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Estimation of carboxylic acid metabolite of clopidogrel in Wistar rat plasma by HPLC and its application to a pharmacokinetic study

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Abstract

A new HPLC method was developed for the estimation of carboxylic acid metabolite of clopidogrel bisulfate in rat plasma using atorvastatin as internal standard. Plasma samples were extracted with a mixture of ethyl acetate and di-chloro methane (80:20, v/v) followed by subsequent reconstitution in a mixture of water:methanol:acetonitrile (40:40:20, v/v). The chromatographic separation was achieved with gradient elution on Kromasil ODS, 250 mm × 4.6 mm i.d., 5 μm analytical column maintained at 30 °C. Carboxylic acid metabolite of clopidogrel as well as the internal standard were detected at a wavelength of 220 nm. The method was validated as per USFDA guidelines. Calibration curves were linear in the concentration range of 125.0–32,000 ng/ml and the correlation coefficient was better than 0.999. The extraction efficiency for the carboxylic acid metabolite of clopidogrel was more than 85.76%. The intra-day accuracy ranged from 98.9% to 101.5% with a precision of 1.30% to 6.06%. Similarly, the inter-day accuracy was between 96.2% and 101.1% with a precision of 3.47% to 4.30%. The drug containing plasma samples were stable at −70 °C for 48 days and at ambient temperature for 24 h. In the auto-sampler maintained at 15 °C, the processed and reconstituted samples were stable for 35 h. The drug containing frozen plasma samples were stable enough to with stand three freeze thaw cycles. The method was successfully applied to the pharmacokinetic study of the two different polymorphs of clopidogrel bisulfate in Wistar rat.

Keywords: Carboxylic acid metabolite of clopidogrel; Rat plasma; Validation and pharmacokinetics

1. Introduction

Clopidogrel is pro-drug, which inhibits platelets aggregation. The active metabolite of clopidogrel selectively inhibits adenylate cyclase, ADP-induced platelet aggregation by direct inhibition of ADP binding to its receptor which further leads to the inhibition of subsequent ADP-mediated activation of GPIIb-IIa complex. [1]. Clopidogrel is similar to ticlopidine in chemical structure but unlike ticlopidine it is devoid of the side effects of serious reduction of white cells. Long term prophylactic use of clopidogrel has been reported [1] to be beneficial in the prevention of ischemic stroke, myocardial infarction and vascular death in patients.

Clopidogrel is inactive in vitro and hepatic biotransformation via cytochrome P450 pathway, primarily by CYP3A4 and CYP3A5 [2]; is essential for its in vivo antiplatelet activity. The active metabolite [3] a thiol compound, is formed by the oxidation of clopidogrel to 2-oxo clopidogrel and subsequent hydrolysis. The active metabolite is highly labile and remains un-detected in plasma. It was isolated in vitro from rat microsomes and the structure elucidation [3] was performed on stabilized acrylonitrile derivative.

Following an oral administration in human, the plasma levels of clopidogrel are very low due to extensive metabolism and difficult to quantify. The main circulating metabolite (the carboxylic acid derivative) is pharmacologically inactive and represents 85% of circulating metabolites in human plasma. Since, neither the parent drug nor the active metabolite is detected in plasma, the measurement of pharmacodynamic effect by estimating platelet aggregation [4] is believed to be a better measure for in vivo estimations. However, the quantitation of inactive carboxylic acid metabolite of clopidogrel which is the most abundant species circulating in blood, has immerged as an indirect approach for studying

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the pharmacokinetics of clopidogrel. Several studies have already been reported earlier where the carboxylic acid metabolite of clopidogrel was measured by GC–MS [5] and chiral HPLC [6]. The LC–MS methods have been reported for the parent compound [7] and also for the carboxylic acid metabolite of clopidogrel [8]. There were no methods reported for the estimation of its carboxylic acid metabolite of clopidogrel by reverse phase HPLC which is the most convenient and common analytical technique so far. Therefore, a new HPLC method was developed for the estimation of inactive carboxylic acid metabolite of clopidogrel in rat plasma. The prime objective of the study was to compare the pharmacokinetics of two different polymorphs [9] of clopidogrel bisulfate after an oral dose of 30 mg/kg in male Wistar rat. The method was validated in accordance with USFDA guideline [10] to assess its specificity, sensitivity, accuracy and precision.

