Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 183–189
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Identification and characterization of a principal oxidation impurity in clopidogrel drug substance and drug product

Arivozhi Mohan *, M. Hariharan [a], E. Vikraman [a], G. Subbaiah [a], B.R. Venkataraman [b], D. Saravanan [c]

[a] *Torrent Research Center, C/O Torrent Pharmaceuticals Ltd., Indira Bridge, Bhat Village, Gandhi Nagar, Pin code 382428, Gujarat, India*
[b] *Department of Chemistry, M.R. Government Arts College, Mannargudi, Pin code 614001, Tamilnadu, India*
[c] *Department of Chemistry, National College, Tiruchirappali, Pin code 620001, Tamilnadu, India*

Received 29 August 2007; received in revised form 15 November 2007; accepted 6 December 2007
Available online 23 December 2007

Abstract

The focus of this study is identification, isolation and characterization of a principal oxidation impurity of clopidogrel which ranged from 0.05 to 0.12% using high performance liquid chromatography. This impurity is considered as principal oxidation impurity as it is observed in oxidative degradation (stress) study. Preparative HPLC with Xterra MS C18 ODB column was used to isolate the impurity. The isolated impurity was co-injected with the sample containing impurities and found the retention time match of the spiked impurities. A thorough study was undertaken to characterize this impurity and based on their spectral data (UV, MS, MS^n $^1H/^{13}C$, DEPT and 2D NMR) the structure was characterized as 5-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-6,7-dihydrothieno[3,2-*c*]pyridin-5-ium with a molecular weight 320 amu.

Keywords: Clopidogrel related compound; Degradation product characterization; LC–MS/MS

1. Introduction

Clopidogrel bisulfate, methyl (+)-(*S*)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-*c*]pyridine-5(4H)-acetate sulfate (1:1), is a potent oral antiplatelet agent often used in the treatment of coronary artery disease, peripheral vascular disease and cerebrovascular disease. It is marketed by Bristol-Myers Squibb and Sanofi-Aventis under the trade name Plavix which is the world's second highest selling pharmaceutical with sales of US$5.9 billion. The mechanism of action of clopidogrel is irreversible blockade of the adenosine diphosphate (ADP) receptor P2Y12 and is important in platelet aggregation, the cross-linking of platelets by fibrin. The blockade of this receptor inhibits platelet aggregation by blocking activation of the glycoprotein IIb/IIIa pathway. Platelet inhibition can be demonstrated 2 h after a single dose of oral clopidogrel, but the onset of action is slow, so that a loading-dose of 300–600 mg is usually administered [1].

Four impurities of clopidogrel have been already identified and documented in the literature [2–6] and named as clopidogrel related compound A, positional stereo isomers of clopidogrel named as clopidogrel related compounds B1 and B2 and a chiral isomer of clopidogrel named as clopidogrel related compound C. It has also been established that these positional stereoisomers (B1 and B2) are process impurities and other impurities are formed during the process and also self-degradation. Marketed samples of Plavix and few batches of drug substances were analyzed using reported method [3]. An Ultron ES-OVM L 57, chiral specific column (Shinwa chemical industries, Japan) with dimensions of 150 mm × 4.6 mm i.d. packed with 5.0 μ particle size was employed for separation. Acetonitrile–potassium phosphate buffer (10 mM) (75:25, v/v) was used as mobile phase and flow rate was kept at 0.8 ml/min with the detection at 220 nm for 30 min. Relative retention time (RRT) of the related compounds A, B1, clopidogrel B2 and C were found, respectively, at 0.46, 0.93, 1.0, 1.1 and 2.10. Related compound D was found at about 2.0 min (RRT of 0.30). As the related compound D elutes in the void volume of the system, various buffer composition, pH, gradients were attempted and found unsuccessful. The main problems that occurred were peak shape, peak purity

* Corresponding author. Tel.: +91 79 23969100; fax: +91 79 23969135.
E-mail address: arivozhimohan@hotmail.com (A. Mohan).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2007.12.021

and blank interference due to peroxide. Hence, conventional, cost effective, new method was developed wherein good peak shape and peak purity were achieved. The new method involves Hypersil BDS C8 column (Thermo Electron Corporation) with gradient conditions for the separations.

