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Short communication

Development and validation of high-throughput liquid chromatography–tandem mass spectrometric method for simultaneous quantification of Clopidogrel and its metabolite in human plasma

Raghunadha Reddy S[a,*], Koteswara Rao.Divi[a], I. Sarath chandiran[b], K.N. Jayaveera[c], Y.K. Naidu[a], M.P. Kalyan Reddy[a,b,c]

[a] Clinical Research and Biosciences (I) Pvt. Ltd., Hyderabad, Andhra Pradesh, India
[b] Gokula Krishna College of Pharmacy, Sullurpet, Andhra Pradesh, India
[c] Jawaharlal Nehru Technological University, Anantapoor, Andhra Pradesh, India

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ABSTRACT

A simple, sensitive and reliable method is described for simultaneous quantification of Clopidogrel and its metabolite in human plasma by using HTLC–MS/MS. The analytical procedure involves on-line coupling of extraction with Cyclone P (50 mm × 0.5 mm 50 μm) HTLC column by injecting 15 μL sample and chromatographic separation is performed with Cohesive Propel C18 (5 μm, 3.0 mm × 50 mm), followed by quantification with mass detector in SRM mode using ESI as an interface. The calibration curves were linear over a concentration range of 0.1–8 ng/mL of Clopidogrel and 70 ng/mL to 6 μg/mL of its metabolite using 20 mL human plasma per batch. The total run time of analysis was 7.5 min and the lower limits of quantification were 0.1 ng/mL for Clopidogrel and 70 ng/mL for its metabolite. The method validation was carried out in terms of specificity, sensitivity, linearity, precision, accuracy and stability. The validated method was applied in bioavailability and bioequivalence study.

1. Introduction

Clopidogrel hydrogen sulfate [1] [methyl (+)-(*S*)-α-(2-chlorophenyl)-6, 7-dihydrothieno [3,2-*c*] pyridin-5(4*H*)-acetate hydrogen sulfate] is a potent oral antiplatelet prodrug. It was routinely used in the treatment of coronary artery disease, peripheral vascular disease, and cerebrovascular disease. It is also used, along with aspirin, for the prevention of thrombosis after placement of intracoronary stent [2]. Clopidogrel, a fast acting novel thienopyridine, has been approved by the FDA for use in patients with acute coronary syndromes. Clopidogrel irreversibly inhibits platelet aggregation [3] by selectively binding to adenylate cyclase-coupled adenosine diphosphate receptors [4] on the platelet surface. Clopidogrel is inactive in vitro and hepatic biotransformation via the cytochrome P450 pathway [5], primarily by CYP3A4 and CYP3A5, 4 is essential for its in vivo antiplatelet activity. The metabolite [6], a thiol compound, is formed by the oxidation of Clopidogrel to 2-oxoclopidogrel and subsequent hydrolysis [7]. The metabolite is highly labile and remains undetected in plasma. The plasma concentrations of the parent drug Clopidogrel are very low (pg/mL levels) due to its extensive metabolism following oral administration in humans. Therefore, the quantification of Clopidogrel in plasma requires a bioanalytical method with high sensitivity. The actual plasma concentrations of parent drug and/or metabolite(s) are of major interest in pharmacokinetic studies. However, the carboxylic acid metabolite of Clopidogrel, which is the most abundant species circulating in blood, was used to document the pharmacokinetic profile of Clopidogrel.

Analytical methods so far reported for quantification of Clopidogrel, employing tandem mass spectrometric detection [8–10] and for quantification of carboxylic acid metabolite, employing HPLC coupled ultraviolet detection [11,12] in human plasma and in human serum [13], mass spectrometric detection [14] and tandem mass spectrometric detection [15]. No method was reported for simultaneous quantification of Clopidogrel and its metabolite. In literature some of the automated methods were reported using high-throughput technique [16].