2. Experimental

2.1. Reagents and chemicals

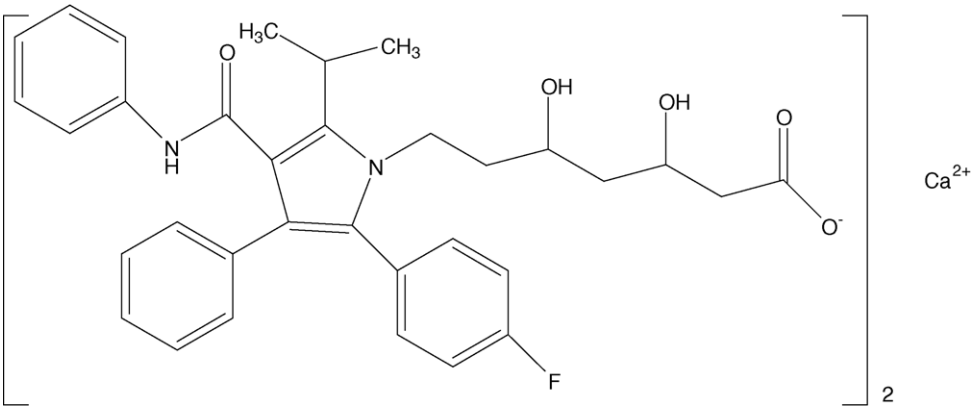
Working standards of carboxylic acid metabolite of clopidogrel (purity 99.87%) (Fig. 1) and atorvastatin (purity 99.75%) were prepared by Zydus Research Centre, Cadila Healthcare Ltd. Acetonitrile Omnisolv® was purchased from Merck, KGaA, Darmstadt, Germany. Trifluroacetic acid was procured from Merck, Germany. Drug free blank plasma was obtained by sacrificing several Wistar rats. HPLC grade type II water from Millipore's Milli-Q System was used throughout the pre-study validation and analysis.

2.2. Instrumentation

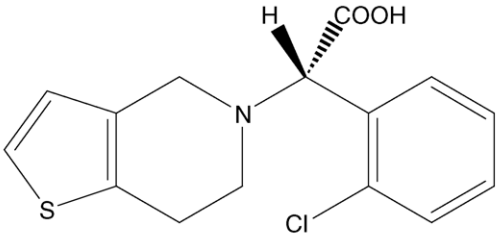
The liquid chromatograph consisted of an integrated HPLC system of Agilent 1100 Series pump, autosampler, degasser and UV/VIS detector. Chemstation Rev.A.09.01. (1206) software, from Agilent technologies, was used to acquire and process all chromatographic data.

2.3. Preparation of stock and working standard solutions

The stock standard solutions of the carboxylic acid metabolite of clopidogrel (320,000 ng/ml) were prepared separately for calibration standards and quality-control (QC) samples, by dissolving appropriate amount of the compound in water:methanol:acetonitrile (40:40:20, v/v) mixture. The stock standard solution was subsequently diluted in the same diluent to obtain working standard solutions in the range 1.25–160,000 ng/ml. A 320,000 ng/ml working solution of internal standard (IS) was prepared in



atorvastatin (IS)



carboxylic acid metabolite of clopidogrel

Fig. 1. Structures of carboxylic acid metabolite of clopidogrel and IS.

water:methanol:acetonitrile (40:40:20, v/v) mixture. All solutions were stored at 2–8 °C.