During the analysis, it has been observed that the new impurity content in clopidogrel tablets, were in the range of 0.05–0.07% (by area percentage) and in drug substance it ranges from 0.08 to 0.12% (by area percentage). Typical chromatograms of clopidogrel drug substance, drug product and drug product spiked with the related compound D were shown in Fig. 1. It is mandatory requirement from regulatory authorities, to identify and characterize any unknown impurity present in it at a level as low as 0.05% [7,8]. The presence of this impurity

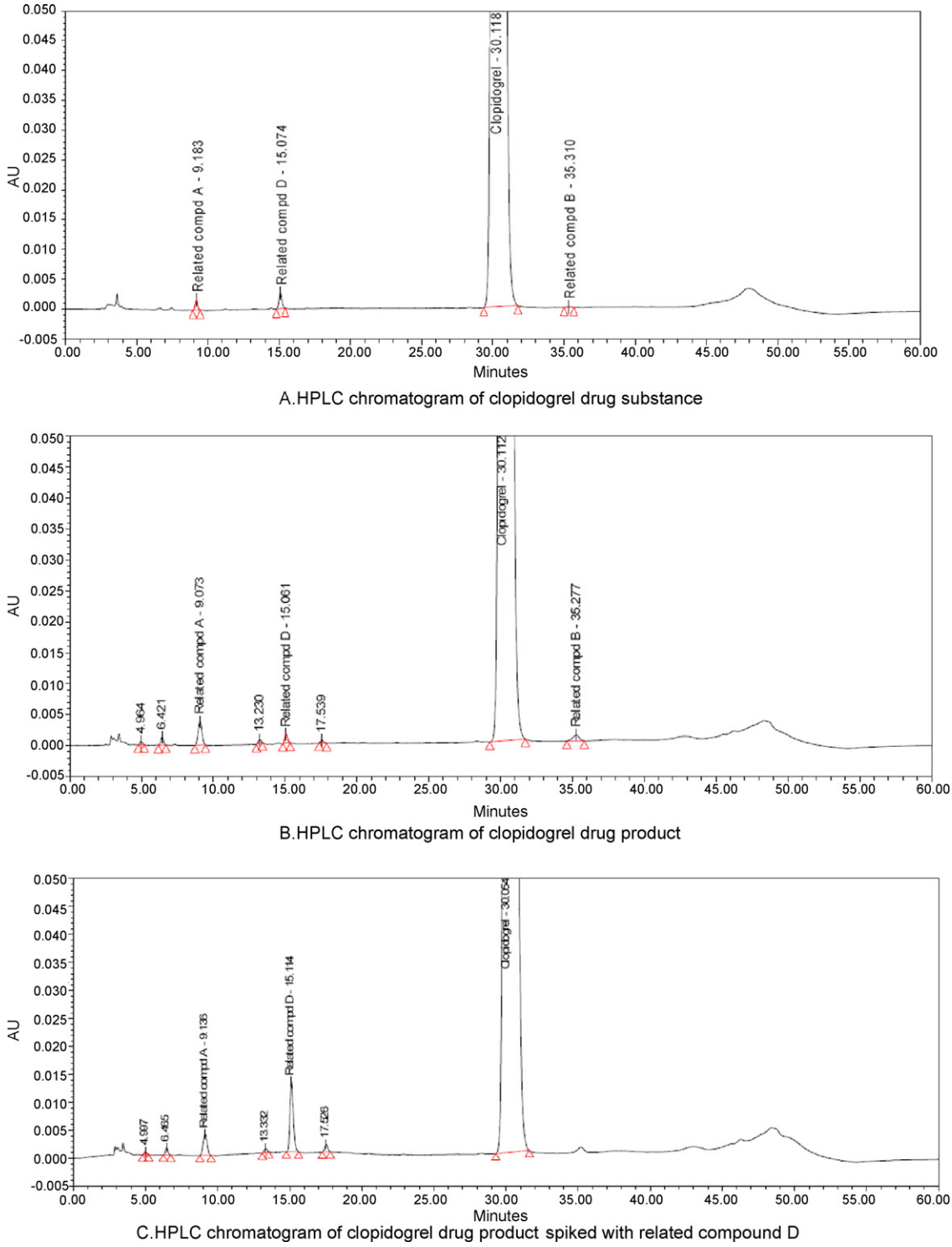


Fig. 1. Typical HPLC chromatograms of (A) clopidogrel drug substance, (B) drug product and (C) drug products spiked with related compound D.

