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# High performance liquid chromatography mass spectrometric (LC-MS/MS) method for the estimation of clopidogrel bisulfate in human plasma by liquid-liquid extraction technique

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## ABSTRACT

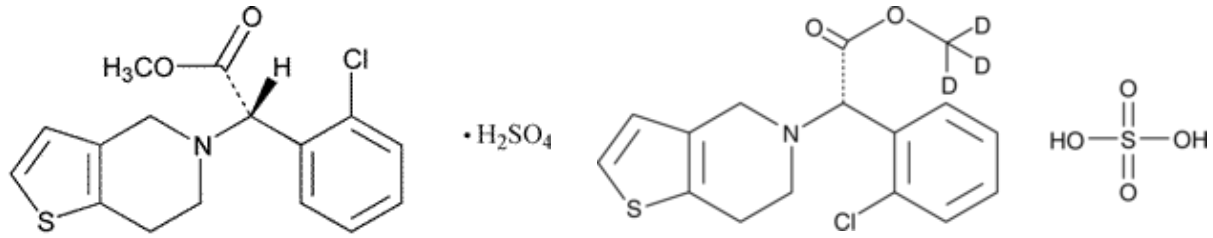
A high performance liquid chromatography mass spectrometric method for the estimation of clopidogrel (CLP), in human plasma in positive ion mode was developed and validated using clopidogrel D3 (CLPD) as internal standard (IS). Sample preparation was accomplished by liquid-liquid extraction technique. The reconstituted samples were chromatographed on Kromasil 100-5C18, 100×4.6 mm, 5µm column using a mobile phase consisting of HPLC grade Acetonitrile: Milli Q/HPLC grade water (90:10, v/v). The method was validated over a concentration range of0.0101 to 5.0315 ng/mL for CLP. This validation report provides the results of selectivity, matrix effect, sensitivity determinations, calibration standards and quality control samples data, precision and accuracy data, the results of recovery, various stabilities, run size evaluation and dilution integrity along with all pertinent supporting documentation.

**Keywords:** LC-MS/MS, Clopidogrel Bisulfate, Clopidogrel D3, Human plasma.

## INTRODUCTION

Analyticalmethodsdevelopmentandvalidationplayimportantrolesinthediscovery,development,andmanufactureofpharmaceuticals. Liquid chromatography accompanied with mass specgtrometry plays an immense role in contemporary analytical methods because of its sensitivity and accuracy [1]. Clopidogrel (CLP) (CAS 120202-66-6; (+)-(*S*)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)acetate) is an oral, thienopyridine class antiplatelet agent used to inhibit blood clots in coronary artery disease, peripheral vascular disease, and cerebrovascular disease (Fig. 1a). Its bisulfate salt has molecular weight 419.9. Clopidogrel D3 Hydrogen Sulfate (CLPD) has molecular weight of 422.93 is a white to off-white powder (Fig. 1b). Both are feely soluble in methanol and dimethyl sulfoxide. CLP is a pro-drug activated in the liver by cytochrome P450 enzymes, including CYP2C19 [2]. CLP requires transformation into an active metabolite by cytochrome P-450 (CYP) enzymes for its

antiplatelet effect. The genes encoding CYP enzymes are polymorphic, with common alleles conferring reduced function [3].




**a** **b**

**Fig. 1: Structure of (a) clopidogrel bisulfate, and (b) clopidogrel D3 hydrogen sulfate**

Several analytical methods including normal phase liquid chromatography (NP-LC) [4], LC/MS/MS [5-8], rapid ultra-high performance liquid chromatography-tandem mass spectrometry (uHPLC-MS/MS) [9]. A high throughput liquid chromatography electrospray ionization tandem mass spectrometric (LC.ESI-MS—MS) methods were developed for the simultaneous estimation of CLP (SR25990C) and its carboxylic acid metabolite (SR26334) in human plasma using glimepiride as internal standard [10-13]. The proposed research aims to develop and validate a High Performance Liquid Chromatography Mass Spectrometric (LC-MS/MS) method for the estimation of CLP in positive ion mode in human plasma using CLPD as internal standard. Liquid-liquid extraction method was employed to isolate the sample from human plasma.

## MATERIALS AND METHODS

### Materials and Reagents

Acetonitrile (HPLC grade), water (HPLC grade), ammonium formate, Dimethyl sulfoxide, formic acid were purchased from Merck, Germany.

### Working standards

Clopidogrel bisulfate (99.36% purity) and clopidogrel D3 hydrogen sulfate (99.9% purity) were gratis samples from Sanofi S.A., France.