We now report a first automated high-throughput liquid chromatography tandem mass spectrometric method developed and validated for the simultaneous quantification of Clopidogrel and its metabolite Carboxylic acid derivative of Clopidogrel in human plasma using Ticlopidine as an internal standard. Robotic liquid handling systems are employed in all liquid transfer steps including the sample preparation procedure as well as in the

* Corresponding author at: Crbio (I) Pvt Ltd, C (3) A, Huda Complex, Tarnaka, Secunderabad 500017, India. Tel.: +91 9912463912.
E-mail address: raghu_nathseelam@yahoo.co.in (R. Reddy S).

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addition/removal of the organic solvent. The current method includes a simple and rapid sample preparation as a result of robotic systems utilization that enabled parallel processing as well as significantly shorter analysis run time compared to previously published methods.

2. Experimental

2.1. Chemicals and reagents

Clopidogrel bisulphate ($C_{16}H_{16}ClNO_2S{\cdot}HCl$) and its metabolite ($C_{15}H_{14}ClNO_2S{\cdot}HCl$) are commercially procured from IDDS, Hyderabad. Ticlopidine HCl ($C_{14}H_{14}ClNS{\cdot}HCl$) is commercially procured from SML, Hyderabad. All the solvents used are HPLC grade. Acetonitrile, and methanol were of HPLC grade and obtained from J.T. Bakers. Formic acid, ammonium formate, isopropyl alcohol and acetone were obtained from Merck. Drug free and healthy human plasma was obtained from Clinical Research (I) Laboratory, Hyderabad. Double distilled water is obtained from Sartorius apparatus.

2.2. Data processing

Chromatograms were acquired on a TSQ tandem mass spectrometry (Thermo Finnigan, Sanjose, CA, USA) equipped with Electrospray ionization (ESI) and connected to a PC run with the standard software Xcalibur 2.0.7 and LC Quan 2.5.6. Mass spectroscopic detection was performed on a Triple quadrapole instrument (Thermo, TSQ Quantum Discovery Max). Robotic liquid handling system is operated using the software package supplied from the cohesive technologies Aria™. The calibration curve is constructed by weighted $1/x^2$ least-square linear regression analysis of the peak area ratio (drug/ISTD) vs. the concentration of drug and (metabolite/ISTD) vs. the concentration of metabolite.

2.3. Standard solutions preparation

2.3.1. Stock solution preparation

Approximately 5 mg of Clopidogrel (A)/10 mg of carboxylic acid derivative of Clopidogrel (B)/2 mg of Ticlopidine (IS) working standard is weighed and transferred to 10.0 mL volumetric flask, to this 5.0 mL of methanol is added and sonicated to aid dissolution and the final volume is made up with methanol.

2.3.2. Preparation of internal standard dilution

The Ticlopidine internal standard (ISTD) dilution of about 100 ng/mL from the ISTD stock solution (IS stock) using (80:20 methanol:water) as the diluent is prepared.

2.3.3. Preparation of calibration curve (CC) standards and quality control (QC) samples

Appropriate dilutions of the stock solutions with diluent were made subsequently in order to prepare the working standard solution in the range of 5–500 ng/mL for A and 3–300 μg/mL for B. All the solutions were stored in a refrigerator between 2 °C and 8 °C. Calibration standards and quality control samples, in the range of 0.1–8 ng/mL for A and 70–6000 ng/mL for B were prepared for calibration. Accuracy and precision, quality control and stability assessment were done by spiking 0.5 mL of drug free plasma with appropriate volume of working solution.

2.4. Solutions used for robotic on-line sample extraction system

10 mM Ammonium formate buffer is used in pump A, pure acetonitrile is used in pump B, 0.1% formic acid is used in pump C and washing solution in the ratio of 65:20:15 (acetonitrile:IPA:acetone) is used in pump D.

Table 1
Source specific and compound specific mass spectrometric parameters.

