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A RAPID AND RUGGED BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF CLOPIDOGREL IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY/ TANDEM MASS SPECTROMETRY

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ABSTRACT

A simple, sensitive and rugged quantitative method for the determination of Clopidogrel in human plasma (K_2EDTA) using liquid chromatography-tandem mass spectrometric (LC-MS/MS) method has been developed and validated. Clopidogrel-d_3 was used as an internal standard. Analyte and the internal standards were extracted from human plasma by liquid-liquid extraction technique using Methyl tertiary butyl ether as extraction solvent and 0.5% formic acid as extraction buffer. The reconstituted samples were chromatographed on a C18 column by using acetonitrile / 5mM ammonium acetate (90/10, V/V) as the mobile phase. The method was validated over the concentration range of 101.98–61028.96 pg/mL. The Quattro Premier XE mass spectrometry was operated under the multiple reaction-monitoring mode (MRM) using the electrospray ionization technique for quantification of ion transitions at m/z 322.13/212.04 and 326.06/215.04 for the drug and the internal standard respectively. The results of the intra and inter batch precision and accuracy studies were well within the acceptable limits. The method has been proved to be simple, sensitive, fast, reliable, rugged and reproducible. A run time of 2.50 min for each sample made it possible to analyze more than 400 plasma samples per day. The proposed method can be applied for the estimation of the drug in real time plasma samples for pharmacokinetic studies.

Key words: Clopidogrel, Validation, Human Plasma, LC-MS/MS, Electrospray ionization.

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INTRODUCTION

Clopidogrel acts as anti-coagulant by inhibition of ADP-induced platelet aggregation, Clopidogrel helps in reduction of atherosclerotic events in patients with atherosclerosis. Clopidogrel [(+)-(*S*)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)acetate] (Figur 1 a & b) is a thienopyridine derivative structurally related to Ticlodipine. Clopidogrel is a prodrug undergoing extensive hepatic biotransformation and converted to active thiol metabolite and an inactive carboxylic acid derivative[1]. Clopidogrel was marketed worldwide as Plavix®/Iscover® ((Sanofi-Aventis, Paris, France).

1(a) **1(b)**

Figure1:Chemical structures of Clopidogrel (1a) and Clopidogrel-d_3 (1b)

For the determination of Clopidogrel in human plasma some methods, Lagorce *et al*., reported a GC–MS method for the analysis of the inactive carboxylic acid metabolite of Clopidogrel in human plasma and serum with a lower limit of quantification (LLOQ) of 5.00 ng/mL[2]. Caplain *et al.*, quantified the inactive metabolite in human plasma and achieved an LLOQ of 25.00 ng/mL using HPLC-UV and an LLOQ of 1.00 ng/mL using GC–MS[3]. Mitakos *et al*,. Proposed an LC–MS method for the determination of inactive metabolite in human plasma with an LLOQ of 100.00 ng/mL[4]. Taubert *et al*., Used LC–MS/MS to analyze unchanged Clopidogrel and its inactive carboxylic acid metabolite in human plasma over the range of 0.50 – 100 ng/mL and 0.50–150.00 µg/mL, respectively[5]. Lainesse *et al*., Described an LC–MS/MS method for the measurement of unchanged Clopidogrel in human plasma (EDTA, anticoagulant) over the range of 20.08–10040.00 pg/mL[6]. Nirogi *et al,*. Used LC–MS/MS and Ticlopidine (internal standard) to analyze unchanged Clopidogrel in human plasma samples (0.5 mL) over the range of 5.00–6000.00 pg/mL[7]. A Robinson *et al*., Proposed an LC/MS/MS method for the estimation of Clopidogrel in human plasma (0. 3mL) over the range of 10.00-12,000.00 pg/mL[8]. Beom Soo Shin *et al*., Described the Determination of Clopidogrel in human plasma (0.5mL) by liquid chromatography/tandem mass spectrometry application to a clinical pharmacokinetic study over the range 10.00-10,000.00 pg/mL[9].

In the present investigation, we have developed a method having a shorter run time with simple liquid-liquid extraction technique. The following are the advantages of the proposed method over those reported earlier: (1) Sample to be collected for time point from individual during the study is reduced significantly. This allows inclusion of additional time points for sample collection; (2) Employing a single step liquid-liquid extraction method simplified the sample extraction procedure , minimizes the chances of errors and saves considerable time; (3) The use of Isotope labeled internal standard, which is physically and chemically identical to the Analyte thus minimizing the errors during sample preparation and mass spectrometer detection.