### Instrumentation

HPLC system (Shimadzu BE-LC-11), API 4000 LC-MS/MS system (BE-MS-05), Freezing centrifuge (Function Line Heraeus, Germany), Millipore water purifier (India).

### Stock solutions

### CLP stock solution

Weighed accurately, about 10 mg of CLP bisulphate working standard and transferred to a 10 mL clean glass volumetric flask, dissolved in dimethyl sulfoxide and made up the volume with the same to produce a solution of 1 mg/mL. Corrected the above concentration of CLP solution accounting for its potency and the actual amount weighed. The stock solutions were diluted to suitable concentrations using a mixture of acetonitrile and Milli Q water (Diluent) in the ratio of (60:40 v/v) for spiking into plasma to obtain calibration curve (CC) standards, quality control (QC) samples and DIQC samples. All other final dilutions (system suitability dilutions, aqueous mixture, etc.) were also prepared in mobile phase.

### CLPD stock solution (internal standard)

Weighed accurately, about 10 mg of CLPD hydrogen sulfate transferred to a 10 mL volumetric flask, dissolved in dimethyl sulfoxide and made up the volume with the same to produce a solution of 1 mg/mL. The above concentrations were corrected CLPD accounting for its molecular weight, potency and the actual amount weighed. The stock solution was stored in refrigerator 2-8°C and used for maximum of six days. The stock solution was diluted to suitable concentration using diluent for internal standard dilution.

### Biological matrix

Eighteen lots of $K_2$ -EDTA human plasma including one hemolytic and one lipemic plasma were screened for selectivity test. All 18 human plasma lots including hemolytic and lipemicplasma

were found free of any significant interference for CLP and internal standard.

### Calibration curve standards and quality control samples

Calibration curve standard consisting of a set of 12 non-zero concentrations ranging from 0.0101 ng/mL to 5.0315 ng/mL of CLP were prepared. Prepared quality control samples consisted of concentrations of 0.0104 ng/mL (LLOQ QC), 0.0302 ng/mL (LQC), 1.1080 ng/mL (MQC1), 2.5180 ng/mL (MQC2), and 4.5290 ng/mL (HQC) for CLP. These samples were stored at –70°C until use.

## SOLUTIONS

### Reconstitution buffer (w/v)

About 126 mg of ammonium formate was transferred to a 1000 mL reagent bottle and made upto the mark with Milli Q water/HPLC grade water. To this 1.0 mL of formic acid was added and mixed well and sonicated in an ultrasonicator for 5 min.

### Mobile phase (90:10, v/v)

A 100 mL of Milli Q water/HPLC grade water was transferred to a 1000 mL reagent bottle and 900 mL of HPLC grade acetonitrile was added to it. It was mixed well, sonicated in an ultrasonicator for 5 min.

### Diluent and rinsing solution (v/v)

A mixture of HPLC grade acetonitrile and Milli Q water/HPLC grade water was prepared in the volume ratio of 50:50 as diluent. It was then sonicated in an ultrasonicator for 5 min.

### Reconstitution solution (v/v)

A mixture of HPLC grade acetonitrile and reconstitution buffer was prepared in the volume ratio of 90:10 as reconstitution solution. It was then sonicated in an ultrasonicator for 5 min. A batch number was provided and the 'Solution Preparation' form was completed. The reconstitution solution was stored at room temperature (20±5 °C) and used within 7 days from the date of preparation.

### System suitability solution

A mixture of analyte and internal standard was prepared for system suitability test. The concentration of analyte corresponds to middle concentration of calibration range (2.5132 ng/mL for CLP) and that of internal standard corresponded to working concentration used for spiking (2.9974 ng/mL for CLPD). The system suitability test solution was injected as an aqueous mixture. Aqueous samples are prepared as per recovery basis. A 50 µL of analyte(50.2639 ng/mL) and 100 µL of working concentration of internal standard (29.9740 ng/mL) were mixed with 850 µL of mobile phase to prepare the system suitability sample.

## BIO ANALYTICAL CONDITIONS

### Chromatographic conditions

Column: Kromasil 100-5C18, 100×4.6 mm, 5µm; Mobile phase: HPLC grade Acetonitrile: Milli Q/HPLC grade Water (90:10, v/v); Rinsing solution: HPLC grade Acetonitrile: Milli Q/HPLC grade Water (50:50, v/v); Flow rate: 1.000 mL/min; Split ratio: 50:50; Sample cooler temp.:10°C; Column oven temp.: N/AP; Injection volume: 20 µL; Needle rinsing volume: 1000 µL; Rinsing mode: Before and after aspiration; Retention time: CLP 2.20 ± 0.3 min, CLPD 2.20 ± 0.3 min; and Run time : 3.50 min.

