subjects 30 patients were selected based on the specificity of the study. Selection was done after Informed consent was taken from each cardiac patient of Mahatma Gandhi Memorial Hospital (MGMH), Warangal. The study was approved by the Human ethical committee of MGMH and Kakatiya Medical College Board Members. Clopidogrel was given to each patient at a dose of 75 mg/kg oral dose which was prescribed by doctor. Blood samples (3ml) were collected at 0, 1, 2, 4, 8 & 12.Plasma was separated immediately by centrifugation and stored at -80^{0}C until analysis.

At the time of analysis the plasma samples are subjected to extraction procedure as stated above and the concentration of the drug in it was determined by using the calibration curve of plasma. The obtained plasma concentration data of Clopidogrel was analyzed to obtain the appropriate pharmacokinetic parameters by applying Two-compartmental open model using Kinetica 5.0 software (Kent scientific).

3. RESULTS AND DISCUSSION

3.1 Method development

The UV-Vis absorbance of Clopidogrel was scanned from wavelength of 200-400 nm on a Shimadzu UV-Vis spectrophotometer (UV 1800). And maximum absorbance was at wavelength of 228 nm in acetonitrile (Fig-1) therefore, wavelength of 228 nm was chosen for HPLC-UV detection in this assay. The mobile phase used for the assay was of very simple and achieved optimal separation of Clopidogrel and I.S. Retinovir without interference from the other components in blank plasma samples (fig-4).The flow rate was selected as 1 ml/min.



Blank Plasma Graph

Figure-1, showing maximum absorbance of Clopidogrel (20 µg/ml) in acetonitrile from range of 200-400nm

3.2. HPLC method validation

3.2.1. Specificity and selectivity

Fig.2 and 3 represents chromatograms of Clopidogrel and I.S. (Ritonasvir) from plasma of cardiac patients after extraction as stated above. No interference of endogenous peaks with Clopidogrel or Retinovir at their respective retention times (Retinovir t_R= 4.85 min, Clopidogrel t_R = 13.03 min) in blank cardiac patients plasma (fig-2)

3.2.2. Sensitivity

The LLOQ of Clopidogrel was found to be 0.05 µg/ml. The LLOD was found to be 0.02 µg/ml. The mean percent accuracy value for plasma samples was 84.13 % and coefficient of variation was below 12% at the LLOQ.

3.2.3 Linearity of calibration curve

The calibration curves of Clopidogrel were linear over the different concentration range in plasma. The correlation coefficient was 0.989

3.2.4 Precision and accuracy

Table-1 shows a summary of intra- and inter-day precision and accuracy. Intra- day accuracy of 10, 100 and 1000 ng/ml was found to be 84.13, 89.60 and 97.32 respectively and inter- day accuracy was found to be 83.0, 89.50 and 94.47 respectively. Therefore, the intra- and inter- day accuracies (% deviation) were within < ±10% for the LLOQ. The intra- and inter-day assay precision (CV) ranged from 11.10 to 171 and 5.52 to 3.34 % respectively. These results indicated that the present assay has good accuracy and precision.

4. CONCLUSION:

A simple, sensitive, accurate and precise HPLC method was developed and validated for the first time to quantify Clopidogrel in healthy subjects plasma. The present method was applied successfully to the pharmacokinetic study of Clopidogrel in cardiac patients, in which all pharmacokinetic parameters were determined. The sample preparation method and the chromatographic condition in the present method will likely facilitate the quantification of Clopidogrel. The final developed method will be used for the study of Popk study of Clopidogrel in South Indian Population.

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Table No.1 Shows intra- and inter-day precision and accuracy

	Trail 1	*Trail 2*	*Trail 3*	*Mean*	*SD*	*Accuracy %*	*CV %*
Intraday							
10	7.40	8.60	9.24	8.41	0.93	84.13	11.10
100	86.40	92.80	89.60	89.60	3.20	89.60	3.57
1000	959.20	968.90	991.60	973.23	16.63	97.32	1.71
Interday							
10	8.8	7.9	8.2	8.30	0.46	83.00	5.52
100	82.5	98.2	87.8	89.50	7.99	89.50	8.92
1000	907.8	958.2	968.2	944.73	32.37	94.47	3.43