The above points low plasma volume, use of isotope labeled internal standard, liquid-liquid extraction and a run time of 2.5 Min. makes the method an attractive procedure in high-throughput bioanalysis of Clopidogrel in human plasma.

MATERIALS AND METHODS

Chemicals and Reagents

The reference samples Clopidogrel hydrogen sulfate (>99.81%) and Clopidogrel-d_3 (>99.80%) were purchased from Varda Biotech Pvt. Ltd (Mumbai, India). Water used for the LC-MS/MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). Acetonitrile, methanol and Methy tertiary butylether (MTBE) were of HPLC grade and purchased from J.T Baker (Phillipsburg, USA). Analytical grade ammonium acetate and formic acid were purchased from Qualigens (Glaxo Mumbai, India). The control human plasma (K_2EDTA) sample was procured from Cauvery Diagnostics and Blood Bank (Secunderabad, India).

Instrumentation and Chromatographic Conditions

A HPLC system (Waters, Alliance LC, 2695 separation module, USA) consisting of a Zorbax Eclipse Plus XDB, C18 column (100 X 4.6 mm, 5 µ; Agilent, USA), were used for the validation. Aliquots of the processed samples (10 µL) were injected onto the column, which was kept at 40 ± 5 ºC. The mobile phase, a 90:10 v/v mixture of acetonitrile and ammonium acetate (5 mM, pH 4.0) was delivered at 0.200 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantitation was achieved with MRM (MS/MS data acquisition mode) in positive ion mode for both the analyte and the internal standard using a Waters Quattro-Premier XE (LC-ESI/MS/MS, Waters, USA). The tuning parameters were summarized in Table 1a &1b. Product ion Mass spectra's were represented in Figure 2a & 2b. Detection of the ions were carried out in the MRM, by monitoring the transition pairs of m/z 322.13 precursor ion to the

m/z 212.04 for Clopidogrel, m/z 326.06 precursor ion to the m/z 215.04 for Clopidogrel-d3. The analysis data obtained were processed by Masslynx software ™ (version 4.1).

Table 1a: Tuning parameters

ES + Source Parameter	Settings	Analyzer Parameter	Settings
Capillary (kV)	1.0	LM Resolution 1	13
Extractor (V)	4	HM Resolution 1	13.0
RF Lens (V)	0.0	Ion Energy 1	1.0
Source Temp (°C)	120	Entrance	2
Desolvation Temp (°C)	500	Exit	2
Cone Flow (L/h)	50	LM Resolution 2	13.0
Desolvation Flow (L/h)	800	HM Resolution 2	13.0
Collision cell Pressure (mbar)	$3.5e^{-3} – 4.5e^{-3}$	Ion Energy 2	1.3
		Multiplier	650

Table 1b: Tuning parameters

Parameter	Setting (Analyte)	Setting (ISTD)
MS Function	MRM 322.13/212.04	MRM 326.06/215.04
Dwell (Secs)	0.3	0.3
Cone voltage (V)	15	15
Collision Energy(eV)	14	14

