GC/MS, FTIR and NMR Studies for the Identification and Characterization of Clopidogrel Bisulfate Degradation Products

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ABSTRACT

Clopidogrel Bisulfate is a thienopyridine derivative. The separation, identification and degradation of Clopidogrel Bisulfate under hydrolytic and oxidative stress conditions according to the International Conference on Harmonization (ICH) guideline Q1A (R2) was performed. TLC using (n-hexan:tetrahydrofuran)(1:1 v/v) as a mobile phase was used to separate the degradation products. Three compounds were isolated then analyzed using RP-HPLC which showed a purity of 99%. Mass fragmentation pathway of the compounds were first established with the help of GC/MS studies. Then, the degradation products were subjected to FTIR and 1H NMR studies. The obtained data were employed to characterize the degradation products and assign structures. The degradation products were identified as (S)-2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl) acetic acid in acidic and basic media, 2-(2-chlorophenyl)-2-oxoacetic acid and 4,5,6,7-tetrahydrothieno [3,2-c] pyridine in oxidative medium.

Key words: Clopidogrel degradation products, FTIR, GC/MS, NMR, Stress studies.

INTRODUCTION

Clopidogrel hydrogen sulfate, methyl (+)-(S)-α-(o-chlorophenyl)-6,7-dihydrothieno [3,2-c] pyridin-5(4H)-acetate hydrogen sulfate, is a novel thienopyridine derivative that irreversibly blocks adenosine diphosphate (ADP) and is important in platelet aggregation, the cross-linking of platelets by fibrin. Clopidogrel bisulphate (Figure 1) is chemically related to Ticlopidine with superior side effects profile and dosing requirements.[1-3]

The empirical formula of Clopidogrel bisulfate is $C_{16}H_{16}ClNO_2S{\bullet}H_2SO_4$ and its molecular weight is 419.9.[4] The molecule of Clopidogrel contains an asymmetrical carbon leading to the existence of two enantiomers (R and S). Studies showed that the active compound Clopidogrel is the S enantiomer.[5,6] Clopidogrel free base was unstable due to a labile proton in the chiral center and was susceptible to racemization and hydrolysis of methyl ester group.[6,7]

It is marketed by Bristol-Myers Squibb and Sanofi- Aventis under the trade name Plavix®, a Plavix tablet contains 75 mg of Clopidogrel bisulfate.[3]

Characterization of Clopidogrel bisulfate degradation under solid stress conditions was performed in previous literature,[8] determination of Clopidogrel bisulfate in active pharmaceutical ingredient by chromatography has also been revealed,[9] a new study for the identification and characterization of a principle oxidation impurity in Clopidogrel drug substances and drug product was conducted,[10] for the quantitative determination of Clopidogrel bisulfate active metabolite in human plasma an LC/MS method was used,[11] and an LC method for the determination of Clopidogrel in pharmaceutical preparations wasvalidated.[12] Simultaneous determination of Clopidogrel and Aspirin in pharmaceutical dosage form was mentioned[13] and RP-HPLC was used

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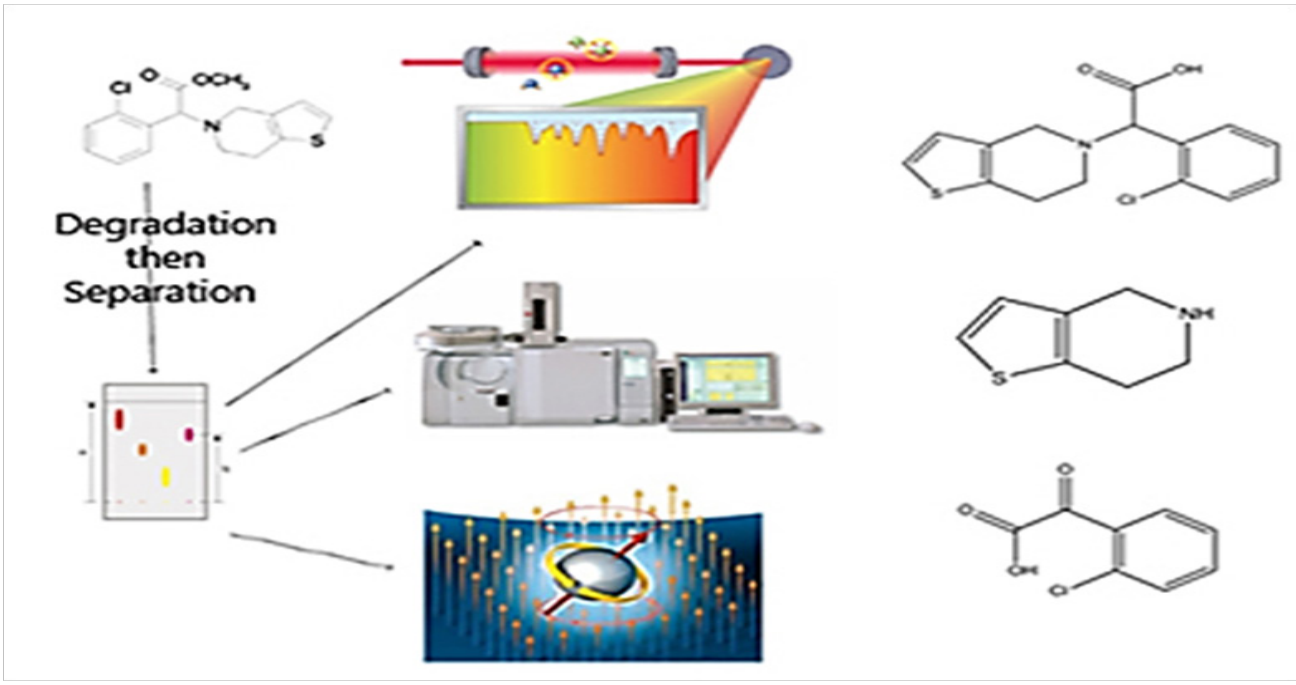