Parameters		MS/MS (SRM)	
Source specific			
Spray voltage		5000	
Auxiliary gas		60 Psi	
Auxiliary gas		5 cm^3 min^{-1}	
Capillary temperature		300 °C	
	Clopidogrel	Clopidogrel metabolite	Ticlopidine (IS)
Compound specific			
Collision energy (CE)	16	19	31
Tube lens offset	105	82	92
Skimmer offset	08	05	08

2.5. Sample preparation

Retrieve the frozen CC, QC and subject samples from the deep freezer and thaw in water bath maintained at room temperature, vortex to mix. Remove the caps from the polypropylene tubes. Aliquot 0.5 mL of CC, QC and subject samples into pre-labelled HPLC vials. Add 50.0 μL of ISTD dilution (100 ng/mL) followed by 0.200 mL of 10 mM ammonium formate buffer of pH 7.5 into vials, cap it, vortex to mix and transfer vials to auto sampler.

3. Results and discussion

3.1. Chromatographic and mass spectrometric conditions

The LC/MS/MS system consisted of four pumps for gradient solvent delivery, and a divert valve to direct LC effluent to the mass spectrometer in the analyte elution window. The analytical column effluent is directed through the divert valve to a thermo electron TSQ quantum discovery mass spectrometer. Source specific and compound specific parameters are presented in Table 1.

The instrument is operated in the positive ion mode. The precursor $[M{\cdot}H]^+$ ions at m/z 321.372, 307.337 and 263.353 for Clopidogrel, its metabolite and Ticlopidine, respectively are selected by the first quadrupole (Q1). After collision-induced fragmentation in Q2, the product ions at m/z 211.870, 197.853 and 124.824 for Clopidogrel, its metabolite and Ticlopidine, respectively, are monitored in Q3. A resolution of one unit (at half peak height) is used for both Q1 and Q3. The full scan of parent and product ion spectra is shown in Figs. 1–3.

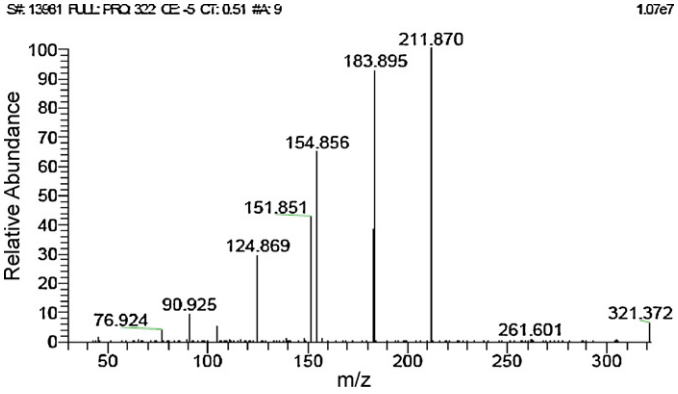

Fig. 1. Mass spectra of the Clopidogrel precursor (321.372 m/z) and major fragment (211.870 m/z).

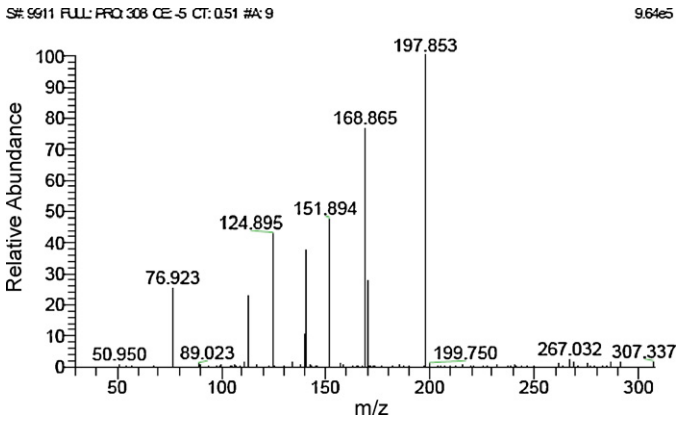

Fig. 2. Mass spectra of the Clopidogrel metabolite precursor (307.337 *m*/*z*) and major fragment (197.853 *m*/*z*).