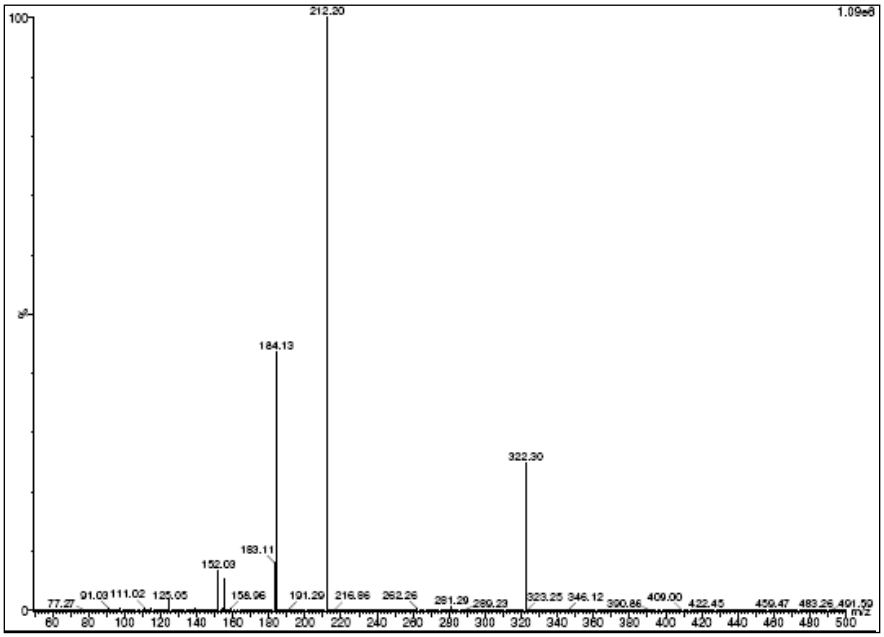

Figure 2a: Product ion mass spectra of $[M+H]^+$ of Clopidogrel

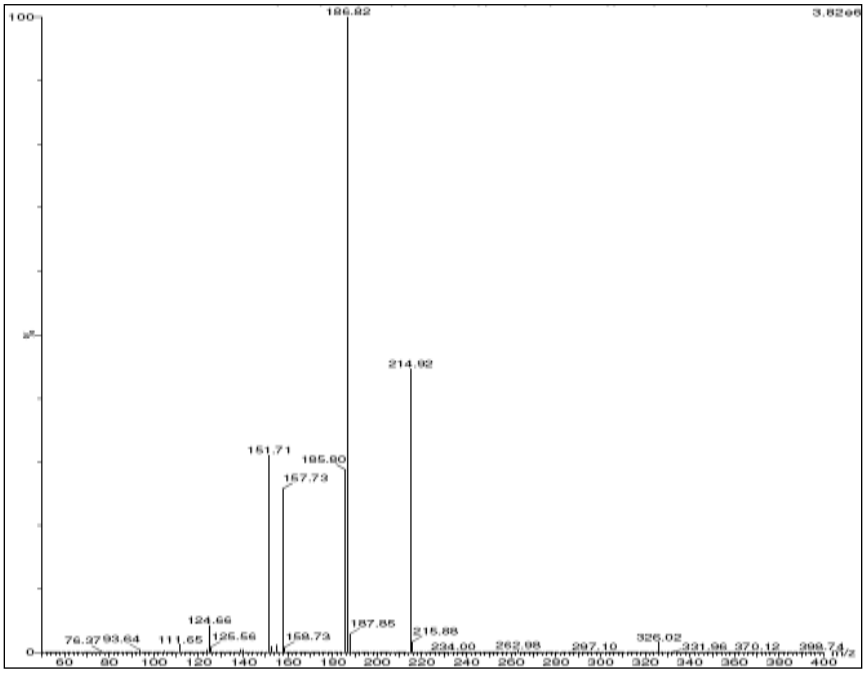

Figure 2b: Product ion mass spectra of $[M+H]^+$ of Clopidogrel-d_3

Standard Solutions

Primary stock solution of Clopidogrel for preparation of standard and quality control (QC) samples were prepared from separate weighing. The primary stock solution 0.5 mg/mL of the analyte was prepared in methanol and stored at 2-8 ºC. From the stock solution, appropriate dilutions were made using a 50:50 v/v mixture of methanol and water as a diluent to produce working standard solutions of 5099.10, 10252.40, 29293.70, 58587.90, 146475.55, 292939.25, 732347.50, 1464699.20, 2441149.10 and 3051448.15 pg/mL of Clopidogrel. These solutions were used to prepare the relevant calibration curve (CC) standards. Another set of working solutions of Clopidogrel were prepared in the diluent (from primary stock) at concentrations of 2320749.10, 1392448.70, 278449.10, 15294.70 and 5210.55 pg/mL to be used as quality control (QC) samples. The primary stock solution of Clopidogrel-d_3 (0.10 mg/mL) was prepared in methanol. A working concentration of the internal standard (50 ng/mL of Clopidogrel-d_3) solution was prepared in the diluent. These working solutions were stored at 2-8 ºC for 7 days.

The calibration curve and quality control samples were prepared by spiking 20 µL of the working solution into 980 µL of control plasma. Calibration samples for Clopidogrel were made at concentrations of 101.98, 205.05, 585.87, 1171.76, 2929.51, 5858.79, 14646.95, 29293.98, 48822.98, 61028.96 pg/mL. Quality control samples for Clopidogrel were prepared at concentrations of 46414.98 (higher quality control, HQC), 27848.97 (middle quality control,

MQC1), 5568.98 (middle quality control 2, MQC2), 305.89, (lower quality control, LQC) and 104.21 (lower limit quality control, LLOQ QC) pg/mL.