2.4. Preparation of calibration standards, quality control and study samples

To 160 μl of the drug free plasma, 20 μl of working solutions of carboxylic acid metabolite of clopidogrel was added to yield final respective concentrations as: 125, 500, 2000, 4000, 16,000 and 32,000 ng/ml of carboxylic acid metabolite of clopidogrel in plasma. To each calibration standard, 20 μl of internal standard solution was added and vortexed. QC samples (250, 1000, 8000 and 24,000 ng/ml) were also prepared in a similar manner. For preparing study samples, 20 μl of working solution of internal standard was added 180 μl of plasma, and vortexed for 60 s.

2.5. Sample preparation procedure

To the calibration standards, QC samples and study samples, 2.5 ml of extraction solvent (ethyl acetate: dichloromethane; 80:20, v/v) was added and vortexed for about 1 min. After centrifuging for 10 min at 3220 × *g*, 2 ml of the supernatant was transferred to the evaporation tube. The supernatant was evaporated to dryness in the thermostatically controlled water-bath maintained at 55 °C under the stream of nitrogen for about 20 min. The dry residue was reconstituted in 100 μl of water:methanol:acetonitrile (40:40:20, v/v) mixture and transferred to 1 ml vial for injection into HPLC.

2.6. Chromatographic conditions

A simple gradient method was used, where the percentage of solvent A, buffer (0.05% trifluoroacetic acid in water) and solvent B–acetonitrile was varied as shown in Table 1. The total run time was 20 min and the total flow rate was 1 ml/min. The separation was achieved on Kromasil ODS, "250 mm × 4.6 mm i.d., 5 μm" analytical column maintained at 30 °C and detection at 220 nm. Fifty microliter of sample, kept in autosampler at 15 °C was injected into HPLC for each chromatographic run.

Table 1
Gradient program used for the separation of carboxylic acid metabolite of clopidogrel

Time (min)	Solvent A (0.05% TFA in water)	Solvent B (acetonitrile)
0.0	90.0	10.0
2.0	90.0	10.0
6.0	50.0	50.0
10.0	30.0	70.0
12.0	10.0	90.0
15.0	10.0	90.0
16.0	60.0	40.0
18.0	90.0	10.0
20.0	90.0	10.0

2.7. Bio-analytical method validation

2.7.1. Linearity (calibration curves)

The linearity of the method was evaluated using freshly prepared spiked plasma samples in the concentration range of 125–32,000 ng/ml. Four such linearity curves were analyzed. Each calibration curve consisted of a blank sample, a zero sample and six calibrator concentrations. Samples were quantified using the ratio of peak area of analyte to that of IS as the assay parameter. Peak area ratio were plotted against plasma concentrations and standard curves were calculated by the equation: $y = mx$ and intercept were forced through zero. A correlation of more than 0.99 was desirable for all the calibration curves.

2.7.2. Limit of detection (LOD) and lower limit of quantitation (LLOQ)

The limit of detection was defined by the concentration with a signal-to-noise ratio of 3. The lowest standard on the calibration curve was to be accepted as the lower limit of quantitation if it complied the acceptance criteria [8] of exhibiting the analyte response five times that of drug free (blank) processed plasma. In addition, the analyte peak in LLOQ sample should be identifiable, discrete, and reproducible with a precision of ±20% and accuracy within 80%–120%. The deviation of standards other than LLOQ should not be more than ±15% of the nominal concentration. It was desirable that a minimum of five non-zero standards, including LLOQ, met the above criteria.

2.7.3. Specificity

Randomly selected drug free plasma samples from six different rats were processed and injected into HPLC to assess the extent to which endogenous plasma components may interfere at retention times of analyte and IS.

2.7.4. Extraction efficiency (recovery)

The extraction efficiency of carboxylic acid metabolite of clopidogrel was evaluated by comparing the mean peak responses of four QC samples of 250, 1000 and 8000 concentrations to the mean peak responses of four plain standards of equivalent concentration. Similarly, the recovery of IS was evaluated by comparing the mean peak responses of four quality control samples to mean peak responses of four plain standards of at the concentration of 16,000 ng/ml. According to the acceptance criteria [8] the recovery of the analyte or IS does not need to be 100%, but should be consistent, precise and reproducible.