Graphical abstract

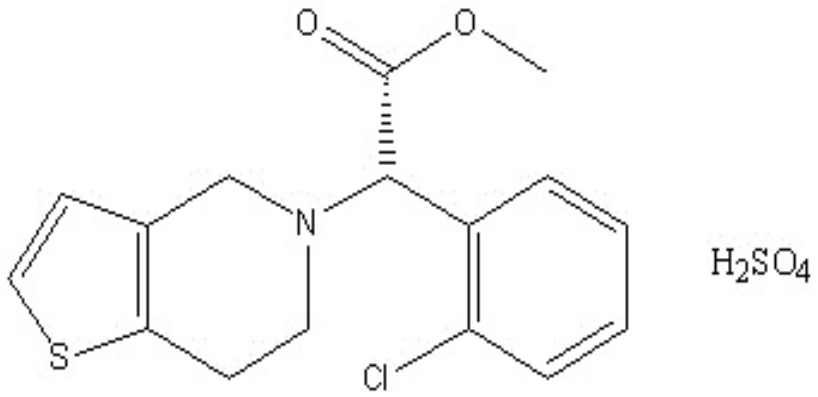


Figure 1: Structural formula of Clopidogrel bisulphate

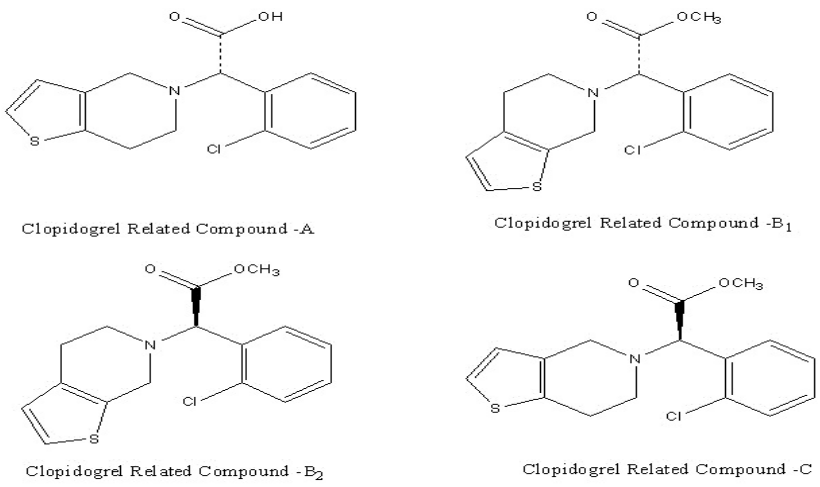


Figure 2: Structural Formula of known Related Compounds of Clopidogrel[17]

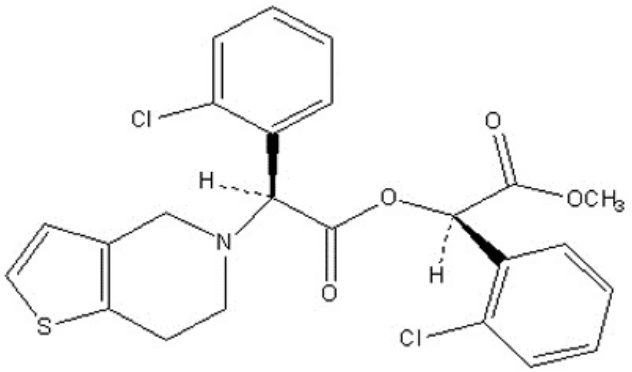


Figure 3: Structural Formula of Related Compound D of Clopidogrel

for the analysis of aspirin and Clopidogrel bisulfate in combination.[14]

Recently, the non-enzymatic and enzymatic chiral inversion of Clopidogrel has been investigated *in vitro* using ^{1}H-NMR and a chiral HPLC procedure.[15] For the analysis of the carboxylic acid metabolite of Clopidogrel in plasma and serum a GC-MS method has also been reported.[16]

United States Pharmacopeia-30 (USP-30)[17] has enumerated related substance method for Clopidogrel tablets in their monograph, the known related compounds of Clopidogrel were given in Figure 2. In addition, British pharmacopeia 2013 has mentioned a related compound named D, as shown in Figure 3.[18] Also literature survey revealed that four impurities of Clopidogrel have been already identified.[12,19]

Though these methods already exist in the literature, none of the methods carried out studies to isolate and characterize degradation products of Clopidogrel bisulfate formed by hydrolysis or oxidation under stress condition according to ICH. An attempt was made towards isolation and characterization of degradation products. Therefore, an endeavor of the present study was to decompose the drug under hydrolytic and oxidation conditions, to resolve the products on preparative TLC and to characterize the major products by NMR, GC/MS and FTIR studies.

MATERIALS AND METHODS

Chemicals

Clopidogrel bisulfate was obtained as gift sample from Al-Razi laboratories (Aleppo, Syria) and was used without further purification. Analytical reagent grade hydrogen peroxide (H_2O_2). Sodium hydroxide (NaOH) was purchased from Hi Media (Mumbai, India). Hydrochloric acid (HCl), Methanol, Dichloromethan, n-hexan, and tetrahydrofuran were supplied by Merck.