Sample processing

A 200-µL volume of the plasma sample was transferred to a 15-mL glass test tube, and 50.0 µL of 50 ng/mL internal standard solution was spiked, vortex for 30 sec. Add 300 µL of extraction buffer (0.5% Formic acid), vortex for another 30 sec, and add extraction solvent (MTBE) 2 mL using Dispensette Organic (Brand GmbH, Wertheim, Germany). The sample was shaken for 10 min using a Multiplus vortexer and Centrifuge all the test tubes at 4500 rpm, at 4 °C for 5 Min, using a Heraeus Megafuse 3SR, Japan centrifuge. The organic layer (1.8 mL) was transferred to a 5-mL glass test tube and evaporated at 50ºC under a stream of nitrogen (Turbo Vap LV, Zymark; Hopkinton, MA, USA). Add 100.0 µL of reconstitution solution to all the tubes and vortex for about 2 min. Transfer 100.0 µL of the reconstituted solution into pre-labeled auto sampler vials and inject 10.0 µL onto LC-MS/MS.

Method validation

The method validation of Clopidogrel was carried out as per the US FDA guidelines (FDA, 2001)[10]. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity, ruggedness, effect of potentially interfering drugs on the method and stability. Selectivity of the method was assessed by analyzing six blank human plasma matrix samples. The responses of the interfering substances or background noises at the retention time of the Clopidogrel and its internal standard were acceptable if they are less than 20% of the response of the lowest standard curve point, and less than 5% of the response of the internal standard respectively.

The Sensitivity of the method was evaluated by analyzing 6 LLOQ samples. At least 67% (4 out of 6) of LLOQ samples should be within 80-120%. Matrix effect was investigated to ensure that precision, selectivity and sensitivity are not compromised by the matrix. Matrix effect was checked with six different lots of K_2EDTA plasma. Three replicate samples each of quality control (low and high) were prepared from different lots of plasma. The QCs should be within acceptance limit 85.00 - 115.00 % (36 QC samples in total).

Linearity was tested for Clopidogrel in the concentration range of 101.98-61028.96 pg/mL. Linearity was determined by using a 1/x2 weighted least square regression analysis of standard plots associated with a ten-point standard curve. To confirm blank interference in each of the standard curves, blank plasma samples were also analyzed. The acceptance limit of accuracy for

each of the back-calculated concentrations is ±15% except LLOQ, where it is ±20%. For a calibration run to be accepted at least 75% of the standards[10], including the LLOQ and ULOQ are required to meet the acceptance criterion otherwise, the calibration curve is rejected. The samples were run from low to high concentration.

Intra-assay precision and accuracy were determined by analyzing six replicates at five different QC levels on two different days. Inter-assay precision and accuracy were determined by analyzing six replicates at five different QC levels. The acceptance criteria included accuracy within ±15% deviation (SD) from the nominal values, except LLOQ QC, where it should be ±20% and a precision of ≤15% relative standard deviation (RSD), except for LLOQ QC, where it should be ≤20%[10].

Recovery of the analyte was determined by comparing the peak areas of the analyte in spiked plasma samples (six each of low, medium, and high QCs) with the those of the analyte in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of the analyte at the step immediately prior to chromatography. Similarly, recovery of the internal standard was determined by comparing the mean peak areas of the extracted QC samples (n=6) with those of the internal standard prepared by spiking the extracted drug-free plasma samples with the same amounts of internal standards at the step immediately prior to chromatography.

The dilution integrity exercise was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above the ULOQ during real time analysis. Dilution integrity experiment was carried out at 2.5 times the ULOQ concentration for the analyte. Six replicates each of dilution factor (DF) 5&10 concentrations were prepared and their concentrations were calculated by applying the DF 5 and 10.

Ruggedness of the method was evaluated by using a different lot of the same column and a different analyst. The precision and accuracy for the quality control samples at HQC, MQC1, MQC2, LQC and LLOQ QC concentration levels found to be within the acceptance limit.

Effect of potentially interfering drugs was evaluated at low and high quality control concentration levels of Clopidogrel for Paracetamol, Ibuprofen, Caffeine, Diphenhydramine, Diclofenac and Chlorphenarimine Maleate, The precision and accuracy at high and low QC levels found to be within the acceptable limit.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8 °C) was performed by comparing the area response of the analyte

(stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability [05 hr(s)], processed samples stability [Autosampler stability for 60 hr(s) 30 min(s), wet extract stability at room temperature 6 hr(s), wet extract stability at refrigerator temperature 65hr(s), dry extracted stability (-28±50C) 64hr(s) 25 min(s), reinjection reproducibility 70 hr(s) 15 min(s) and freeze and thaw stability (Four cycles)] were performed at low and high QC levels using six replicates at each level and stability of analyte in blood has been proven at room temperature [4hr(s) 10 min(s)] and refrigerator temperature [4hr(s) 15 min (s)]. Long term stability (50 days) was performed at low and high QC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and precision (≤15% RSD).