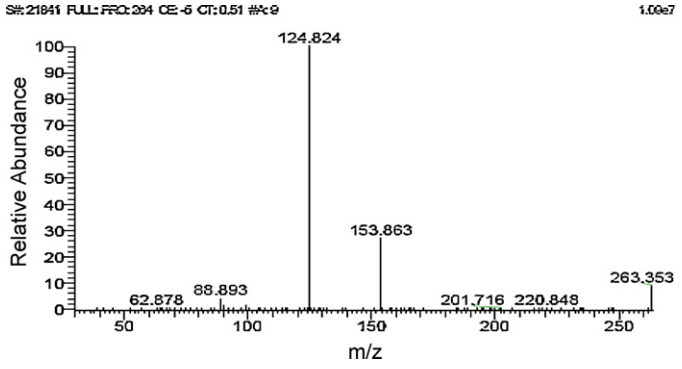

Fig. 3. Mass spectra of the Ticlopidine precursor (263.353 *m*/*z*) and major fragment (124.824 *m*/*z*).

3.1.1. Steps involved in on-line robotic method development

A typical two-column setup featuring two six-port switching valves as described by Herman [16] is employed for method development. The procedure consisted of four steps:

(1) The eluent loop is filled with 50% acetonitrile in 10 mM ammonium formate.
(2) 15 μL sample is loaded onto the cyclone P (50 mm × 0.5 mm, 50 μm) HTLC column at a flow rate of 2 mL/min during 60 s.
(3) The eluent loop is discharged at 0.5 mL/min for 60 s to transfer the analytes from HTLC column onto the Cohesive Propel C18 (50 mm × 2.1 mm i.d., 5 μm) column and 0.5% aqueous formic acid at 0.2 mL/min in added post column.
(4) LC–MS/MS is performed using ballistic gradient at 2.0 mL/min (10–90% acetonitrile in 0.5% formic acid).

3.2. On-line sample extraction

The gradient program accomplished a cyclone HTLC column for sample extraction, elution with four pumps as reported in Table 2. TLX turbo flow on-line technique is employed for separation of analyte from sample molecules. The mechanism involved in sample preparation may be affinity. The small drug molecules bind to the HTLC column, and molecules that have lower binding affinity quickly diffuse into the column particles and large sample molecules are flushed to waste, then the mobile phase elutes the analyte molecules that are bound at HTLC column to analytical column, from this analytical column analytes are entered to mass detector. To achieve required chromatograms with consistency we have performed different combinations of the solvents and gradient system. Finally we succeeded with the solution combinations as mentioned in Table 2 and analyzed more than 150 samples without overloading of the chromatographic columns with improved real throughput efficiency.

3.3. Ion suppression

One important factor that can affect the quantitative performance of a mass detector is ion suppression. Sample matrix, coeluting compounds and cross-talk can contribute to this effect. Ionization suppression typically observed in sample extracts from biological samples is not likely to be caused ionization suppression is the result of high concentrations of nonvolatile materials present in the spray with the analyte. The exact mechanism by which the nonvolatile materials inhibit release of analyte into the gas phase has not been clearly demonstrated, although a likely list of effects relating to the attractive force holding the drop together and keeping smaller droplets from forming should account for a large portion of the ionization suppression observed with ESI. Once nonvolatile materials have been removed from sample preparation, there is no guarantee that suppression of ionization will no longer be a problem, other mechanisms such as impairing agents (e.g. trifluoro acetic acid) may play a role in ionization suppression. Bonfiglio et al. [17] reported the effects of sample preparation methods on the variability of ESI response. According to their results precipitation method showed the greatest amount of ESI response suppression followed by solid-phase extraction while liquid–liquid extracts demonstrated the least. In our study robotic liquid handling system was employed for sample extraction from plasma and 0.1% formic acid was employed as mobile phase additive to minimize ion suppression.

