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Identification, isolation and characterization of potential degradation products in pioglitazone hydrochloride drug substance

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During stress degradation studies of pioglitazone hydrochloride, one major unknown oxidative degradation impurity and two major unknown base degradation impurities were identified by LC-MS. These impurities were isolated using preparative liquid chromatography. Based on the spectral data (¹H NMR, ¹³C NMR, MS and IR), oxidative degradation impurity, base degradation impurity-1 and base degradation impurity-2 were characterized as pioglitazone N-oxide, 3-(4-(2-(5-ethylpyridine-2-yl) ethoxy) phenyl)-2-mercaptopropanoic acid and 2-(1-carboxy-2-{4-[2-(5-ethylpyridine-2-yl)-ethoxy] phenyl}-ethyl disulfanyl)-3-{4-[2-(5-ethylpyridine-2-yl)-ethoxy] phenyl} propanoic acid, respectively. The formation and mechanism of these impurities were discussed and presented.

1. Introduction

Pioglitazone hydrochloride is an antidiabetic agent, which is used to manage a certain type of diabetes like NIDDM (non-insulin-dependent diabetes mellitus, type 2 diabetes). It is used to decrease insulin resistance. Its chemical designation is 5-(4-(2-(5-ethylpyridin-2-yl) ethoxy) benzyl) thiazolidine-2,4-dione hydrochloride. Pioglitazone activates peroxisome proliferator-activated receptor gamma (PPAR-gamma), a ligand-activated transcription factor, thereby inducing cell differentiation and inhibiting cell growth and angiogenesis. Pharmacological studies indicate that pioglitazone improves sensitivity to insulin in muscle and adipose tissue and inhibits hepatic gluconeogenesis. It improves glycemic control while reducing circulating insulin levels (Sohda et al. 1990).

Several articles were published on analytical methods of pioglitazone hydrochloride and its metabolites for its biological studies (Ho et al. 2004; Berecka et al. 2005; Sripalakit et al. 2006). Some papers were published on analytical methods for the bulk drug and formulation of pioglitazone hydrochloride (Chandna et al. 2005; Sane et al. 2004; Jyothi et al. 2006). Kumar et al. (2008) reported a stability-indicating high-performance liquid chromatographic method for pioglitazone hydrochloride with stress degradation studies. Some other publications and patents were published on process related impurities preparation and characterization of pioglitazone (Richter et al. 2007; Balwant et al. 2007; Radhakrishna et al. 2002; Dolitzky et al. 2006). The present research work describes the identification, isolation and characterization of three unknown impurities (major degradation products) formed in the oxidative and base degradation studies (ICH Q3BR2 2006).

2. Investigations, results and discussion

The objective of this work is to identify and characterize the major unknown degradation products, formed in oxidative and

base stress degradation studies. The analytical HPLC chromatograms of pioglitazone hydrochloride, stressed samples in base and oxidative degradations are shown in Fig. 1. Pioglitazone eluted with a retention time of about 7 min (Fig. 1a). In the chromatogram of the base stressed sample two prominent degradation impurities 1 and 2 were eluted at relative retention times of 1.05 and 1.12 respectively with respect to the pioglitazone peak (Fig. 1b). In the chromatogram of the oxidative stressed sample one prominent degradation impurity eluted at 1.45 relative retention time with respect to pioglitazone peak (Fig. 1c). To get further structural insight, LC-MS analysis was carried out on the stressed samples. The mass spectra thus obtained showed the protonated molecular ion of the oxidative degradation impurity at m/z 373, whereas the pioglitazone displayed protonated molecular ion at m/z 357. The base degradation mass spectra showed the protonated molecular ions of the impurities –1 and 2 at m/z 332 and 661 respectively, whereas the pioglitazone displayed protonated molecular ion at m/z 357. Thus the oxidative degradation impurity and base degradation impurity-2 has 16 amu and 304 amu more than the molecular ion of pioglitazone and base degradation impurity-1 has 25 amu less than the molecular ion of pioglitazone.