Method development

Mass Spectrometer parameters were tuned in positive ionization mode using electrospray ionization for the analyte and the internal standard. For the data acquisition MRM mode was used to get better selectivity.

Chromatography was optimized using various combinations of acetonitrile and buffer with varying contents of each component on different columns like C8 and C18 of different makes like Luna, Xbridge, Chromolith, Hypersil, ACE and Intertsil etc. Use of a buffer with desired pH (4.0) helped in achieving good response for MS detection in the positive ionization mode. A mobile phase consisting of acetonitrile and 5 mM ammonium acetate (90:10 v/v; pH 4.00± 0.05) was found suitable, as the analyte was protonated and well separated in this phase. Zorbax Eclipse Plus XDB, C18 column (100 X 4.6 mm, 5 µ; Agilent, USA), column gave a good peak shape for both analyte and internal standard and at LLOQ level signal to noise ratio was found to be good. The mobile phase was operated at a flow rate of 1.0 mL/min with 80% split (0.200 mL into MS). The retention time of analyte and the internal standard were 2.03 and 2.02 respectively.

Liquid-liquid extraction (LLE) technique was employed for the extraction of drug and internal standard. LLE is helpful in producing a spectroscopically clean sample when compared to protein precipitation and avoiding the introduction of plasma components and non-volatile materials onto the LC and MS system. Clean samples are essential for minimizing the matrix effect in LC-MS/MS. Among the different solvents checked MTBE was found to be optimal, which produced a clean chromatogram for a blank sample and yielded the highest recovery for the analyte from the plasma.

An internal standard must mimic the analyte during extraction as well as during the ionization. For LC-MS/MS analysis, use of stable isotope-labeled molecule as internal standard proved to be helpful when there is a significant matrix effect. In Clopidogrel method development and validation, Clopidogrel-d_3 was used as the internal standard. An isotopically labeled internal standard best compensates for sample-to-sample variability and recovery also.

Selectivity and Chromatography

The specificity of the LC-MS/MS method was established by screening the standard blanks of different lots from commercially available human plasma. Ten different lots of plasma were screened for the specificity experiment. Of the ten, seven batches were intended anticoagulant plasma (K_2EDTA), one each of haemolytic plasma, and lipidemic plasma and one lot containing heparin as anticoagulant. All the investigated human plasma lots were found to be free of interferences at the retention time of drug and the internal standard. Area of the peak at the retention time of drug in standard blank samples was ≤ 20.00% of the area of the analyte in the extracted LLOQ sample; area of the peak at the retention time of internal standard in standard blank samples was ≤ 5.00 % of the area of the internal standard in the extracted LLOQ sample. Representative chromatograms of Standard Blank, Standard Zero (Standard Blank with Internal Standard), ULOQ standard, LLOQ standard were mentioned in Figure: 3a, 3b, 3c and 3d respectively.

Sensitivity

The Sensitivity of the method was evaluated by analyzing 6 LLOQ samples. The LLOQ was 101.98 pg/mL for Clopidogrel. The precision and accuracy for Clopidogrel at LLOQ level were found to be 4.78 % and 96.03 % respectively.

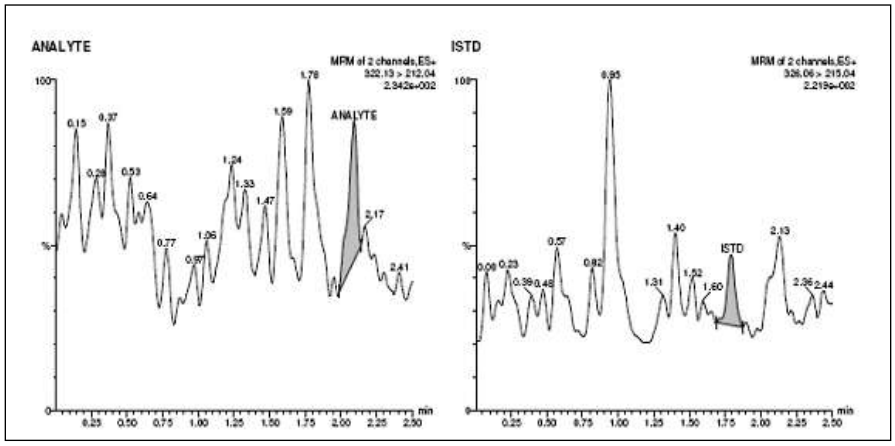

Figure 3a: Typical MRM chromatograms of Clopidogrel (left panel) and IS (right panel) Standard Blank