2.7.5. Accuracy and precision

Intra-day accuracy and precision were evaluated from replicate analysis ($n = 6$) of quality-control samples containing the carboxylic acid metabolite of clopidogrel at different concentrations (LLOQ, low, medium and high) on the same day. Inter day accuracy and precision were also assessed from

the analysis of the same QC samples on four separate occasions in replicate ($n = 6$). QC samples were analysed against calibration curves.

The evaluation of precision was based on the criteria [8] that the, relative standard deviation for each concentration level should not be more than ±15% except for the LLOQ, for which it should not exceed ±20%. Similarly for accuracy, the mean value should not deviate by ±15% of the nominal concentration except for the LLOQ where it should not deviate by more than ±20% of the nominal concentration.

2.7.6. Stability

2.7.6.1. Long term stability. Four aliquots each of, low and high QC samples were kept in deep freezer at −70 ± 5 °C for 48 days. Thereafter, the samples were processed and analyzed using freshly prepared calibration standards. The concentrations thus obtained were compared with the theoretical value of QC samples to determine the long-term stability of carboxylic acid metabolite of clopidogrel in rat plasma. The samples were to be considered stable if the percentage change (bias) in the concentration of the stability samples was not more than ±15% of the theoretical value.

2.7.6.2. Short term stability. Four aliquots each of the low and high unprocessed QC samples were kept at ambient temperature (20–30 °C) for 24 h in order to establish the short-term stability of carboxylic acid metabolite of clopidogrel in rat plasma. Thereafter, 20 μl of working solution of IS was added and the samples were processed and analyzed. The concentrations thus obtained were compared with the theoretical value of QC samples. The percentage deviation in the concentration of the stability samples from the theoretical values was calculated and the stability was to be concluded if the bias was within ±15%.

2.7.6.3. Autosampler stability. In order to establish the autosampler stability of carboxylic acid metabolite of clopidogrel in reconstituted extracts, four aliquots each of low and high QC samples were stored at 15 °C in auto-sampler for 35.0 h. Thereafter, samples were injected into HPLC and concentrations were compared with the theoretical value. The samples were to be considered stable if the bias was within ±15%.

2.7.6.4. Solution stability. In order to determine the stability of carboxylic acid metabolite of clopidogrel in solution, working standard solution of 6000 ng/ml was kept at 2–8 °C for 68 days. Thereafter, the mean areas of carboxylic acid metabolite of clopidogrel from five replicate chromatographic runs were compared to that of mean area of freshly prepared solutions of same concentration. The samples qualified the criteria of stability if the deviation was within ±2%.

2.7.6.5. Freeze thaw stability of frozen plasma samples. Four aliquots each of low and high unprocessed QC samples were stored at −70 ± 5 °C. Thereafter, the frozen plasma samples were subjected to three freeze thaw cycles. After the completion of third cycle the samples were analyzed. The concentrations thus obtained were compared with the theoretical value of QC samples, and the samples qualified the test if the deviation from the nominal value was within ±15%.

2.7.6.6. Dried extract stability. Three aliquots each of, low and high QC samples were extracted and kept in deep freezer at −70 ± 5 °C, without reconstitution i.e., in dried state. After 24 h, the samples were brought to ambient temperature, reconstituted, analyzed and concentrations obtained were compared with theoretical values. A deviation of more than ±15% was undesirable.

2.7.6.7. Effect of dilution on sample integrity. During the course of study the probability of encountering samples with concentrations above the upper limit of quantitation (ULOQ) cannot be ruled out and therefore they have to be diluted with drug free plasma to bring them within the calibration range. To establish the effect of dilution on the integrity of samples, four aliquots of 40,000 ng/ml were prepared. The samples were further subjected to five-fold dilution with drug free rat plasma to bring them within the calibration range. The samples were processed, analyzed and the concentrations obtained were compared with theoretical values. The effect of dilution was to be considered insignificant if the percent deviation in concentration of the stability samples from theoretical values was within ±15%.