### State file information (API 4000 in positive ion mode)

The samples were detected in Positive ion mode (API 4000). The detection for CLP m/z – 322.00 (parent) and 212.00 (product) and that of CLPD m/z – 326.00 (parent) and 215.10 (product). The state file parameters may be modified to optimize the response and the State file parameters 0.5 units may change from instrument to instrument.

### Sample preparation

The samples were thawed at room temperature and vortexed to ensure complete mixing of the contents. 500 µL of the plasma sample was pipetted into glass stoppered tubes, 50 µL of 30 ng/mL CLPD dilution was added to it and vortexed, except in blank plasma samples where 50 µL diluent was added and vortexed. A 5 mL of methyl tert butyl ether was added and shaken for 20 min on

reciprocating shaker at 200 rpm. Samples were centrifuged at 4000 rpm for 10 min at 4 °C. Then supernatant organic layer (4.0 mL) was transferred to prelabelled glass dry test tubes and evaporated to dryness under gentle stream of nitrogen at 40 °C. The samples were reconstituted in 500 µL of mobile phase and injected into the LC-MS/MS system.

### Validation of the assay method

The following experimental design is drawn in order to prove the test method is capable to yield consistent, reliable and reproducible results within the pre-determined acceptance limits. Acceptance criteria for the validation parameters are specified in individual experimental design. Observations and results were recorded in individual method validation data sheets. The parameters such as selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery and stabilities have been validated.

The precision of the assay was measured by the percent coefficient of variation over the concentrations of LLOQ QC, LQC, MQC1, MQC2, and HQC samples during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low, middle and high quality control samples to their respective nominal values, expressed in percentage.

Recovery studies were conducted by processing 18 blank plasma samples and spiked with six sets each of (25 µL of drug and 50 µL of IS) LQC, MQC2 and HQC final dilutions along with internal standard, which represent 100% extraction of analyte and internal standard (non-extracted samples). Six sets of LQC, MQC2 and HQC samples were processed and injected (extracted samples). The extracted samples of CLP were compared with the non-extracted samples of LQC, MQC2 and HQC. The extracted samples of internal standard were compared with the response of internal standard in the entire non-extracted eighteen quality control sample.

Dilution Integrity for 24 sets of dilution samples were prepared by spiking 1.70 time's of highest standard concentration (8.5548 ng/mL). Six sets of dilution integrity samples were processed by diluting them twice and another six sets were processed by diluting them four times. These quality control samples were analysed along with a processed calibration curve standards (undiluted) of concentration range equivalent to that used for the calculation of precision and accuracy (PA-2). The quality control sample concentrations were calculated using appropriate dilution factor. Ruggedness was performed for one precision and accuracy batch (PA-3) and analyzed using different column of same make and different solutions.

## STABILITY STUDIES

Room temperature (20 ± 5 °C) spiking solution stability was carried out at 13 h for CLP and CLPD (Internal Standard) by injecting six replicates of prepared stock dilutions of CLP equivalent to final MQC2 quality control concentration and CLPD at final working concentration from stock solution. Comparison of the mean area response of stability samples of CLP and CLPD at 13 h was carried out against the comparison samples (freshly prepared) [14].

Refrigerated stock solution stability of CLP was carried out by injecting six replicates of stock dilution at final MQC2 concentration level. The stock solutions were found to be stable for 5 days.

The stability of clopidogrelin human plasma was determined during four freeze-thaw cycles. Six replicates, each of LQC and HQC were analysed after four freeze-thaw cycles. The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for the calculation of precision and accuracy.

Bench Top Stability of CLP using six sets each of LQC and HQC was determined at 11 h. The quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for calculation of precision and accuracy.

In assessing the auto sampler stability of CLP, six sets of QC samples (LQC and HQC) were processed and placed in the auto sampler. These samples were injected after a period of 52 h and were quantified against freshly spiked calibration curve standards of concentration range equivalent to that used for calculation of precision and accuracy.