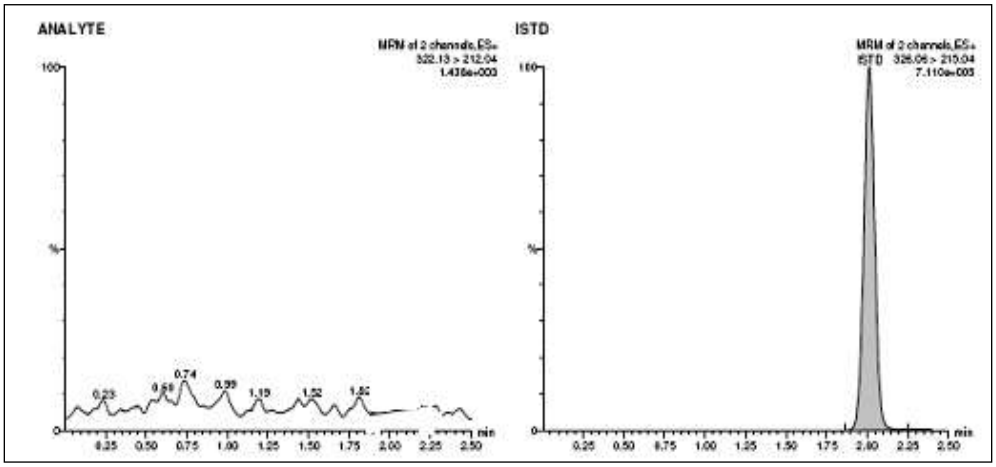

Figure 3b: Typical MRM chromatograms of Clopidogrel (left panel) and IS (right panel) Standard Zero sample Chromatogram

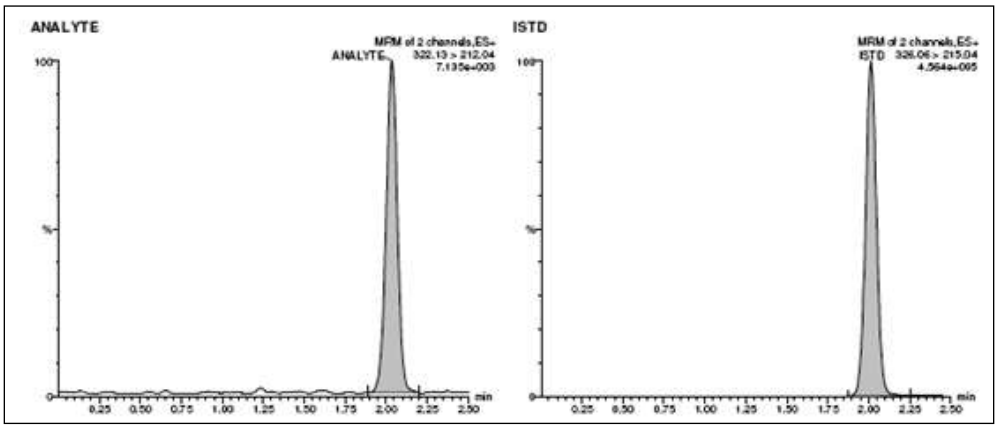

Figure 3c: Typical MRM chromatograms of Clopidogrel (left panel) and IS (right panel) Lower Limit of Quantitation

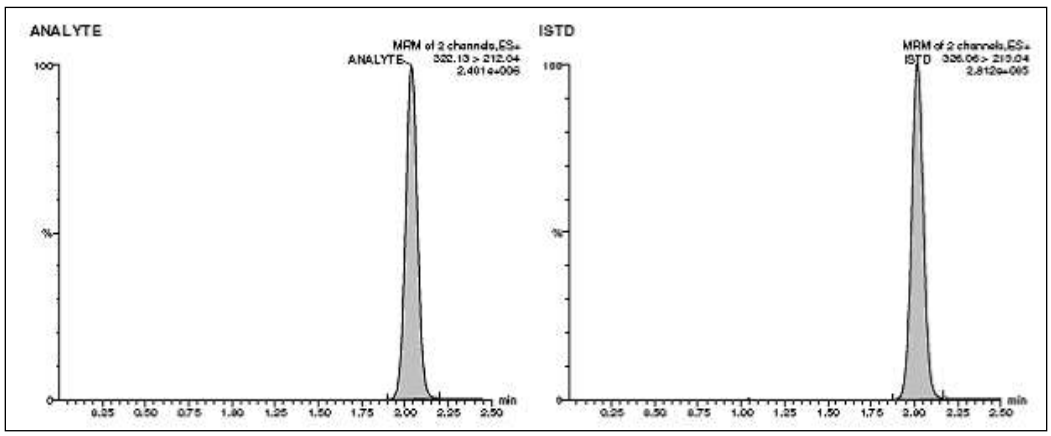

Figure 3d : Typical MRM chromatograms of Clopidogrel (left panel) and IS (right panel) Upper Limit of Quantitation