As the molecular ion information is not just enough to arrive at the structure, the drug substance was stressed and subjected to purification by preparative HPLC to isolate more quantities of the oxidative degradation impurity, the base degradation impurity-1 and impurity-2 for spectroscopic studies. After isolation oxidative and base degradation products (impurity-1 and impurity-2) were spiked to pioglitazone and the spiked HPLC chromatogram was shown in Fig. 1d.

3. Experimental

3.1. Chemicals and reagents

The investigated samples of pioglitazone hydrochloride were received from Process Research Department of Custom Pharmaceutical Services of

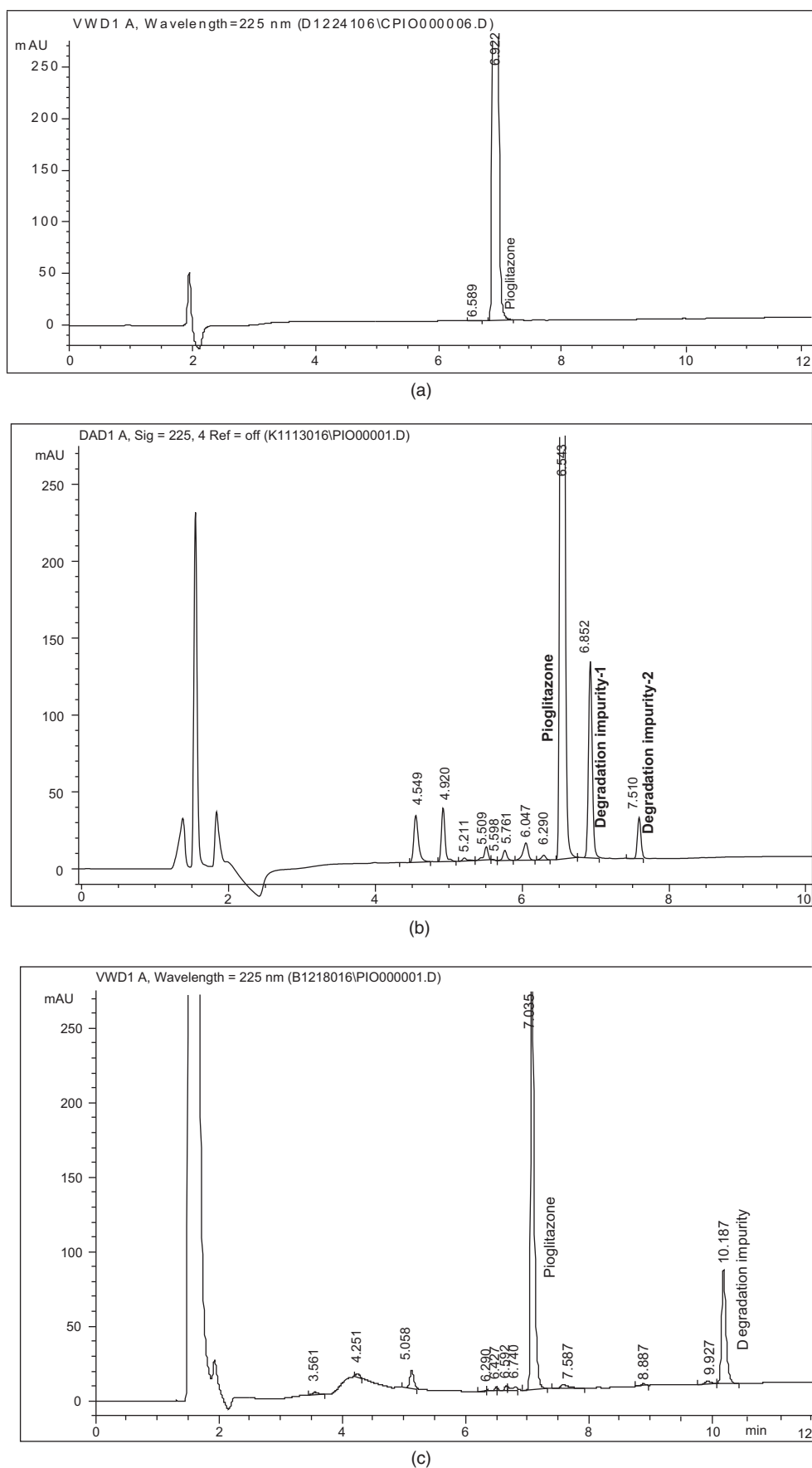


Fig. 1: Typical HPLC chromatograms of (a) pioglitazone drug substance, (b) stressed sample by base hydrolysis condition, (c) stressed sample by oxidative condition, (d) pioglitazone spiked with N-oxide, impurity-1 and impurity-2