3.4. Assay validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose (International Conference on Harmonization Guideline Q2A) “Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use” (US Food and Drug Administration Draft Guidance for Industry, 2000).

3.4.1. Specificity and selectivity

Six human plasma samples from six individual healthy donors receiving no medication were extracted and analyzed for the assessment of potential interferences with endogenous substances.

Table 2
Steps involved in on-line robotic method.

Step	Start	S	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0.00	30	2.00	Step	0.0	100.0	0.0	0.0	–	Out	0.80	Step	20.0	80.0
2	0.50	90	0.40	Step	50.0	0.0	50.0	0.0	T	In	0.80	Step	20.0	80.0
3	2.00	30	2.00	Step	0.0	0.0	0.0	100.0	–	In	0.80	Ramp	20.0	80.0
4	2.50	60	2.00	Step	0.0	0.0	0.0	100.0	–	In	0.80	Step	20.0	80.0
5	3.50	60	2.00	Step	50.0	50.0	0.0	0.0	–	In	0.80	Step	20.0	80.0
6	4.50	30	2.00	Step	50.0	50.0	0.0	0.0	–	In	0.80	Step	20.0	80.0
7	5.00	60	2.00	Step	0.0	100.0	0.0	0.0	–	Out	0.80	Step	20.0	80.0

Table 3
Back calculated concentrations from calibration curves.

Nominal conc. Clopidogrel (pg/mL)	102	285	816	1632	3265	4472	6577	8433
Mean accuracy (%)	99.8	102	96.5	99.2	95.2	100.8	99.8	106.7
Precision (%)	0.5	4	5.2	9.6	5.6	4	5.4	3.7
Nominal conc. metabolite (ng/mL)	73	203	581	1163	2327	3188	4689	6012
Mean accuracy (%)	101.7	96	96.7	100.6	100.8	100	100.6	103.5
Precision (%)	2.1	5.6	10.4	5.2	7.1	3	2.9	3.5

Accuracy: 100% measured concentration/nominal concentration. Precision: coefficient of variation (100% standard deviation/mean).