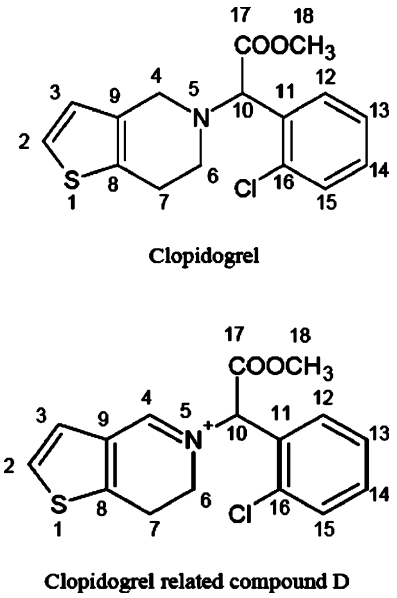


Fig. 2. Chemical structures of clopidogrel and clopidogrel related compound D.

in tablets can have a significant impact on quality and safety of the important drug. The isolation of any impurity is required to find the response in an analytical method and also to validate the analytical procedure for its quantitative estimation. Even though the same impurity is formed during oxidation condition, there is no report on the isolation and characterization. Hence comprehensive study was undertaken to identify and characterize the oxidative impurity. The new oxidation impurity in this paper referred as related compound D. The chemical structures of clopidogrel, and related compound D were shown in Fig. 2.

2. Experimental

2.1. Materials and methods

Clopidogrel bisulphate was purchased from Dr. Reddys Laboratories Ltd., Hyderabad, India and clopidogrel tablets of brand name Plavix manufactured by Sanofi Pharma Bristol Myers Squibb Inc. were used. Potassium phosphate and ammonium acetate, GR grade was obtained from E. Merck, India. Methanol, acetonitrile of HPLC grade were obtained from E. Merck, India. Purified water was collected through Milli-Q water purification system (Millipore, USA). Dimethylsulphoxide-*d*6 (DMSO-*d*6) was purchased from Aldrich Chemical Co., USA and all other chemical used were of analytical grade.

2.2. Analytical methods

Analytical method was developed using Waters HPLC system consisting Alliance integrated hardware of quaternary solvent delivery module, auto sampler and PDA detector. Data was processed through Waters Empower software Version 1.63. Hypersil BDS C8 column (Thermo Electron Corporation) with dimensions of 250 mm × 4.6 mm i.d. packed with 5.0 μ particle size was employed along with gradient conditions for the separations. The gradient involves two mobile phases consisting of acetonitrile–potassium phosphate buffer (pH 2.3; 10 mM) (20:80, v/v) as solvent A and acetonitrile–potassium phosphate buffer (pH 2.3; 10 mM) (80:20, v/v) as solvent B. The gradient program employed with a timed gradient program of *T* (min)/%*B* (v/v): 0.01/0, 5/0, 15/15, 40/30, 45/0, 60/0 for the separations. Flow rate was kept at 1.0 ml/min and the column eluent was monitored at 220 nm for 60 min.

2.3. Preparative HPLC method

Preparative HPLC system used was a Waters system equipped with W 600 quaternary solvent delivery module Delta prep 2487 dual wavelength UV detector. Data was processed through Waters empower software. An Xterra MS C18 ODB HPLC column (Waters, Ireland) with dimensions 100 mm × 30 mm packed with 5.0 μ particle size was used for preparative work. The gradient conditions employed for the separations with a timed gradient program of *T* (min)/%*B* (v/v): 01/10, 06/10, 16/95, 17/95, 18/100, 23/100, 28/10, 32/10. Flow rate was kept at 20 ml/min and the column eluent was monitored at 220 and 300 nm for about 32 min.

2.4. Infra-red spectroscopy

The IR spectra were recorded in the solid state as KBr as dispersion using Shimadzu FT-IR 8700 with DRS technique.