Stability in whole blood was studied by spking 12 mL each with CLP at a concentration of LQC (0.0303 ng/mL) and HQC (4.5299 ng/mL) level in pre-labelled tubes. Then the prepared samples was

divided in to two aliquots (6 mL each), one was kept at room temperature and the another aliquot was centrifuged at 4000 rpm, 10 min at 4°C and plasma was separated. After 3 h the whole blood samples kept at room temperature was centrifuged at 4000 rpm 10 min at 4°C and plasma was separated. The six replicates of LQC and HQC level samples of plasma from each was processed as per the extraction procedure described earlier and compare the response ratio of samples kept at room temperature for a period of minimum 3 h with the fresh (Immediately centrifuged) samples. Stability duration was calculated as the start time of aliquot one samples centrifugation, less than start time of aliquot two samples centrifugation [14].

## RESULTS AND DISCUSSION

Representative chromatograms of extracted blank plasma from the batches of plasma screened are given in Fig. 2 there was no significant interference from endogenous components observed at the mass transitions of CLP and internal standard showing that the method is selective. The lowest limit of reliable quantification for CLP in human plasma was set at the concentration of the LLOQ, 0.0101 ng/mL. The precision and accuracy for CLP at this concentration was found to be 1.84 % and 100.50 % which shows that the method is sensitive.

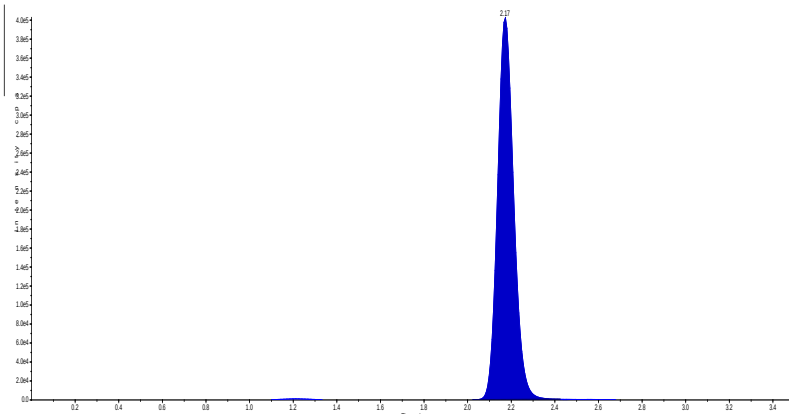



**Fig. 2: A representative chromatogram of blank plasma sample of CLP**

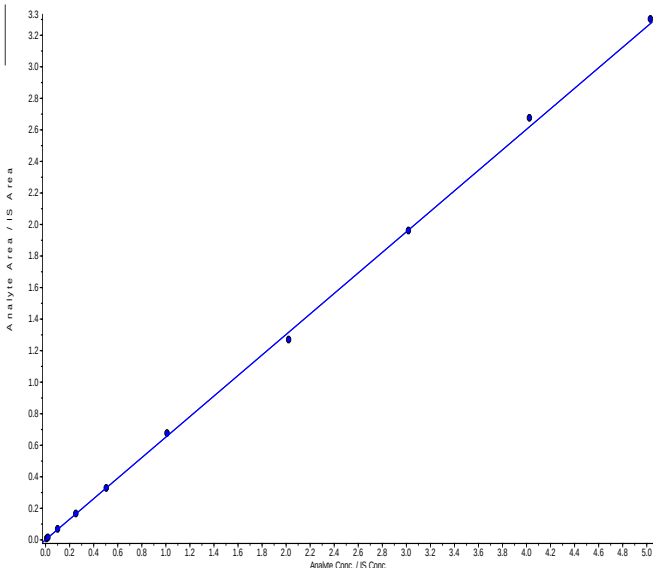



**Fig. 3: Calibration curve for regression analysis of CLP**

No significant matrix effect was observed in all the fourteen and high (HQC) concentrations. The precision for IS normalized matrix factor at LQC and HQC level was found to be 1.20% and 0.48%, respectively and IS normalized factor was 0.982 for LQC and 0.996 for HQC.

A regression equation with a weighting factor of 1/(concentration ratio) $^{2}$ of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for CLP in human plasma. The representative calibration curve for regression analysis is illustrated in Fig. 3. Correlation coefficient (r) was greater than 0.99 in the concentration range of 0.0101 ng/mL to 5.0315 ng/mL for CLP.

Within-batch precision for LLOQ QC, LQC, MQC1, MQC2, and HQC ranged from 1.12% to 2.81%, 1.67% to 2.83%, 0.55% to 0.96%, 0.38% to 0.44%, and 0.32% to 0.64%, respectively.Within-batch accuracy for LLOQ QC, LQC, MQC1, MQC2, and HQC ranged from 96.79% to 100.16%, 96.25% to 99.72%, 93.32% to 94.36%, 93.52% to 94.61% and 92.88% to 93.64%, respectively.