Matrix effect

No significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high QC concentrations. The precision and accuracy for Clopidogrel at low QC concentration was found to be 3.59% and 102.33%, and at high QC level was 2.28% and 101.88% respectively.

Linearity

The linearity was determined by using a 1/x2 weighted least square regression analysis of standard plots associated with a ten-point standard curve. All the four calibration curves analyzed during the course of validation were found to be linear over the concentration ranging from 101.98–61028.96 pg/mL. The correlation coefficient (r) was observed to be ≥ 0.9994. The overall % mean accuracy for the CC standards was found to be in between 97.70–108.52 % and the overall precision was ≤ 10.93 %.

Precision and Accuracy

As shown in Table 2a and 2b, The precision and accuracy of the method was evaluated by the % CV and % accuracy respectively, at different concentration levels corresponding to LLOQ QC, LQC, MQC2, MQC1 and HQC during the course of validation. The precision and accuracy of analyte in the intra-batch and inter-batch runs were within ± 15% and within ± 20% at LLOQ QCs.

Table 2a: Intra-batch precision and accuracy

Spiked QC Concentration (pg/mL)	Concentration found (mean; pg/mL)	Precision (%)	Accuracy (%)
104.21	100.21	3.97	96.17
305.89	306.86	2.26	100.32
5568.98	5745.18	1.52	103.16
27848.97	27242.42	3.90	97.82
46414.98	44926.34	3.41	96.79

Table 2b: Inter-batch precision and accuracy

Spiked QC Concentration (pg/mL)	Concentration found (mean; pg/mL)	Precision (%)	Accuracy (%)
104.21	103.27	6.03	99.10
305.89	302.64	2.09	98.94
5568.98	5690.70	0.49	102.19
27848.97	27721.74	1.81	99.54
46414.98	45569.68	1.23	98.18

Extraction Efficiency

Six replicates at low, medium and high quality control concentration for Clopidogrel was prepared for recovery determination. The recovery comparison samples of analyte compared against the response of analyte in the mid QC level. The mean recovery of Clopidogrel at different levels of HQC, MQC1, MQC2 and LQC were found to be 59.23, 58.07, 58.41 and 57.33 % respectively. The mean recovery of internal standard was found to be 61.90%.

Dilution Integrity

The upper limit of quantitation can be extended to 152572.50 pg/mL for Clopidogrel. The dilution integrity of the method was evaluated for DF 5 and 10 with screened human blank plasma. The precision for DF 5 and 10 was found to be 1.10 and 1.91 %, and the % mean accuracy for DF 5 and 10 was found to be 99.32 and 98.53 % respectively, which are within acceptance limit of 85.00 - 115.00 %. The results are summarized in the Table 3.

Table 3: Dilution Integrity

DI Spiked Standard conc 152572.50pg/mL				
Dilution Factor	**DIQC (spiked concentration (pg/mL)**	**Concentration found (mean; pg/mL**	**Mean Accuracy (%)**	**Precision (%)**
1/5	30514.50	30305.80	99.32	1.10
1/10	15257.25	1532.58	98.53	1.91

Ruggedness

Ruggedness was performed by using a different lot of the same column and a different analyst. The precision and % mean accuracy for the quality control samples at HQC, MQC1, MQC2 and LQC concentration levels found to be within acceptance limit 15.00 %. For all the samples of LLOQ QC was found to be within the acceptance limit of ≤ 20.00 %. The results are summarized in the Table 4.

Effect of Potentially Interfering Drugs

Effect of potentially interfering drugs was performed at low and high QC concentration levels of Clopidogrel for Paracetamol, Ibuprofen, Caffeine, Diphenhydramine, Diclofenac and Chlorphenarimine Maleate. At least 67 % (2 out of 3) of samples at each level (HQC & LQC) were within 85.00-115.00 % of potential interfering drugs (i.e. Paracetamol, Ibuprofen, Caffeine, Dipheny hydramine, Diclofenac and Chlorphenarimine Maleate). The results of quality control samples are summarized in the Table 5.