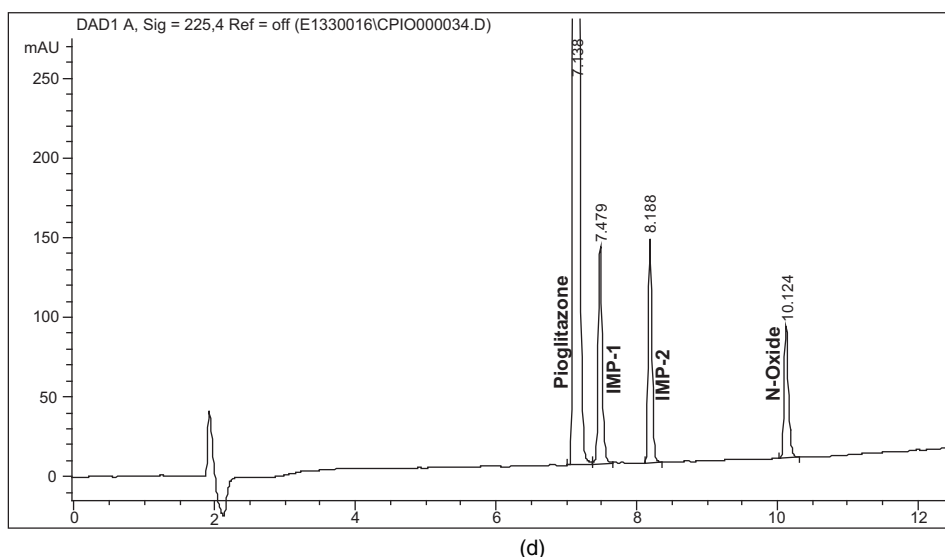


Fig. 1: (Continued)

Dr. Reddy's Laboratories Limited, Hyderabad, India. The chemicals used in the present analysis are trifluoroacetic acid (Across), sodium hydroxide (Rankem), hydrochloric acid (Rankem), hydrogen peroxide (S.D. Fine Chemicals) and HPLC-grade acetonitrile (Rankem). Water used in the analysis and isolation was purified using Milli-Q plus purification system.

3.2. Instrumentation

3.2.1. High-performance liquid chromatography

An Agilent 1100 series HPLC with photodiode array detector with chemstation data handling system was used. The analysis was carried out on Zorbax Bonus RP18 column with 150 mm length, 4.6 mm internal diameter and 3.5 μ m particle size. Mobile phase A was water and trifluoroacetic acid in the ratio of 100:0.05 (v/v) and mobile phase B was acetonitrile and trifluoroacetic acid in the ratio of 100:0.05 (v/v). UV detection was carried out at 225 nm and flow rate was kept at 1.0 mL/min. Column oven temperature was maintained at 30 °C. The gradient program: time/% of MP-B was 0/10, 12/62, 16/65, 17/10 with post run 5 min.

3.2.2. High-performance liquid chromatography (preparative)

An Agilent 1100 series preparative liquid chromatography equipped with a photodiode array detector system was used. Data was processed through chemstation software. Zorbax SB C18 (250 mm long $\times$ 9.4 mm i.d.) preparative column packed with 5 μ m particle size was employed for isolation of the impurities. Mobile phase A was water and trifluoroacetic acid in the ratio of 100:0.05 (v/v) and mobile phase B was acetonitrile and trifluoroacetic acid in the ratio of 100:0.05 (v/v). The flow rate was kept at 10 mL/min and the UV detection was carried out at 225 nm. The gradient program employed with T (min)/% mobile phase-B (v/v) as 0/20, 5/25, 7/70, 10/70, 11/20 with post run time of 5 min.