APPARATUS AND EQUIPMENT

Analytical HPLC was performed as mentioned in our previous study.[20]

GC-MS

GC-MS analyses were performed on Shimadzu -GCMS-QP2010 Plus device equipped with META-5X column (30.0 m X 0.32 mm X 0.25 mm), carrier gas was He, and gas flow rate 1.27 ml/min. Mass spectra were obtained by electron impact (EI) ionization at 70 eV with an emission current of 400 mA. The scan time was 1 s and the scan range was m/z 29–600. The ion source temperature was maintained at 280ºC. The identity confirmed by fragmentation pattern and by NIST &WILEY mass spectral libraries. The temperature program was as follows:

- 80ºC, hold for 5 min;
- Temperature rise from 80ºC to 200ºC at a rate 20ºC /min;
- Temperature rise from 200ºC to 295ºC at a rate 10ºC/min and hold for 5 min.

^{1}H NMR Spectroscopy

About 10 mg of the tested substances were each dissolved in 0.6 mL of DMSO-$_{d6}$ and were immediately analyzed by NMR spectroscopy. The one dimensional

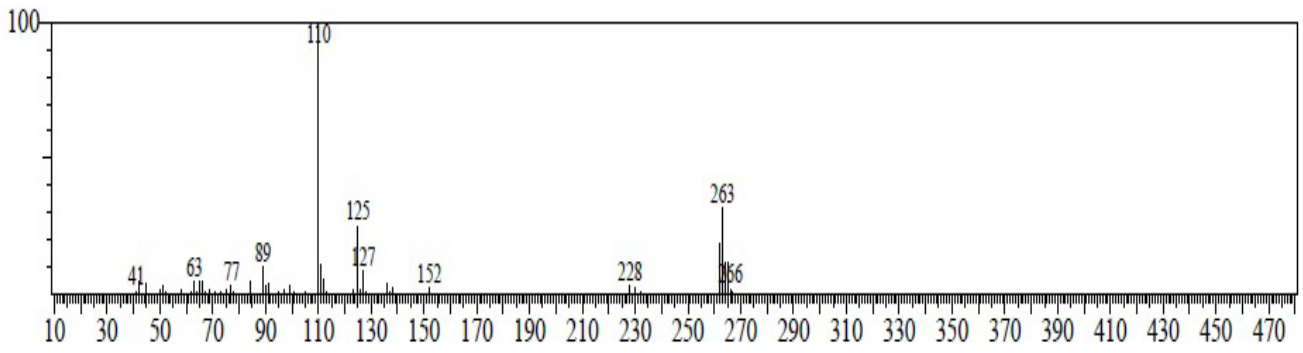
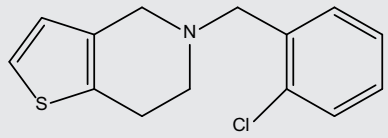
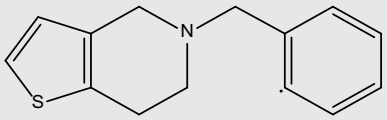
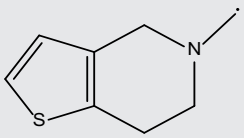
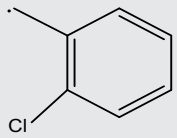
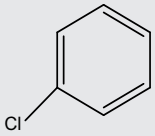


Figure 4: MS spectrum of compound A (Clopidogrel Acid)

Table 1: The Mass fragment of compound A (Clopidogrel acid)

Major fragment	m/z
	263/265
	228/229
	152/153
	125/127
	110/111

NMR measurements were performed on a BRUKER AVANCE III NMR spectrometer (Bruker, Rheinstetten, Germany) with 400 MHz for ^1H, employing the manufacturer's pulse programs. The ^1H chemical shift values were reported on the δ scale in ppm. Standard Bruker pulse sequences were applied by running ACD/Labs (ACD/NMR Processor Academic Edition) software version 12.01.

IR Spectroscopy

The IR spectrum was recorded in the solid state as a KBr disk, and in Nujol as a dispersion medium, using the FT-IR (Bruker, alpha) spectrophotometer, the wave length resolution was set to 4 cm^{-1}, the IR spectrum was collected in a range of 400–4000 cm^{-1}, with Bruker Opus 5.5 software.

Preparative TLC Method

A mobile phase of (n-hexan:tetrahydrofuran)(1:1 V) was used, 20 X 20 cm glass TLC plates coated with (SIL. G. $UV_{254+366}$) were purchased from MACHEREY-NAGEL GmbH & Co. KG, Germany.

Preparation of degradation samples of Clopidogrel

Acid and base degradation

Accurately weighed 500 mg of Clopidogrel was dissolved in 50 ml of methanol. The drug was subjected to accelerated degradation under acidic and basic conditions by refluxing with (10 ml) 1N HCl and (10 ml) 1N NaOH, respectively, at 70°C for a period of 3 and 1 hr, respectively. The accelerated degradation in acidic and basic media was performed in the dark in order to exclude the possible degradation effect of light on the drug.[19]

Peroxide degradation

Accurately weighed 500 mg of drug was dissolved in 50 ml of methanol. Subsequently, 10 ml of hydrogen peroxide 30.0% v/v was added and the solution was heated in boiling water bath for 1 hour.[19]

Data Analysis

Structure formulae were generated and processed by Chem Bio Draw Ultra 12.0 Software.