2.5. Mass spectrometry

The isolated compound was dissolved (about 0.05 mg/ml) in methanol containing of 0.1% formic acid (v/v) and infused into the ion source by the syringe pump at the rate of 10 μl/min. The mass spectrum of the isolated degradation product was acquired on a Finnegan LCQ instrument from Thermoquest (San Jose CA) in positive spray ionization (ESI+) mode. The spray potential was set at 5.6 kV and the capillary temperature at 220 °C. Mass range was scanned between 100 and 500 amu. The mass spectrum was also recorded in negative spray ionization (ESI−) mode. The spray potential was set at 5.6 kV and the capillary temperature at 220 °C.

2.6. Nuclear magnetic resonance

^{1}H (400.13 MHz) and ^{13}C (100.62 MHz) NMR spectra of isolated related compound D was recorded on an Avance DPX-400 MHz spectrometer Bruker (Germany). The probe was a ^{1}H/^{13}C 5 mm, 3 axis gradients (*x*, *y*, *z*), optimized for inverse detection. Spectra were recorded in DMSO-*d*6 (5-mm tubes) at 300 K. Sample concentration was 0.6 mg in 0.6 ml. The residual protonated resonance of the solvent (DMSO-*d*6) was used as an internal chemical shift standard, which was related to tetramethylsilane with chemical shifts of 2.5 and 39.2 ppm, respectively, for ^{1}H and ^{13}C. Processing of the raw data were performed using Bruker XWinNmr software. The pulse conditions were 90° pulse, 9.4 μs (attenuation 0db) for ^{1}H and 30° pulse, 11.75 μs (attenuation 0db) for ^{13}C. Gradient pulses

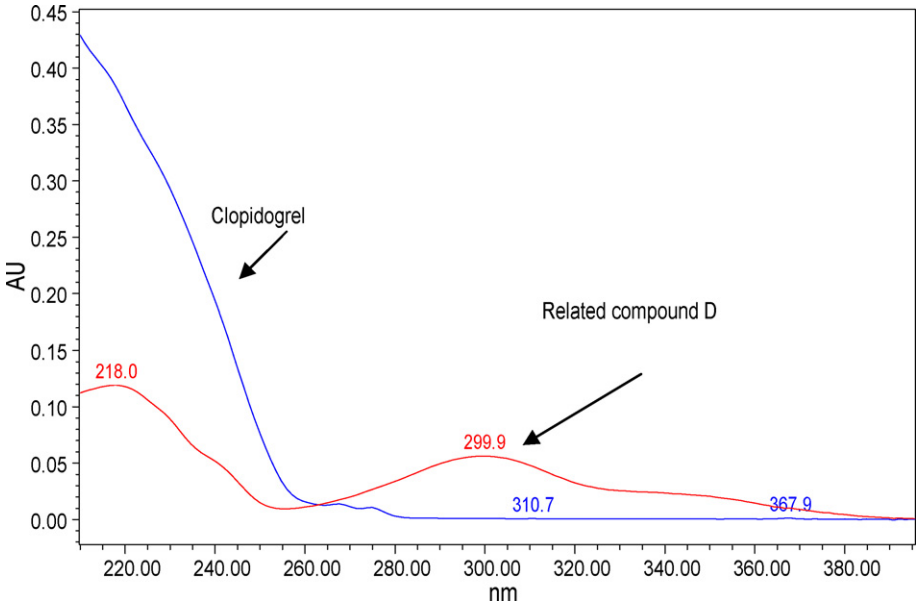


Fig. 3. UV spectral overlay of clopidogrel and related compound D.

used in this study were all shaped to a sine envelope with 1 ms duration (DQF-COSY, and $^{1}H/^{13}C$ HSQC). Spectral width was 5431.88 Hz for proton and 18111.66 Hz for carbon.

2.7. Preparation of degradation samples of clopidogrel

2.7.1. Acid and base degradation

A solution of clopidogrel bisulphate (500 mg) in 50 mL of 0.1 N hydrochloric acid was kept at 80 °C for 60 min. Another sample was prepared in a similar manner by treating clopidogrel bisulphate (500 mg) in 50 mL of 0.1 N sodium hydroxide.