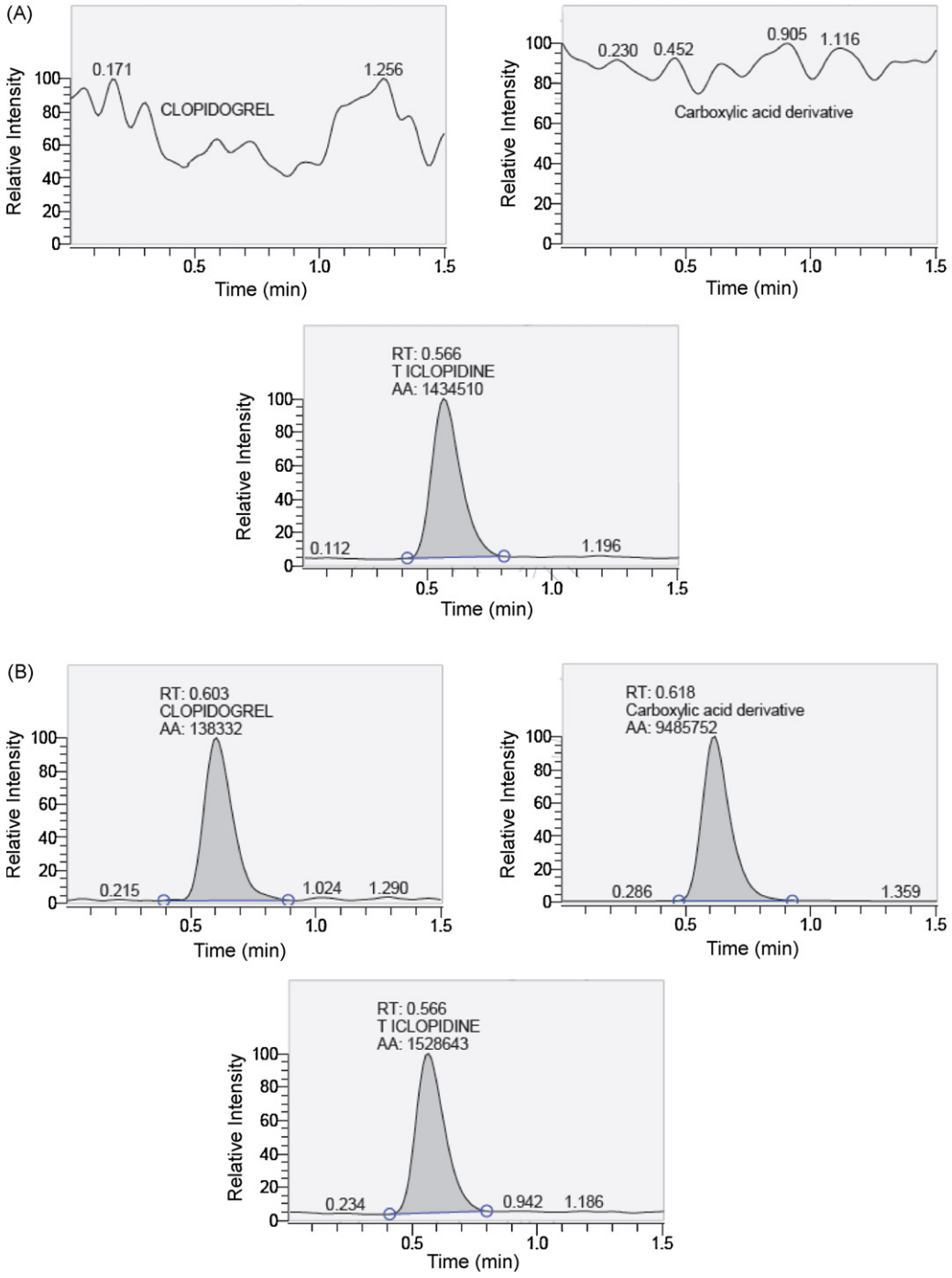

Fig. 4. (A) Representative chromatograms from an extract of human blank plasma spiked with Ticlopidine as IS. (B) Representative chromatograms from an extract human blank plasma spiked with Clopidogrel, its metabolite and Ticlopidine (as IS).

Table 4
Assessment of accuracy and precision of the method.

Nominal conc. Clopidogrel (pg/mL)	5548	2774	299	125
Intra-day accuracy (%) (day 1)	95.8	97.7	97.0	92.8
Intra-day precision (%) (day 1)	2.9	7.9	3.9	10.8
Intra-day accuracy (%) (day 2)	101.8	93.9	95.7	104.9
Intra-day precision (%) (day 2)	6.7	5.3	5.7	5.3
Intra-day accuracy (%) (day 3)	111.0	95.0	95.2	100.6
Intra-day precision (%) (day 3)	10.6	13.5	12.3	15.1
Overall accuracy (%)	102.8	95.5	96.0	103.3
Overall precision (%)	7.6	2.0	1.0	2.7
Number of determinations	18	18	18	18
Nominal conc. metabolite (ng/mL)	3955	1977	213	89
Intra-day accuracy (%) (day 1)	94.5	93.6	91.8	80.8
Intra-day precision (%) (day 1)	2.8	6.9	3.0	6.4
Intra-day accuracy (%) (day 2)	100.4	92.2	91.9	93.9
Intra-day precision (%) (day 2)	4.3	5.9	3.2	5.6
Intra-day accuracy (%) (day 3)	110.4	94.7	90.8	103.2
Intra-day precision (%) (day 3)	4.5	3.3	5.5	3.2
Overall accuracy (%)	101.8	93.5	91.5	92.6
Overall precision (%)	7.9	1.3	0.6	12.2
Number of determinations	18	18	18	18