Intra-day precision for LLOQ QC, LQC, MQC1, MQC2, and HQC was 2.53%, 2.52%, 0.94%, 0.64% and 0.42%, respectively. Intra-day accuracy for LLOQ QC, LQC, MQC1, MQC2, and HQC was 97.36%, 96.96%, 93.84%, 93.98%, and 93.12%, respectively. Inter day or between batch precision for LLOQ QC, LQC, MQC1, MQC2, and HQC was 2.52%, 2.60%, 0.94%, 0.65%, and 0.55%, respectively.

Between batch accuracy for LLOQ QC, LQC, MQC1, MQC2, and HQC was 98.29%, 97.88%, 93.98%, 94.19%, and 93.29%, respectively. Within-batch precision and accuracy for stabilities (Freshly Spiked QC's) of LQC, MQC1, MQC2, and HQC was 7.83%, 4.23%, 0.84%, and 2.47%, respectively. Within batch accuracy for LQC, MQC1, MQC2, and HQC was 108.97%, 88.97%, 93.60% and 89.48%, respectively.

Recovery studies showed that the mean overall recovery of CLP was 50.27% with a precision range of 0.26% to 7.33%. The mean recovery of internal standard CLPD was 52.56% with a precision ranging from 1.39% to 4.56%.

Dilution integrity test for CLP results demonstrate acceptable dilution integrity for two and four time's dilution. CLP precision and accuracy, for a dilution factor of 2 was 2.01% and 97.47%, respectively. Similarly, CLP precision and accuracy, for a dilution factor of 4 was 0.61% and 93.75%, respectively.

Within batch precision for LLOQ QC, LQC, MQC1, MQC2, and HQC was 2.81%, 2.83%, 0.55%, 0.44%, and 0.36%, respectively. Within batch accuracy for LLOQ QC, LQC, MQC1, MQC2, and HQC was 97.92%, 97.68%, 94.36%, 94.43% and 93.36%, respectively indicated that the method is rugged.

## STABILITY STUDIES

Stability studies showed that the precision of room temperature (20 ± 5 °C) stock solution stability of CLP at 0 h and 13 h 4.08% to 9.23%, respectively and percentage of stability was found to be 99.88%. The results CLPD at 0 h and 13 h was 4.38% to 9.93%, respectively and percentage of stability was found to be 101.50%. Refrigerated stock solution stability of CLP showed the precision ranged from 0.27% to 0.42%% and percentage of stability was found to be 100.73%. Refrigerated stock solution stability of CLPD was carried out by injecting six replicates of stock dilution at final working concentration level. The stock solutions were found to be stable for 5 days. The precision ranged from 0.29% to 0.53% and percentage of stability was found to be 100.94%.

The stability of CLPin human plasma showed the percent nominal ranged from 91.22% to 102.65% and the precision ranged from 2.25% to 5.99% for four freeze thaw cycles. Bench top stability of CLP was found to be stable up to 11 h. The percent nominal ranged from 90.96% to 103.59% and the precision ranged from 3.09% to 5.67%. Auto sampler stability results demonstrate that the processed samples were stable for 52 h. The percent nominal at 52 h ranged from 92.10% to 104.53% and precision ranged from 3.89% to 5.17%.

Stability of CLP in whole bood demonstrates that the whole blood samples were stable for 3 h at room temperature (20±5 °C). The percent stability at LQC level was found to be 100.00% with the precision range of 1.27% to 1.44% and at HQC level was found to be 99.65% with the precision range of 0.21% to 0.54% for 3 h.

## CONCLUSIONS

HPLC mass spectrometric method for the estimation of CLP, in human plasma in positive ion mode was successfully developed and validated using CLPD as internal standard (IS). Sample preparation was accomplished by liquid-liquid extraction technique. The reconstituted samples were chromatographed on Kromasil 100-5C18, 100×4.6 mm, 5µm column using a mobile phase consisting of HPLC grade Acetonitrile: water (90:10, v/v). The method was validated over a concentration range of 0.0101 ng/mL to 5.0315 ng/mL for CLP. This validation report provides the results of selectivity, matrix effect, sensitivity determinations, calibration standards and quality control samples data, precision and accuracy data, the results of recovery, various stabilities, run size evaluation and dilution integrity along with all pertinent supporting documentation. The developed method could be employed for the determination of CLP in human plasma.

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