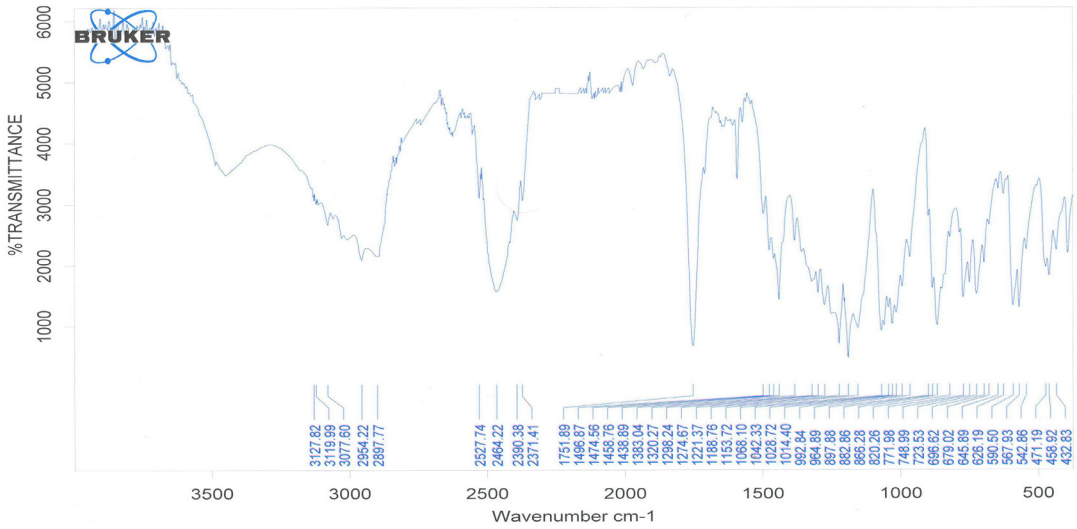


Figure 5: IR spectrum of compound A (Clopidogrel acid)

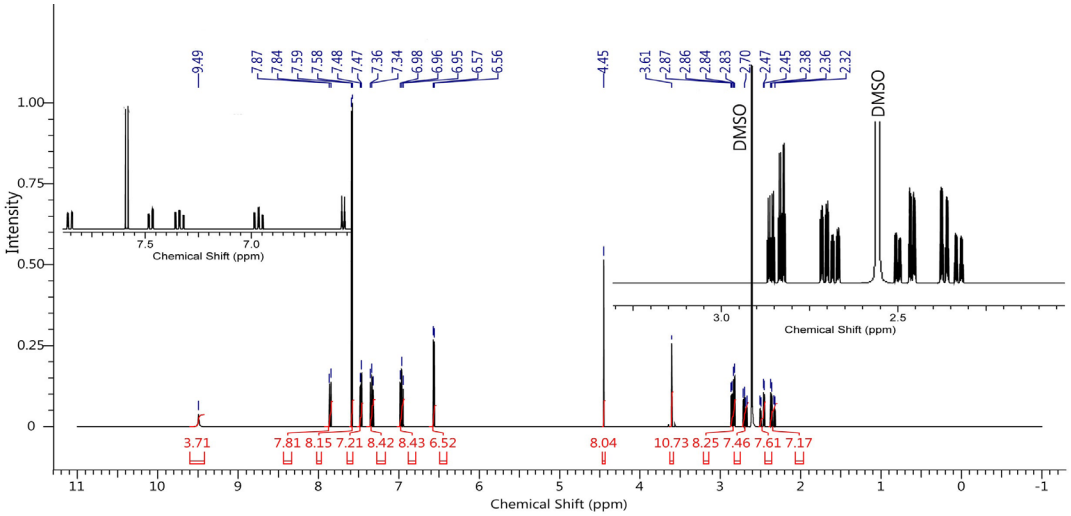


Figure 6: 1H NMR spectrum of compound A (Clopidogrel acid)

RESULTS AND DISCUSSION

Isolation of Degradation Product(s) by Preparative TLC

The resultant solutions after acid, base and oxidative degradation were isolated as follows, the aqueous layer was washed with dichloromethane to remove Clopidogrel, then the aqueous layer was subjected to preparative TLC, the bands were visualized using UV_{254} lamp, the desired band was scratched with a spatula, extracted with methanol which was finally evaporated. The resulted solid was analyzed using RP-HPLC[20] and the purity of the compound was found to be 99% which was good enough for carrying out the spectroscopic experiments.

Characterization of the Degradation Product

Characterization of the compounds was performed using analytical data obtained from IR, GC/MS and ^{1}H NMR spectrum experiments.

Table 2: Bands and assignments of compound A (Clopidogrel acid)

Frequency cm^{-1}	Assignment
3450	OH Carboxylic
2954, 3077, 3119	Chlorophenyl CH Stretch
2464	C-S-C stretch
1752	C=O carboxylic
1474,1496	Chlorophenyl ring stretch
1439, 1383	Pyridine methylene wag
1188	C-O carboxylic acids
1298, 1275	Methylene twist
1221	Chlorophenyl C-Cl stretch and bends
1154	Pyridine ring stretch
1068, 1028, 1014, 993	Pyridine-methylene rock
749, 724, 697	Chlorophenyl spatial bend

Elucidation of the Structure of Degradation Product Resulted from hydrolytic stress Conditions (compound A)

It was found that there was one degradation product (compound A). The MS, IR, and NMR spectra of product A were recorded. The major Mass fragments obtained by GC–MS analysis are given in Table(1).

As shown in Figure (4) and Table (1) that the main fragment of the product A was m/z: 263/265 which corresponds to Clopidogrel acid when it loses the carboxylic group. Other fragments such as m/z: 125/127 and 110/111 refer to O-chlorophenyl molecule.

FTIR, and ^{1}H NMR spectral data are given in Figure (5), Figure (6) and Tables (2). On the basis of these data it was inferred that the methoxy group of Clopidogrel was not present in compound A.

The degradation product was formed by hydrolysis of ester group of Clopidogrel to form methanol and Clopidogrel acid.

As shown in Figure(5) and table (2), the main functional groups of compound A (Clopidogrel acid) appeared clearly; the absence of ester group, the presence of –OH carboxylic at 3450 cm^{-1}, and the presence of -C-O-carboxylic at 1188 cm^{-1} .