2.7.2. Peroxide degradation

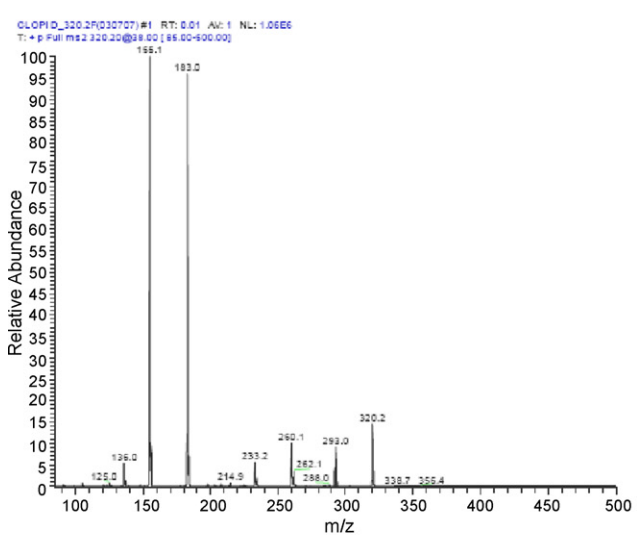
A solution of clopidogrel bisulphate (500 mg) in methanol (0.5 mL) and hydrogen peroxide (5% in water, 5 mL) was kept at 60 °C for 3 h. Similarly 10 tablets of clopidogrel bisulphate were powdered (equivalent to 500 mg of clopidogrel bisulphate) was dissolved in 0.5 ml of methanol and 5 ml of hydrogen peroxide (5%) and the solution was kept at 60 °C for 3 h.

2.7.3. Thermal degradation

Clopidogrel bisulphate (1.0 g) was moistened with water and was kept in an oven maintained at 120 °C for 24 h.

2.8. Analysis of degradation samples by analytical LC

The degradation samples were diluted to the required concentration analyzed with analytical LC. 2 and 7% of related compound D was found in the drug substance and drug product (Plavix), respectively, at oxidative condition. In other con-



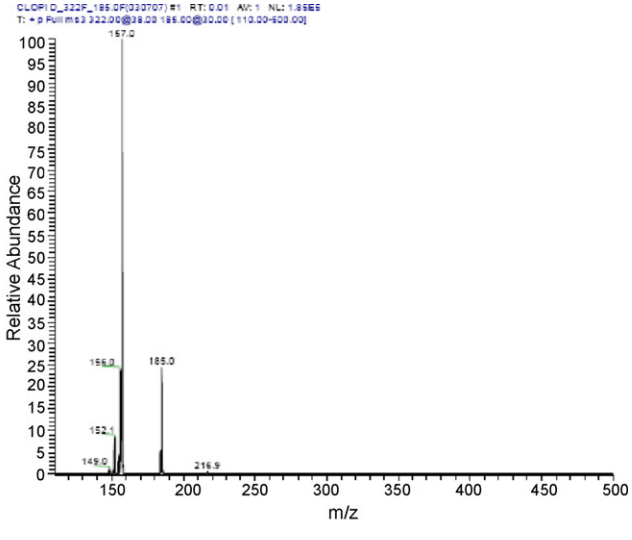
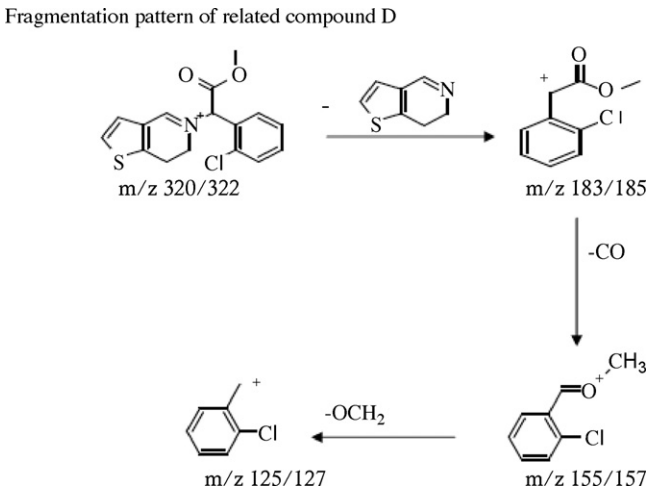


Fig. 4. MS–MS spectrum of related compound D's chlorine isotope.



Scheme 1. Fragmentation pattern of related compound D.