2.8. Pharmacokinetic study in rat

Two groups of male rats 8–10 weeks of age; were used for the study. Each group consisted of six animals. All animals were fasted for 18 h prior to the administration of the drug. Food was supplied after 4 h of drug administration and there was free access to water through out the study. Each group of animals received a single oral dose of 30 mg/kg of different polymorphs (forms I and form II) of clopidogrel. A homogenous suspension of clopidogrel bisulfate was prepared in a vehicle comprising of 0.5% (w/v) carboxy methyl cellulose (CMC) in water and polyethylene glycol (PEG) 400 (90:10, v/v). 0.3 ml of blood was withdrawn from the retro-orbital plexus of a rat into heparinized eppendorf tubes at various time points as; predose, 0.08, 0.17, 0.33, 0.67, 1, 2, 4, 6, 8, 24 and 48 h after dose administration. Samples were kept on ice until centrifugation. Plasma was separated by centrifugation at approximately 3220 × g for 5 min at 25 ± 5 °C and immediately analyzed. The pending samples were stored in the deep freezer at −70 ± 5 °C until analyzed. The study adheres to "Principles of Laboratory Animal Care" and is approved by the animal care committee IAEC/CPSEA—Institutional animal ethics committee/Committee for the purpose of control and supervision of experiments on animals.

The pharmacokinetic parameters namely: maximum plasma concentration (C_{max}), time point of maximum plasma concentration (T_{max}), area under the plasma concentration–

time curve from 0 to the last measurable concentration (AUC_{0-t}), area under the plasma concentration–time curve from 0 to infinity ($AUC_{0-\infty}$), elimination rate constant (λ_Z) and half-life of drug elimination during the terminal phase ($t_{1/2}$) were estimated using non-compartmental analysis of WinNonlin Professional Software Version 4.0.1 (Pharsight Corporation, USA).

3. Results and discussion

3.1. Bio-analytical method validation

3.1.1. Linearity

All calibration curves were found to be linear over the calibration range of 125–32,000 ng/ml. The mean correlation coefficient was 0.999. *F*-test on lack of fit at 95.0% confidence level was performed and it exhibited significance. The results are presented in Table 2.

3.1.2. Limit of detection and lower limit of quantitation

The LOD was 75 ng/ml. The LLOQ was 125 ng/ml with coefficient of variation of 7.3% and accuracy of 97.6%. The ULOQ was 32,000 ng/ml with coefficient of variation of 1.9% and accuracy 101.4%. Results are presented in Table 2.

3.1.3. Specificity

No significant interfering peaks were observed at the retention times of either analyte or internal standard in six different lots of drug free rat plasma samples used for analysis. The chromatographic separation has been exhibited in Fig. 2.

3.1.4. Recovery (extraction efficiency) from plasma matrix

The extraction efficiency of carboxylic acid metabolite of clopidogrel from rat plasma at the concentrations of 250, 1000, 8000 and 24,000 ng/ml was found to be 88.5%, 88.1%, 85.8% and 87.0% with precision of 8.7%, 1.5%, 0.2% and 4.9%, respectively. The mean recovery for internal standard was 90.5% ± 5.2%.

3.1.5. Accuracy and precision

The intra-day accuracy ranged between 98.9% and 101.5% with a precision of 1.3% to 6.1%. (Table 3). The inter-day accuracy was between 96.2% and 101.1% (Table 3) with a precision of 3.5% to 5.5%. One-way analysis of variance (ANOVA) was carried out with grouping variable "day" to assess precision at 95% level. The result was non-significant when data for each day was compared with other days and within the same day.