As shown in Figure 7, the ^{1}H NMR of compound A show the following data (400 MHz, DMSO-d6) δ ppm 2.24 - 2.58 (m, 2 H, 4) 2.61 - 2.95 (m, 2 H, 3) 3.61 (ddt, J=2.33, 1.58, 0.75, 0.75 Hz, 2 H, 6) 4.44 - 4.46 (s, 1 H,10) 6.57 (d, J=5.10 Hz, 1 H, 7) 6.97 (td, J=7.50, 1.20 Hz, 1 H, 15) 7.35 (t, 1 H, 16) 7.47 (d, J=6.30 Hz, 1 H, 14) 7.58 (d, 1 H, 10) 7.86 (d, 1 H, 17) 9.49 (s, 1 H, 14).

On the basis of these data it was concluded that compound A was (S)-2-(2-chlorophenyl)-2-(6,7-dihydrothi-

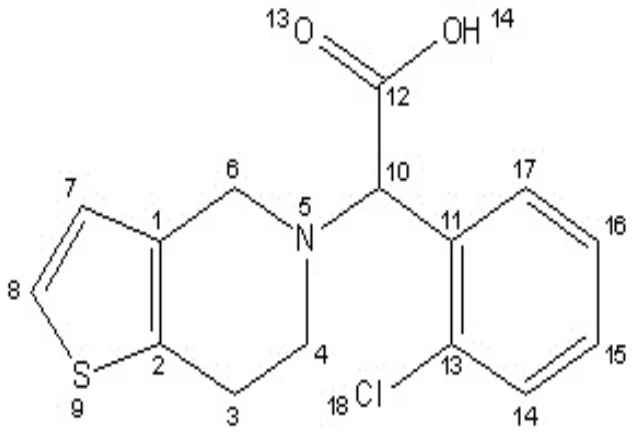


Figure 7: Atom number of compound A (1H NMR)

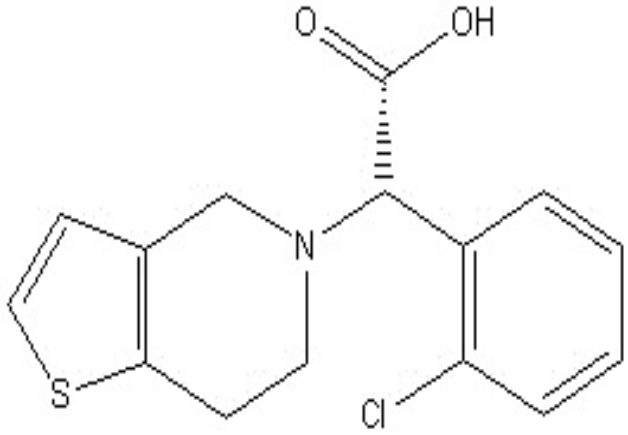
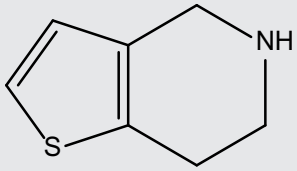
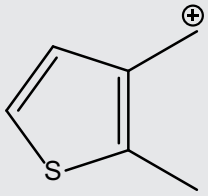
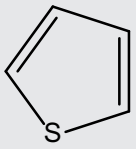


Figure 8: Structure of compound A (Clopidogrel Acid)