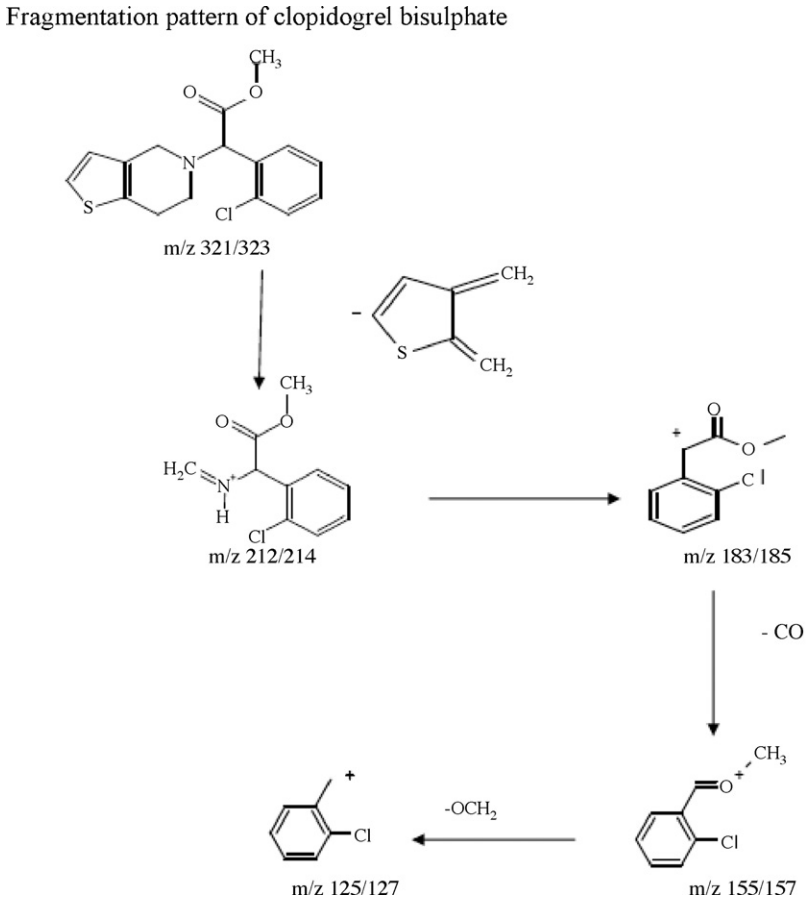
ditions, formation of related compound D was not noticed. The purity angle of all the impurities was found less than that of purity threshold and thus the peak purity of the impurities were confirmed. The spectrum of the related compound D and clopidogrel were extracted from PDA detector in the range of 210–400 nm. The UV spectra are presented in Fig. 3.

2.9. Isolation of degradation product(s) by preparative LC

Clopidogrel bisulphate (5 g) was treated with hydrogen peroxide (5%, 15 mL) and kept at 80 °C for 3 h. The aqueous layer was washed with dichloromethane to remove clopidogrel. The aqueous layer was subjected to preparative LC as described in Section 2.4 and as many as 10 fractions were collected separately. Purity of all these fractions were analyzed by analytical LC and found to be in the range of 99%. The fractions were pooled together, 100 mg of sulfuric acid was added and the solvent was evaporated. The resulted solid was reanalyzed on analytical LC and the purity of the same was found to be 99% which was good enough for carrying out the spectroscopic experiments.

2.10. Characterization of the degradation product

The isolated related compound D was injected in both the analytical HPLC methods. The retention time and UV spectrum obtained in the PDA detector matches with that of targeted impurity. Characterization of the related compound D was performed using analytical data obtained from IR, UV, Mass, MS^n experiments, $^1H/^{13}C$ NMR spectrum, DEPT and 2D NMR experiments. The MS–MS spectrum is presented in Fig. 4.



Scheme 2. Fragmentation pattern of clopidogrel bisulphate.

3. Results and discussion

In forced oxidative degradation study 2 and 7% of related compound D was found in the drug substance and drug product (Plavix), respectively. Hence the experiment was used to enrich the impurity. It was also confirmed that related compounds A and C are the degradation products and related compounds B1 and B2 are process impurities based on their trend. The higher level of related compound D in the drug product reveals that the susceptibility is more in the drug product than that in drug substance.