The apparent responses at the retention time of drug, metabolite and internal standard were compared to the response at the lower limit of quantification (LLOQ) for drug, metabolite and to the response at the working concentration for internal standard. Observed retention times were about 0.6 min (Clopidogrel), 0.7 min (Clopidogrel metabolite) and 0.5 min (Ticlopidine) respectively. No additional peak due to endogenous substances that could have interfered with the detection of the compounds of interest was observed. Representative chromatograms from an extract of human blank plasma spiked with internal standard (Ticlopidine) and from extract human blank plasma spiked with Clopidogrel, its metabolite and internal standard (Ticlopidine) are shown in Fig. 4A and B.

3.4.2. Linearity

Linearity means that the assay provides test results that are proportional to the concentration of the analyte in the sample either directly or via a mathematical transformation. The relationship between the experimental response value and known concentrations of the analyte is referred to as calibration curve. In our study calibration curve is constructed by weighted $1/x^2$ of the peak area ratio (drug/IS) vs. the concentration of drug and (metabolite/IS) vs. the concentration of metabolite with the above calibration standards to generate a calibration curve. Linear calibration curves were obtained with a coefficient of correlation (r^2) usually higher than 0.995. For each calibration standard level, the concentration was back calculated from the linear regression curve equation. The mean accuracy and precisions for back calculated concentrations of each standard calculated from calibration curves were tabulated as Table 3.

3.4.3. Recovery

Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations with unextracted standards that represent 100% recovery. Recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise and reproducible. The recoveries of Clopidogrel, its metabolite and Ticlopidine were evaluated with 6 replicates at 3 different concentration levels. In our method we got 94%, 88% and 90% recovery for Clopidogrel, its metabolite and Ticlopidine, respectively, which are within the acceptance criteria.

3.4.4. Precision and accuracy

Intra-day accuracy and precision were evaluated by analysis of quality control samples at 4 different levels ($n = 6$ at each level) on the same day. These levels were chosen to demonstrate the performance of the method and to determine the lower limit of quantification of the method. The upper limit of quantification was given by the highest level of the calibration curve. Samples with concentration above this upper limit of quantification should be diluted prior to reanalysis. To assure the interday accuracy and precision, the intra-day assays were repeated on 3 different days. The overall performance was calculated. The results were found to be quite comfortable as per international guidelines.

The accuracy and precision for interday and intra-day were tabulated for both drug and metabolite in Table 4.

3.4.5. Stability

According to FDA guidelines, stability assessments i.e. Freeze-thaw, bench top, short-term, long-term, stock solution and post preparative stabilities are evaluated as a part of Bioanalytical method validation. In our study quality control plasma samples are used subject to bench top (8 h), in injector (10–85 h), freeze-thaw (−20 to +20 °C) cycles, short-term (24 h) at room temperature and long-term (25 days) at deep freezer (at −20 °C) tests are performed. The values obtained for present stability studies

Table 5
Stability results.

Experiment	Clopidogrel				Metabolite			
	Accuracy[a]		Precision[a]		Accuracy[a]		Precision[a]	
	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC
Freeze-thaw stability	97.6	93.2	5.2	11.5	103.0	95.0	3.9	3.2
Bench top Stability	101.0	97.2	3.0	3.7	101.6	100.4	5.7	4.1
Auto sampler Stability	99.4	101.0	8.5	3.0	98.8	104.0	6.4	4.5
In injector stability	99.8	95.9	4.3	12.8	96.6	85.1	2.9	2.9

[a] Mean of six determinations.

Table 6
Pharmacokinetic parameters of Clopidogrel and its metabolite.