Plasma samples containing carboxylic acid metabolite of clopidogrel at concentrations of 40,000 ng/ml could be

Table 2
Summary of calibration standards of carboxylic acid metabolite of clopidogrel in rat plasma

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	Recovery (%)	R.S.D. (%)	*n*
125	122	97.6	7.3	4
500	503	100.6	3.2	4
2000	2085	104.3	5.1	4
4000	4005	100.1	2.3	4
16000	15378	96.1	3.1	4
32000	32438	101.4	1.9	4

Calibration curve	*Y* intercept	Slope	Correlation (r^2)	*F*-test	
				F	*p*-value
1	0	0.0161	0.9988	30.90	<0.05
2	0	0.0135	0.9983	24.49	<0.05
3	0	0.0131	0.9999	24.23	<0.05
4	0	0.0157	0.9991	20.72	<0.05
Mean	0	0.0146	0.999	–	–

Table 3
Intra- and inter-day precision and accuracy of the method

	Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	Precision (%)	Accuracy (%)	*n*
	125	124	3.4	98.9	6
	250	254	6.1	101.5	6
Intra-day	1000	1001	4.5	100.0	6
	8000	8017	1.3	100.2	6
	24000	24077	2.5	100.3	6
	125	123	3.5	98.0	24
	250	253	4.3	101.1	24
Inter-day	1000	995	3.8	99.5	24
	8000	7694	4.3	96.2	24
	24000	24029	5.54	100.12	24