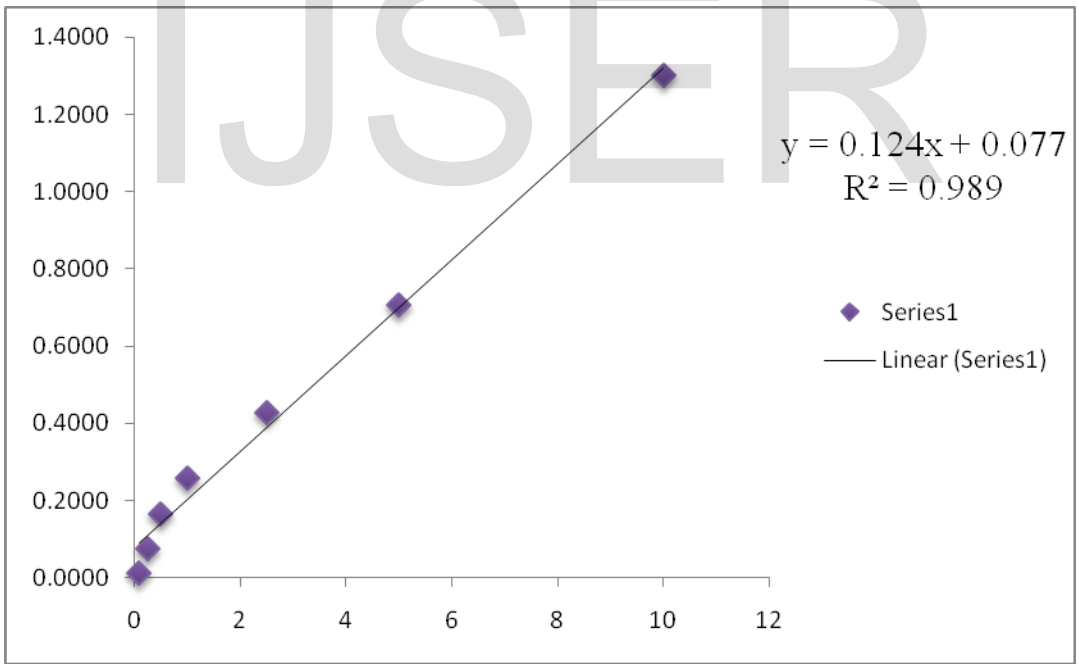

Figure-2,Standard Graph

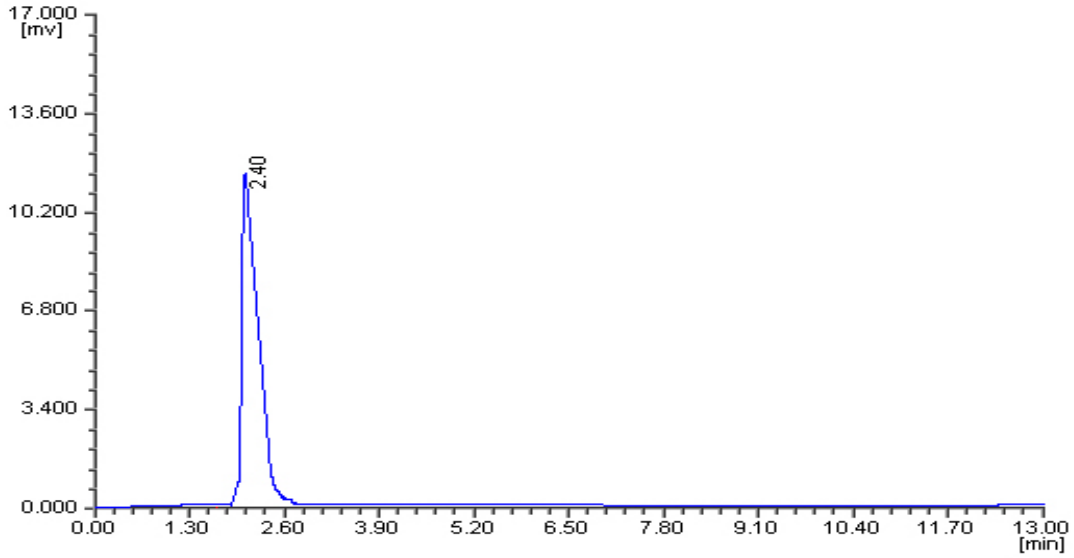

Figure-3, showing blank plasma

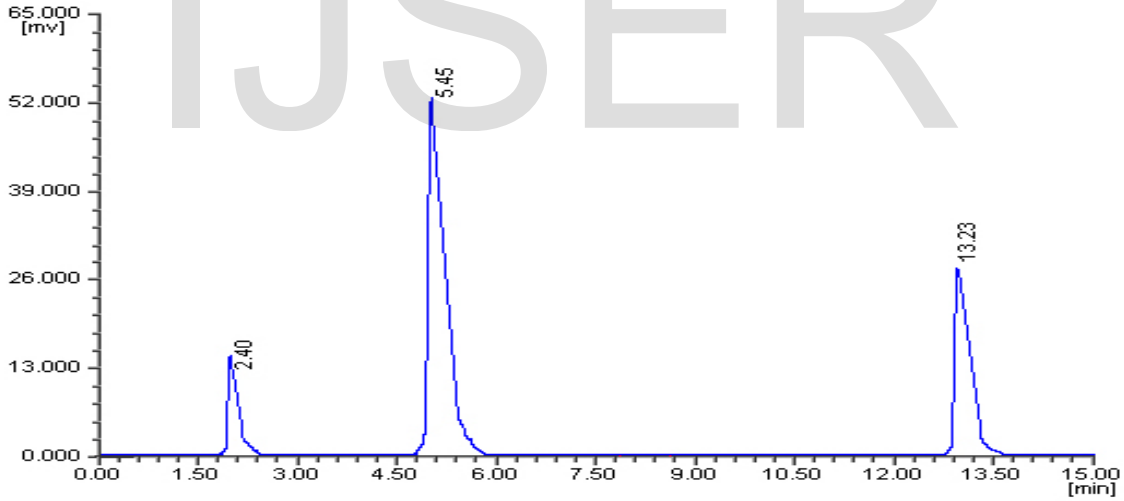

Figure-4, showing Clopidogrel (5µg/ml) and Internal standard (10µg/ml) spiked in healthy subjects plasma.