The isolated related compound D was found as off white powder and shows UV absorbance maxima at 299.9 nm which is higher than that of clopidogrel (220 nm). The +ve ES–MS spectrum of the related compound D showed peaks at *m/z* 320 and 322 corresponding to the ^{35}Cl and ^{37}Cl isotope, respectively. The compound does not form Lithium adduct ion and −ve ES–MS spectrum showed no peaks states that the molecular ion obtained is positively charged, i.e., *m/z* 320/322 is due to M^+. In comparison with clopidogrel, the related compound D corresponds to 1 atomic mass unit (amu) less which can be presumed to have similar structure as that of clopidogrel but with short of one hydrogen atom.

Two daughter ions were obtained at *m/z* 183 and 155 when the molecular ion M^+320 fragmented in MS/MS experiments. Both the daughter ions were contains chlorine atom as it was confirmed by MS/MS experiments of the chorine isotope molecular ion M^+322 and the m/z values of the daughter ions were 185 and 157. As the peak intensity ratios are nearly identical, it was confirmed that the eliminated neutral fragments contains no chlorine atom. Further MS^2 experiments of daughter ion (*m/z* 183) showed the formation of a fragmentation ion at *m/z* 155 and MS^3 shows the formation of a ion at *m/z* 125. Similar experiments were performed with the ^{37}Cl isotope of daughter ion of *m/z* 185 and fragmentation ions of *m/z* 157 and 127 were found. Based on the fragmentation data, the structure for related compound D was assigned as shown in Fig. 1 and its probable fragmentation pathway is given in Scheme 1.

The fragmentation pathway of related compound D was compared with the clopidogrel fragmentation pathway given in Scheme 2 [9]. It was found that *m/z* value of the clopidogrel daughter ion is 212/214 but the same was not observed in the case of related compound D. The fragmentation ion obtained from MS^2 experiments of clopidogrel were 183/185 and 155/157 which is similar to that of the daughter ion of related compound D. Similar fragmentation was not observed in related compound D, due to the double bond associated with nitrogen atom. However, the fragmentation ion obtained from MS^2 experiments of clopidogrel were similar to that of the daughter ion obtained from related compound D. This was confirmed by MS^3 and MS^4 experiments.

^1H NMR spectrum of related compound D is slightly different from that of clopidogrel ^1H NMR spectrum and exhibits one hydrogen less than that of clopidogrel. Comparison of ^1H, ^{13}C and 135 DEPT NMR data with that of clopidogrel (Table 1) shows that the related compound D has one methylene proton less and one methine proton more than that of clopidogrel. The methine protons at 2nd and 3rd positions are deshielded to the extend of 0.3, 0.7 ppm, respectively. The methylene protons at 6th and 7th positions are deshielded to the extend of 0.5, 0.4 ppm, respectively. The presence of methylene protons at 6th and 7th positions was confirmed by irradiation (proton decoupling) experiment. Irradiation of protons at chemical shift δ 4.30 ppm (m) affects the multiplicity of protons at δ 3.94 and 3.80 ppm which further reveals that the double bond position. The appearance of one singlet at δ 9.19 ppm at 4th position and the deshielding of methine proton at 10th position from δ 5.60 to δ 6.66 ppm were also noticed. All the above deshielding con-

Table 1
^1H and ^{13}C NMR assignments for clopidogrel bisulphate and related compound D

Position[a]	Clopidogrel bisulphate					Related compound D				
	^1H	δ (ppm)	*J* (Hz)	^{13}C	DEPT	^1H	δ (ppm)	*J* (Hz)	^{13}C	DEPT
1	–	–		–	–	–	–		–	–
2	1H	7.43/m	4.8	125.72	CH	1H	7.69–7.71/d	5.2	128.66	CH
3	1H	6.88/d	4.8	126.03	CH	1H	7.55–7.57/d	5.2	128.94	CH
4	2H	4.24/bs		50.89	CH_2	1H	9.21/s		162.51	CH
5	–	–		–	–	–	–		–	–
6	2H	3.43/bs		49.62	CH_2	2H	3.88–3.91, 4.23–4.30/m	7.6 and 14.8	48.49	CH_2
7	2H	3.06/bs		22.63	CH_2	2H	3.45/t	7.6 and 14.8	23.31	CH_2
8	–	–		134.83	–	–	–		155.46	–
9	–	–		128.62	–	–	–		128.88	–
10	1H	5.60/s		65.87	CH	1H	6.65/s		70.94	CH
11	–	–		128.07	–	–	–		127.93	–
12	1H	7.51/m[b]		131.22	CH	1H	7.58–7.61/m[b]		132.79	CH
13	1H	7.69/t	7.2	129.12	CH	1H	7.65/d	7.2	128.88	CH
14	1H	7.57/m[b]		132.08	CH	1H	7.50–7.54/m[b]		133.14	CH
15	1H	7.65/t	7.2	130.86	CH	1H	7.66/d	7.2	131.07	CH
16	–	–		132.98	–	–	–		134.98	–
17	–	–		167.71	–	–	–		167.23	–
18	3H	3.75/s		54.33	CH_3	3H	3.85/s		54.46	CH_3