Table 3: The Mass fragment of product B

Major fragment	m/z
	139/140
	111/112
	84/85

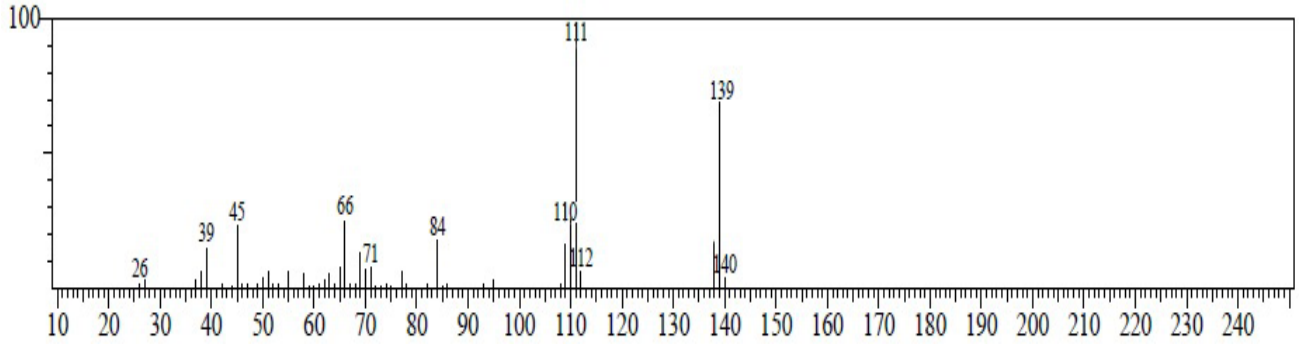


Figure 9: MS of product B

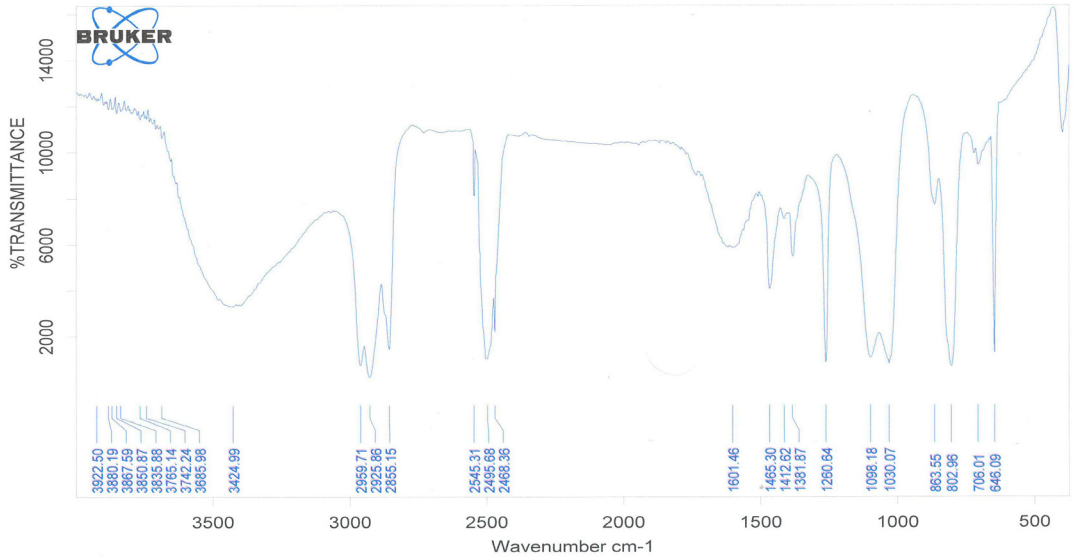


Figure 10: IR spectrum of product B

Table 4: Bands and assignments of product B	
Frequency cm^{-1}	**Assignment**
3425	N-H stretch
2960	Sym C-H stretch
2926	C-H stretch
2855	sym C-H stretch
2496	C-S-C stretch
1601	sym wag-stretch
1465	sym C-H wag
1382	C–N stretch amine
1261	Pyridine ring stretch
1098	C-H wag
1030	in plane C-C wag
863	N–H wag
706	C-H bend
646	planar ring distortion

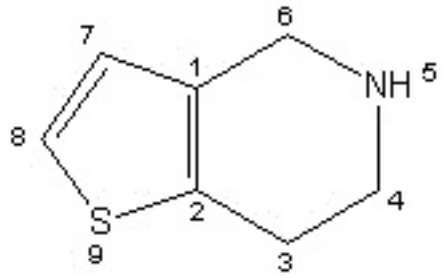


Figure 11: Atom number of compound B (^{1}H NMR)

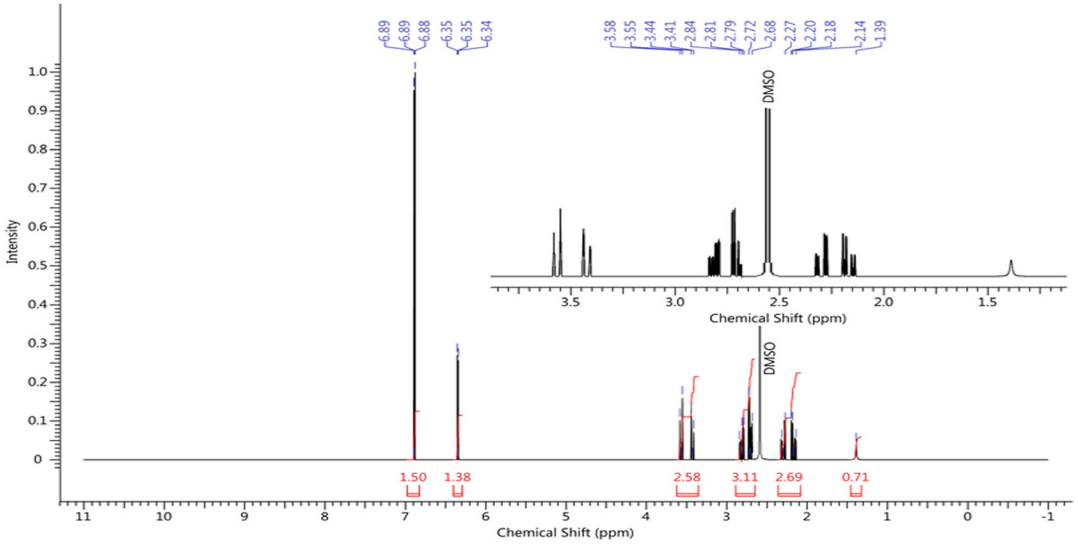


Figure 12: ^{1}H NMR spectrum of compound B

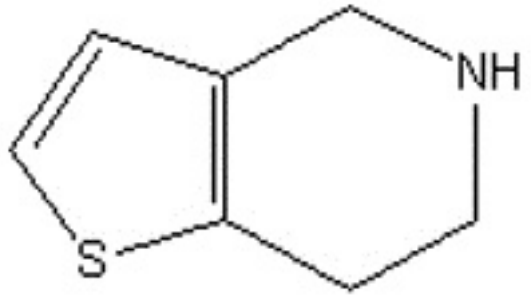


Figure 13: Structure of product B (4,5,6,7-tetrahydrothieno[3,2-c]pyridine)

eno [3,2-c] pyridin-5 (4H)-yl) acetic acid which is (Clopidogrel acid) Figure (8).

Elucidation of the Structure of Degradation Products Resulted from oxidative stress conditions

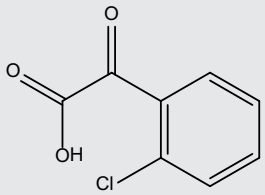
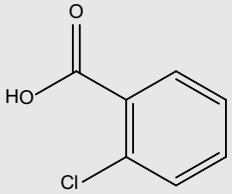
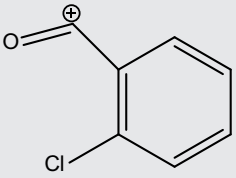
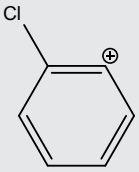
It was found that there were two degradation products (compound B and compound C). The MS, IR, and NMR spectra of product B were recorded. The major Mass fragments for product B is given in Table (3).