Table 4: Ruggedness Precision and Accuracy

Experiment Name	QC (spiked concentration (pg/mL)	Concentration found (mean; pg/mL	Mean Accuracy (%)	Precision (%)
Different column	104.21	112.73	108.17	7.04
	305.89	307.95	100.67	2.56
	5568.98	5734.93	102.98	1.12
	27848.97	28948.81	103.95	1.33
	46414.98	47805.92	103.00	2.52
Different analyst	104.21	110.00	105.56	5.91
	305.892	310.01	101.35	4.18
	5568.98	5634.93	101.18	1.79
	27848.97	28353.55	101.81	1.89
	46414.98	47143.26	101.57	1.27

Table 5: Effect of Potential interfering drugs

Compound Name	QC (spiked concentration (pg/mL)	Concentration found (mean; pg/mL	Mean Accuracy (%)	Precision (%)
Paracetamol	305.892	286.95	93.81	1.71
	46414.98	47460.80	102.25	1.88
Ibuprofen	305.89	297.33	97.20	3.22
	46414.98	47612.65	101.45	1.84
Caffeine	305.89	310.38	101.47	8.44
	46414.98	47088.35	101.40	0.43
Diphenhydramine	305.89	310.21	101.41	4.25
	46414.98	47065.66	101.40	0.35
Diclofenac	305.89	298.06	97.44	3.37
	46414.98	47082.69	101.44	1.04
Chlorphenarimine Maleate	305.89	306.43	105.09	6.73
	46414.98	47031.93	101.33	1.44

Stability

In the different stability experiments carried out viz. bench top stability (05 hrs), auto sampler stability (60 hr(s), 30 min(s)), repeated freeze-thaw cycles (4 cycles), reinjection reproducibility (70 hr(s), 15 min(s)), wet extract stability at room temperature (6 hr(s)), wet extract stability at refrigerator temperature (65 hr(s)) dry extract stability (64 hr(s), 25 min(s) -28±5 0C) and stability of the analyte in blood at room temperature (4 hr(s), 10 min(s)) and at refrigerator temperature (4 hr(s), 15 min(s)) have been proved. The mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their low and high QC levels thus, the results were found to be within the acceptable limits during the entire

validation. Long term stability at –70 °C for 50 days the mean % nominal values of the analyte was found to be within ±15% of the predicted concentrations for the analyte at their low and high QC levels. The results are summarized in Table 6.

Table 6: Stability Samples Results for Clopidogrel

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Accuracy/ Stability (%)	Precision (%)
Autosampler [a]	305.892	296.91 ± 8.51	97.06	2.86
	46414.98	47437.57 ± 463.82	103.55	0.97
Wet extract (RF) b	305.892	297.99 ± 6.77	97.42	2.27
	46414.98	46884.99 ± 500.38	101.01	1.60
Wet extract (RT)[c]	305.892	304.50 ± 10.62	99.55	3.49
	46414.98	46720.57 ± 433.20	100.66	0.93
Bench top[d]	305.892	306.61 ± 12.35	100.24	4.03
	46414.98	47018.88 ± 621.58	101.30	1.32
FT[e]	305.892	311.43 ± 16.78	101.81	5.39
	46414.98	46949.94 ± 406.14	101.15	0.87
Dry extract[f]	305.892	302.42 ± 6.95	98.86	2.30
	46414.98	46959.86 ± 560.98	101.17	1.19
Long-term[g]	305.892	326.85 ± 20.19	106.85	6.18
	46414.98	45656.87± 572.69	98.37	1.25

[a] 60 hrs,30 min in autosampler at 5±3°C; [b] after 65 hrs in refrigerator at 2-8°C; [c] after 6 hrs at room temperature; [d] 5 hrs on bentch; [e]after four freeze and thaw cycles; [f] processed sample stability 64 hrs , 25 Min at -28±5^{0}C; 50 days stable in Plasma [g]

CONCLUSION

The use of LC/MS/MS technology can enable the performance of highly accurate analysis. The LC-MS/MS method development and validation presented in this paper is simple, sensitive, fast, reliable, specific and rugged for quantification of Clopidogrel in human plasma and is validated according to FDA Guidance for industry on bioanalytical method validation. The method was suitable for pharmacokinetic studies in humans. The extraction method is found to be consistent and reproducible. The cost-effectiveness, simplicity of the assay and usage of liquid-liquid extraction, and sample turnover rate of less than 2.5 min per sample, make it an attractive procedure in high-throughput bioanalysis of Clopidogrel.

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