[a] Refer structural formula (Fig. 1) for numbering: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad.
[b] Unresolved.

firmed that there is a structural change in the piperidine ring. It was also confirmed by ^{1}H–^{1}H COSY spectrum. There is no appropriate change in the chemical shift of aromatic ring which confirms the presence of the aromatic rings in the related compound D. ^{13}C and DEPT 135 NMR spectra results indicate the presence of one methyl carbon, two methylene carbons, eight methine carbons and five quaternary carbons. Related compound D shows five quaternary carbons like that of clopidogrel but one methylene carbon less and one methine carbon more.

The methylene signal at δ 49.62 ppm disappeared and a new methine signal at δ 162.51 ppm is observed. The high deshielding chemical shift value of the methine signal indicates that carbon may be attached to an electronegative atom or attached to aromatic ring or under the anisotropic influence of an aromatic ring. This led to the hypothesis of existence of C=N in the piperidine ring of related compound D structure. The position of the double bond was assigned to 4th carbon due the possibility of extended conjugation explained for the shifting of UV absorbance maxima to the higher wavelength. This was also confirmed by the deshielding of aliphatic methine carbon at 10th position to about 5 ppm and the deshielding of one of the quaternary carbon at 8th position to about 20 ppm. The presence of C=N was further confirmed by the IR characteristic absorption peak at 1469 cm^{-1}.

All the above observations can be well explained on the basis of the proposed structure (Fig. 1) with quaternary nitrogen and a double bond. All proton signals were assigned on the basis of ^{1}H NMR and ^{1}H–^{1}H COSY spectral results. Carbon signals were assigned on the basis of DEPT135 and ^{1}H–^{13}C HSQC spectral results.

Based on the above spectral data, the structure was characterized as 5-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-6,7-dihydrothieno [3,2-*c*] pyridin-5-ium with a molecular weight of 320 amu.

4. Conclusions

The major oxidative degradation product related compound D in clopidogrel drug substance as well as drug product was isolated by preparative LC and was characterized by using spectroscopic techniques namely NMR, MS and MS^{n}.

Acknowledgements

The authors wish to thank the management of Torrent Pharmaceuticals Ltd. and Torrent Research Center for allowing us to carryout the present work. The authors are also grateful to the Director Dr. C. Dutt of Torrent Research Center for his constant encouragement. The authors also wish to thank friends and the other colleagues of Torrent Research Center for their cooperation.

References

[1] http://www.Rxlist.com.
[2] H. Agrawal, N. Kaul, A.R. Paradkar, K.R. Mahadik, Talanta 61 (2003) 581–589.
[3] United states Pharmacopoeia 30, pp. 1802–1803.
[4] J.M. Pereillo, M. Maftouh, A. Andrieu, M.F. Uzabiaga, O. Fedeli, P. Savi, M. Pascal, J.-M. Herbert, J.-P. Maffrand, C. Picard, Am. Soc. Pharmacol. Exp. Ther. 30 (2002) 1288–1295, DMD 30.
[5] Y. Gomez, E. Adams, J. Hoogmartens, J. Pharm. Biomed. Anal. 34 (2004) 341–348.
[6] A. Mitakos, I. Panderi, J. Pharm. Biomed. Anal. 28 (2002) 431–438.
[7] ICH harmonized tripartite guideline, Impurities in New Drug Substances Q3A(R2), current step 4 version dated 25 October 2006.
[8] ICH harmonized tripartite guideline, Impurities in New Drug products Q3B(R2), current step 4 version dated 2 June 2006.
[9] W. Danikiewicz, M. Swist, J. Mass Spectrom. 42 (2007) 405–406.