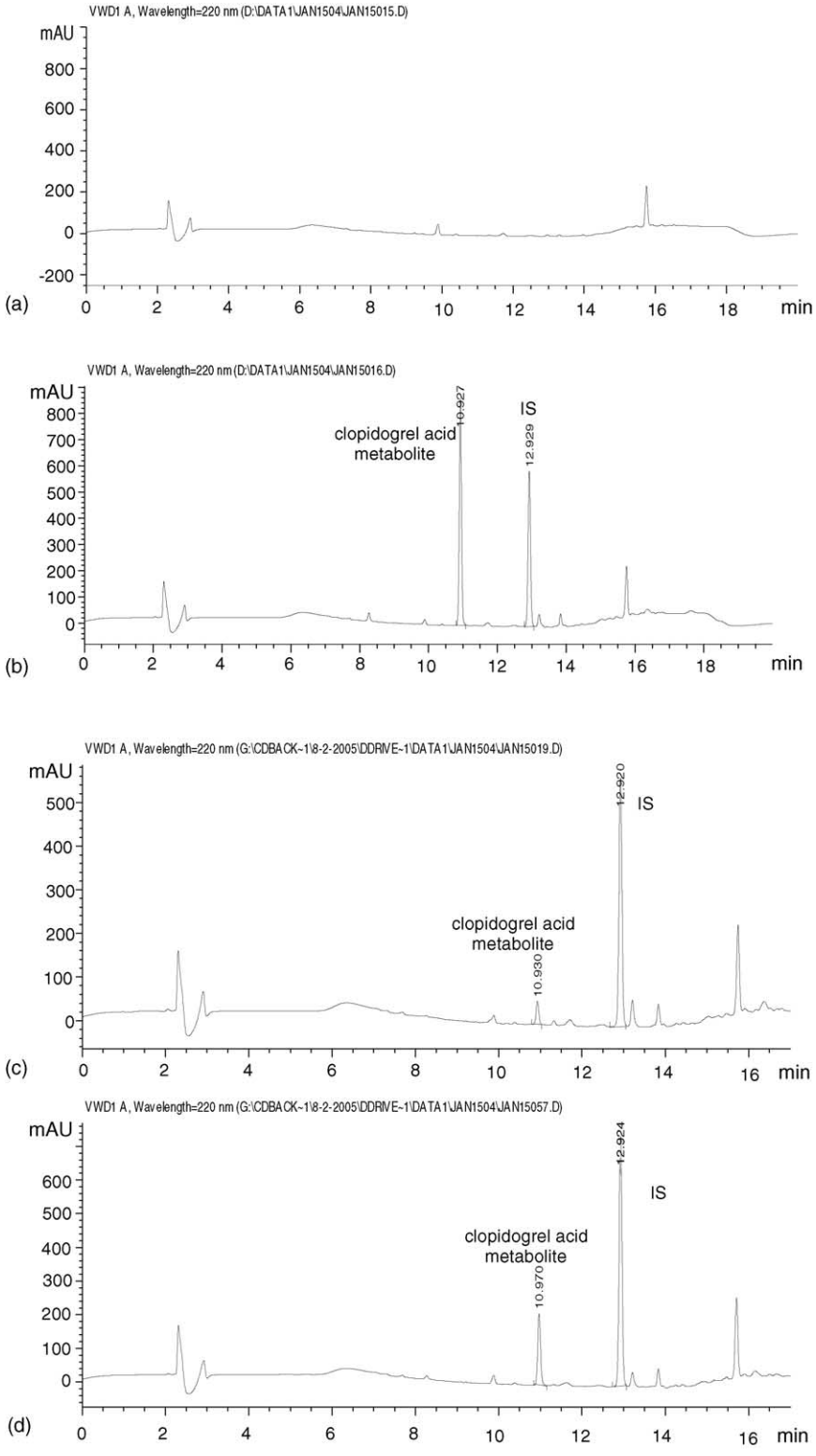


Fig. 2. (a) Representative HPLC chromatogram of blank plasma. (b) Representative HPLC chromatogram of mid QC plasma sample containing carboxylic acid metabolite of clopidogrel (1000.0 ng/ml), and atorvastatin (IS). (c) Representative HPLC chromatogram of LLOQ plasma sample containing carboxylic acid metabolite of clopidogrel (125 ng/ml) and atorvastatin (IS). (d) Representative HPLC chromatogram of 20 min plasma sample containing carboxylic acid metabolite of clopidogrel and atorvastatin (IS).

Table 4
Summary of stability of carboxylic acid metabolite of clopidogrel in rat plasma

Stability	Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	Recovery (%)	R.S.D. (%)	Bias (%)	*n*
Long term (48 days, −70 °C)	250	244	97.7	2.1	−2.3	4
	8000	7822	97.8	1.7	−2.2	4
Short term (24 h, 20°–30 °C)	250	251	100.4	2.1	0.4	4
	8000	8062	100.8	1.1	0.8	4
Auto sampler (35 h, 15 °C)	250	246	98.3	1.7	−1.8	4
	8000	7892	98.7	0.4	−1.4	4
Freeze-thaw (3 cycles, −70 °C)	250	240	95.9	1.9	−4.1	4
	8000	7705	96.3	1.6	−3.7	4
Dry-extract (24 h, −70 °C)	250	249	99.8	0.4	−0.2	4
	8000	7953	99.4	0.9	−0.6	4

accurately quantified after five-fold dilution with drug free plasma and bringing them within the calibration range. The back calculated average concentration was 7948 ng/ml (precision, 0.7%) with the accuracy of more than 99.0%.

3.1.6. Stability

The carboxylic acid metabolite of clopidogrel was stable at −70 ± 5 °C for 48 days in rat plasma. The percent bias for carboxylic acid metabolite of clopidogrel ranged from −2.2% to −2.3% at the concentrations of 250 and 8000 ng/ml, respectively (Table 4). Carboxylic acid metabolite of clopidogrel was found to be stable for 24 h in rat plasma at ambient temperature (20–30 °C). The percent bias observed were 0.4% and 0.77%, at the two QC levels (Table 4).

In the autosampler maintained at 15 °C, the reconstituted extracts were stable for 35 h and the percent bias for the drug concentrations were −1.8 and −1.4% at the two QC levels (Table 4) studied. Plasma samples containing carboxylic acid metabolite of clopidogrel were stable even after subjecting to three-freeze thaw cycles. The percent bias observed after three freeze thaw cycles was −4.1% and −3.7% at low and high QC levels as depicted in Table 4.

Dried residues containing carboxylic acid metabolite of clopidogrel were stable at −70 ± 5 °C for 24 h (Table 4), with a percentage bias of −0.2% and −0.6%, respectively. Which implies that if the analysis was held due to some unanticipated reasons, the processed samples could be safely stored for 24 h without reconstitution. Working solutions of carboxylic acid metabolite of clopidogrel and IS found to be stable for 68 days at 2–8 °C.