The degradation products were formed by fragmentation of Clopidogrel to form product B and product C. GC-MS analysis of product B revealed a molecular ion peak at m/z: 139/140 and the fragmentation pattern also confirmed the structure given in Figure (9).

IR spectrum and bands and assignments of product B are also shown in Figure (10) and Table (4); respectively.

As shown in Figure(10) and table(4), the main functional groups of product B appeared clearly; the presence of N-H stretch at 3425cm^{-1}, the aromatic C-H stretch at 2855 cm^{-1}, the presence of C-S-C stretch at 2496cm^{-1}, the presence of C–N stretch and Pyridine ring stretch at 1382, 1261cm^{-1}; respectively.

Table 5: The Mass fragment of product C

Major fragment	m/z
	184/186
	156/157
	139/141
	111/113

As shown in Figure 11, the ^{1}H NMR spectrum of product B was (400 MHz, DMSO-d6) δ ppm 1.39 (s, 1 H, 5) 2.15-2.35 (m, 2 H, 3) 2.7-2.85 (m, 2 H, 4) 3.4 - 3.6 (m, 2 H, 6) 6.35 (d, J=5.10 Hz, 1 H, 7) 6.89 (d, J=5.10 Hz, 1 H, 8). Figure (12).

On the basis of these data it was concluded that the product B was 4,5,6,7-tetrahydrothieno[3,2-c]pyridine Figure (13).

The MS, IR, and NMR spectra of product C were recorded. The major Mass fragments for product C is given in Table (5).

GC-MS analysis of product C revealed a molecular ion peak at m/z: 184/186 and the fragmentation pattern

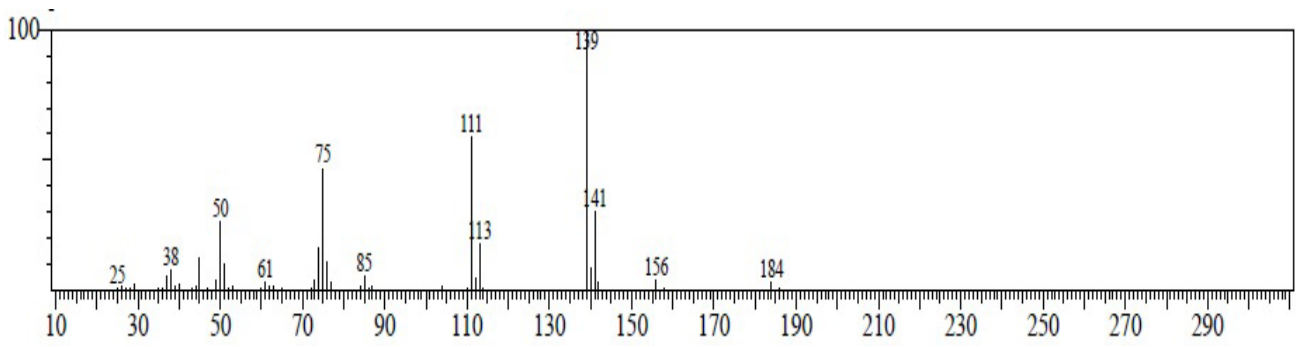


Figure 14: MS of product C

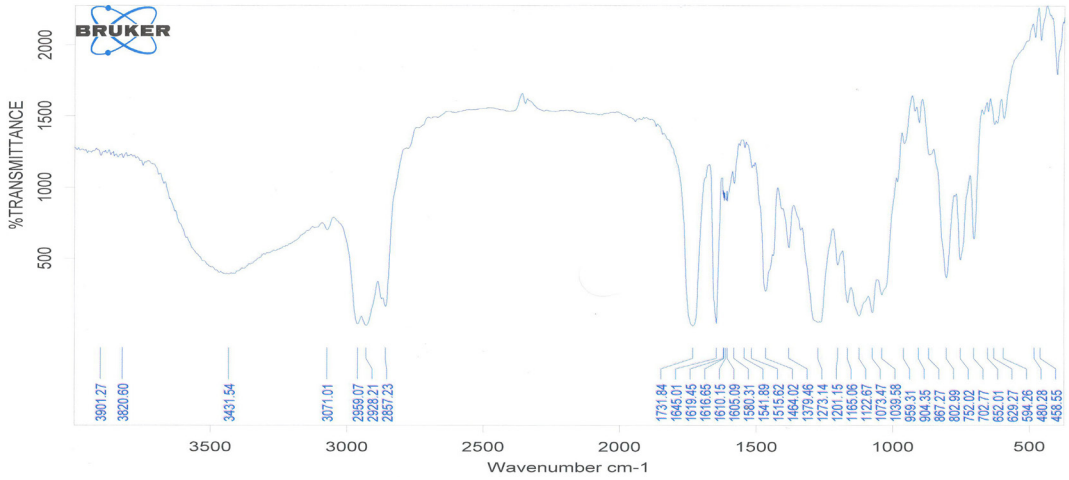


Figure 15: IR spectrum of product C

Table 6: Bands and assignments of product C	
Frequency cm^{-1}	**Assignment**
3431	OH carboxylic
2959, 2928	Chlorophenyl C-H Stretch
1732	C=O carboxylic
1645	C=O ketone
1605,1580	Chlorophenyl ring stretch
1201	Chlorophenyl C-Cl bend
803, 752, 703	Chlorophenyl spatial bend