Clopidogrel			Clopidogrel metabolite		
PK Parameters	Formulation		PK Parameters	Formulation	
	Test	Reference		Test	Reference
C_{max} (pg/mL)	1704.37	1599.97	C_{max} (ng/mL)	654.341	680.356
AUC0-t (pg h/mL)	3330.29	3356.44	AUC0-t (ng h/mL)	1050.84	1093.55
AUC0-inf (pg h/mL)	4144.9	4089.38	AUC0-inf (ng h/mL)	1384.34	1408.26
T_{max} (H)	1.166	1.361	T_{max} (H)	1.138	0.945
Kel (H-1)	0.418	0.491	Kel (H-1)	0.425	0.406
T1/2 (H)	2.443	2.387	T1/2 (H)	2.167	2.083

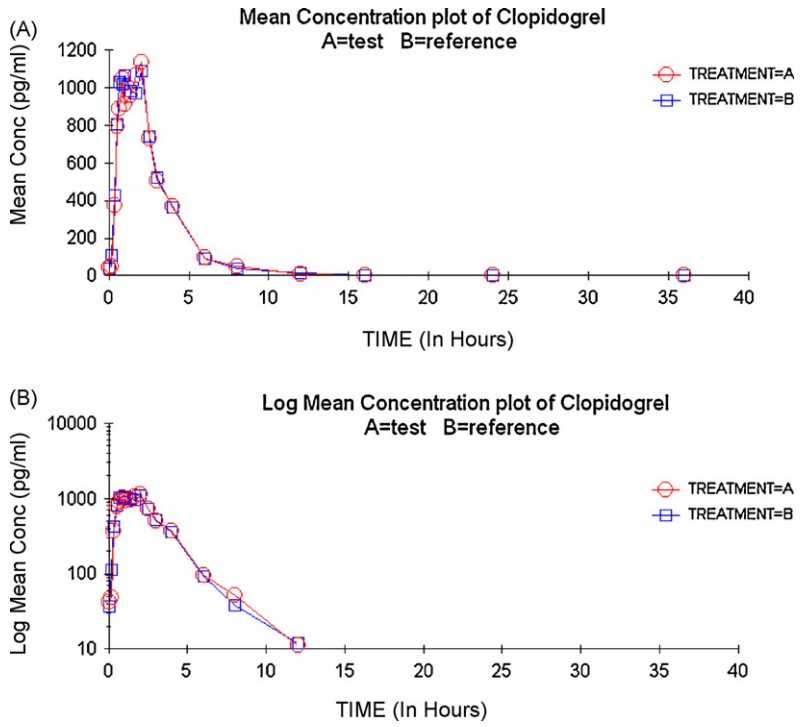

Fig. 5. (A) Mean plasma concentration–time profiles of Clopidogrel. (B) Mean plasma concentration–time profiles of Clopidogrel metabolite.

are tabulated (Table 5), which are within the acceptance criteria.

3.5. Application of the method

The present method was applied for a randomized cross-over bioequivalence study of two different Clopidogrel preparations in 12 healthy male volunteers. After single oral administration of the drug blood samples were collected at a suitable time intervals up to 36 h. This method was successfully used to measure the plasma concentrations of Clopidogrel and its metabolite. Various pharmacokinetic parameters established and compared for the both of the preparations were given in Table 6. Plasma concentration–time profiles were given as graph (Fig. 5).

4. Conclusion

On-line coupling requires some modifications to the off-line extraction techniques. The coupling is most commonly performed with the help of multiport valves and one or more pumps for the dynamic extraction or transfer of the extract to the chromatographic system, the extraction can be performed in either static or dynamic mode or as a combination of these so long as the extraction system allows the on-line transfer of the extract to the chromatographic system. In on-line systems, the whole extract is transferred to the chromatographic column, in contrast to traditional off-line techniques where only a small part is injected. This means that the sensitivity of the on-line method is much better. However, the high sensitivity easily leads to overloading of the analytical column. Miniaturisation of the extraction system is often required to avoid this. In our method miniaturisation is achieved with small extraction in extraction vessels and the total analysis means sample extraction, chromatographic separation and mass spectrometric detection has been completed within 7.5 min for one sample quantitation.

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