3.2. Pharmacokinetic study

The oral pharmacokinetics of the polymorphs of clopidogrel bisulfate was compared in terms of extent (AUC_{0-t} and $AUC_{0-\infty}$) and rate (C_{max} and T_{max}) of absorption.

The mean C_{max} for the two polymorphs; form I and form II, of clopidogrel bisulfate were 12.5 ± 1.4 and 8.0 ± 4.0 μg/ml, respectively. The mean T_{max} for form I and form II were 1.0 ± 0.0 and 1.0 ± 0.2 h, respectively.

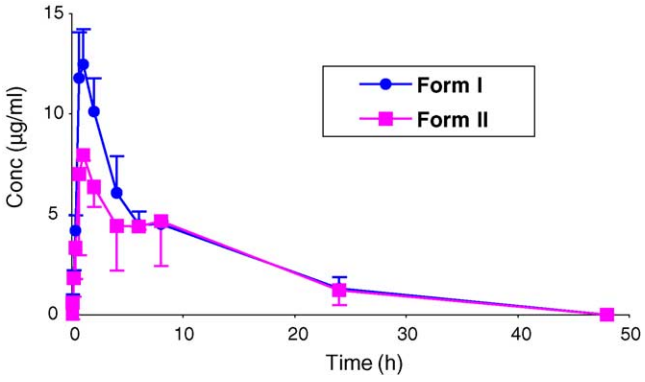


Fig. 3. Mean plasma concentrations vs. time plots of carboxylic acid metabolite of clopidogrel after a single oral dose (30.0 mg/kg) of two different polymorphs of clopidogrel bisulfate in male Wistar rat under fasting condition. (Form I and Form II are the two polymorphs of clopidogrel as described in US patent no US 6,429,210 B1).

The mean $AUC_{(0-t)}$ for form I and form II was 109.8 ± 18.5 and 97.1 ± 26.7 μg h/ml, respectively. The $AUC_{(0-\infty)}$ was found to be 124.2 ± 21.3 and 111.8 ± 33.2 μg h/ml. The mean concentration versus time graphs for the two polymorphs are shown in Fig. 3. The data indicates that form I was superior to form II in terms of both rate and extent of absorption.

4. Conclusions

The simple bio-analytical methodology described in this manuscript allowed the estimation of carboxylic acid metabolite of clopidogrel up to 125 ng/ml in rat plasma with adequate accuracy and precision. The method was validated and was successfully applied to bio-analysis involving the pharmacokinetic study of clopidogrel bisulfate in Wistar rat. The analysis of pharmacokinetic parameters obtained for the two polymorphs confirmed that the form I of clopidogrel bisulfate salt was orally more bioavaliable than form II. A comparison of the present HPLC method with the reported method [8] for the estimation of carboxylic acid metabolite of clopidogrel

shows that the LLOQ of the present method is quite comparable the LLOQ of 93.0 ng/ml in the reported LC–MS method. The present method shows a extraction efficiency more than 85.0% by simple liquid–liquid extraction technique where in the reported method [8] an extraction efficiency of 74.6% was achieved after sample pre-concentration by solid phase extraction technique. The liquid-liquid method used in the present method is one step procedure and is cost effective as compared to solid phase extraction. The only limitation of the present method is the long run time of 20.0 min as compared to the short run time of 6.5 min in the reported [8] method. However, Lagorce et al. [5] have reported a far more sensitive GC–MS method for the estimation of the carboxylic acid metabolite of clopidogrel, in human plasma and serum; with a LLOQ of 5 ng/ml. The analytical procedure is not simple and involves multiple steps of robotic liquid–liquid extraction, followed by solid–liquid extraction and finally derivatization of the analyte using *n*-ethyl di-isopropyl ethylamine and -bromo-2,3,4,5,6-pentafluoro toluene prior to GC–MS analysis.

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