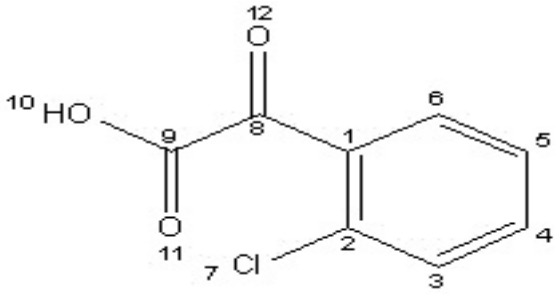


Figure 16: Atom number of compound B (^{1}H NMR)

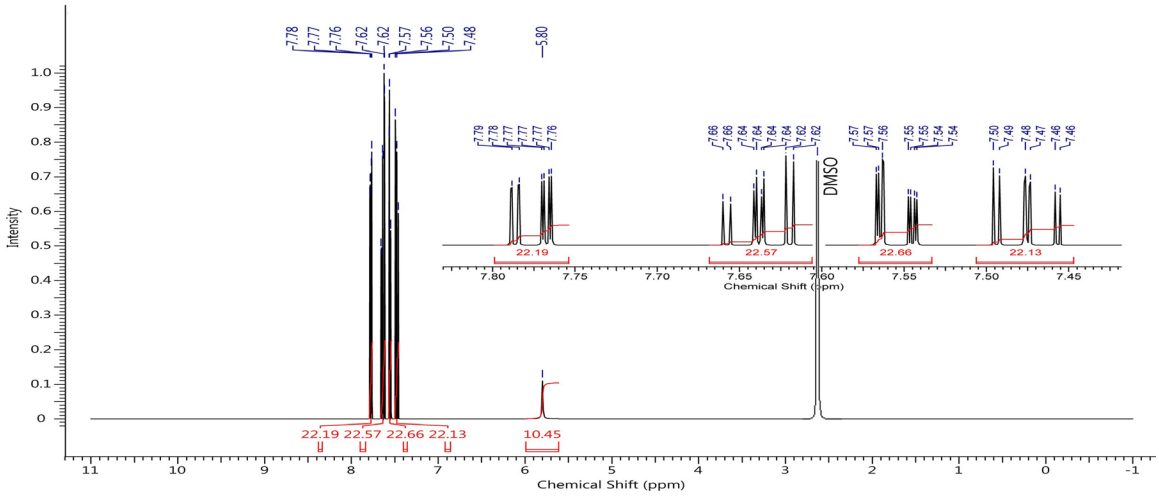


Figure 17: ^{1}H NMR spectrum of compound C

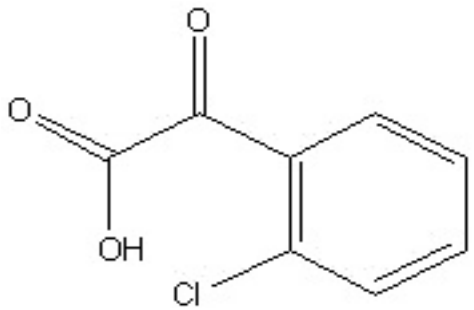


Figure 18: Structure of product C (2-(2-chlorophenyl)-2-oxoacetic acid)

which is shown in Table (5) also confirmed the structure given in Figure (14).

IR spectrum and bands and assignments of product C are also shown in Figure (15) and Table (6); respectively.

As shown in Figure (15) and table(6), the main functional groups of 2-(2-chlorophenyl)-2-oxoacetic acid appeared clearly; the presence of OH carboxylic at 3431 cm^{-1}, the aromatic C-H stretch at 2959 & 2928 cm^{-1}, the presence of C=O carboxylic at 1732 cm^{-1} , the presence C=O ketone at 1645 cm^{-1} , and C-Cl bend 1201 cm^{-1}.

As shown in Figure 16, the ^{1}H NMR spectrum of product C was (400 MHz, DMSO-d6) δ ppm 5.80 (s, 1 H, 10) 7.44 - 7.52 (td, 1 H, 4) 7.52 - 7.59 (dd, 1 H, 3) 7.60 - 7.69 (td, 1 H, 5) 7.77 (dd, J=7.80 Hz, 1 H, 6), Figure (17).

On the basis of these data it was concluded that product C was 2-(2-chlorophenyl)-2-oxoacetic acid Figure (18).

CONCLUSION

The hydrolytic and oxidative degradation product of Clopidogrel bisulfate was isolated by preparative TLC and was characterized using spectroscopic techniques namely NMR, IR, and MS. The degradation products were identified as Clopidogrel acid which is chemically: (S)-2-(2-chlorophenyl)-2-(6,7-dihydrothieno [3,2-c]pyridin-5(4H)-yl) acetic acid in acidic and basic media, 4,5,6,7-tetrahydrothieno [3,2-c] pyridine and 2-(2-chlorophenyl)-2-oxoacetic acid in oxidative medium.

ABBREVIATIONS

GC/MS : Gas Chromatography/Mass Spectrometry
FTIR : Fourier transform infrared spectroscopy
NMR : Nuclear magnetic resonance
ICH : International Conference on Harmonization
TLC : Thin Layer Chromatography
ADP : Adenosine Diphosphate
LC/MS : Liquid Chromatography/ Mass Spectrometry
RP-HPLC : Reversed Phase High Performance Liquid Chromatography
USP : United States Pharmacopoeia
EI : Electron Impact
DMSO-$_{d6}$: Deuterated Dimethyl sulfoxide

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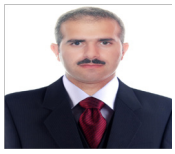
Highlights of Paper

- Clopidogrel was subjected to stress degradation according to ICH.
- Degradation products were isolated by preparative TLC.
- GC/MS, FTIR, 1H NMR techniques were used for characterization.
- Structures of Degradation products were elucidated.

Author Profile



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