3.2.3. LC-MS

LC-MS was carried out on the degraded drug substance of pioglitazone. The mobile phase used was water: trifluoroacetic acid in the ratio of 100:0.05 (v/v) as a mobile phase - A and acetonitrile: trifluoroacetic acid in the ratio of 100:0.05 (v/v) as a mobile phase - B using the following gradient/elution (T/%B: 0/10, 12/62, 16/65, 17/10) flow rate of 1.0 mL/min and monitored at 225 nm. A Zorbax Bonus RP18 column (150 mm $\times$ 4.6 mm i.d., with a particle size of 3.5 μ m) was used. The injected volume was 10 μ L. Zero air was used in nebulizer and curtain gas and high pure nitrogen was used as a collision assisted dissociation gas. The following MS parameters were used for data acquisition: Nebulizer: 8.00 psi, curtain gas 8.00 psi, ion spray voltage 4500 V, Temperature 0 °C, declustering potential 70 V, focusing potential 180 V, entrance potential 10 V.

3.2.4. High resolution mass

All samples were analyzed on the Micromass LCT Premier mass spectrometer equipped with an ESI Lockspray source for accurate mass values. Leucine enkephalin was used as internal reference compound which was introduced via the Lockspray channel using the Waters reagent manager. The mass range was calibrated with the cluster ions of sodium formate using a fifth order polynomial fit. Data were acquired using the W mode.

The mass spectrometer was equipped with a Waters Acquity system. Pioglitazone and impurities were dissolved in water: acetonitrile (50:50 v/v) diluent at a concentration level of 0.25 mg/mL. The sample solutions were introduced to the mass spectrometer via a HPLC column. The chromatographic conditions used were: A: water: trifluoroacetic acid in the ratio of 100:0.05 (v/v); B: acetonitrile and trifluoroacetic acid in the ratio of 100:0.05 (v/v); using the following gradient elution (T/%B: 0/10, 12/62, 16/65, 17/10) with a flow rate of 1.0 mL/min and monitored at 225 nm. A Zorbax Bonus RP18 column with 150 mm length, 4.6 mm internal diameter and 3.5 μ m particle size was used.

3.2.5. NMR spectroscopy

The ^1H and ^{13}C NMR experiments for pioglitazone and impurities were performed on Mercury plus 400 MHz FT NMR spectrometer using DMSO- d_6 as solvent. The ^1H chemical shift values were reported on the δ scale in ppm, relative to TMS (δ = 0.00 ppm), while ^{13}C chemical shifts relative to DMSO- d_6 (δ = 39.50 ppm) as internal standards.

3.2.6. ESI-MS/MS

The MS/MS experiments were performed on a PESCIEX API 3000. The sample was introduced through a turbo ion spray interface in positive ionization mode using infusion pump. The nebulizer and curtain gases used were zero air and nitrogen respectively, ion spray voltage was maintained at 4500 V. Focusing potential, declustering potential and entrance potential were kept at 180 V, 70 V and 10 V respectively.

3.2.7. FT-IR spectroscopy

FT-IR spectra were recorded as KBr pellet on Perkin-Elmer instrument model—spectrum one.

3.3. Preparation, analysis and isolation of degradation samples

3.3.1. Preparation of degradation samples

3.3.1.1. Base degradation. A solution of pioglitazone hydrochloride (250 mg) in 25 mL of 0.5 N sodium hydroxide was kept for 48 h.

3.3.1.2. Oxidative degradation. A solution of pioglitazone hydrochloride (250 mg) in 25 mL of 5% w/v hydrogen peroxide was kept at 60 °C for 24 h.

3.3.2. Analysis of degradation samples by analytical HPLC

The degradation samples were diluted to required concentration and analyzed with analytical LC.3.2.1. In base degradation two major unknown degradation impurities were found (one is with about 9.0% and another was about 4.5% by area normalization). At oxidative degradation one major unknown degradation impurity was found about 3.0% by area normalization.

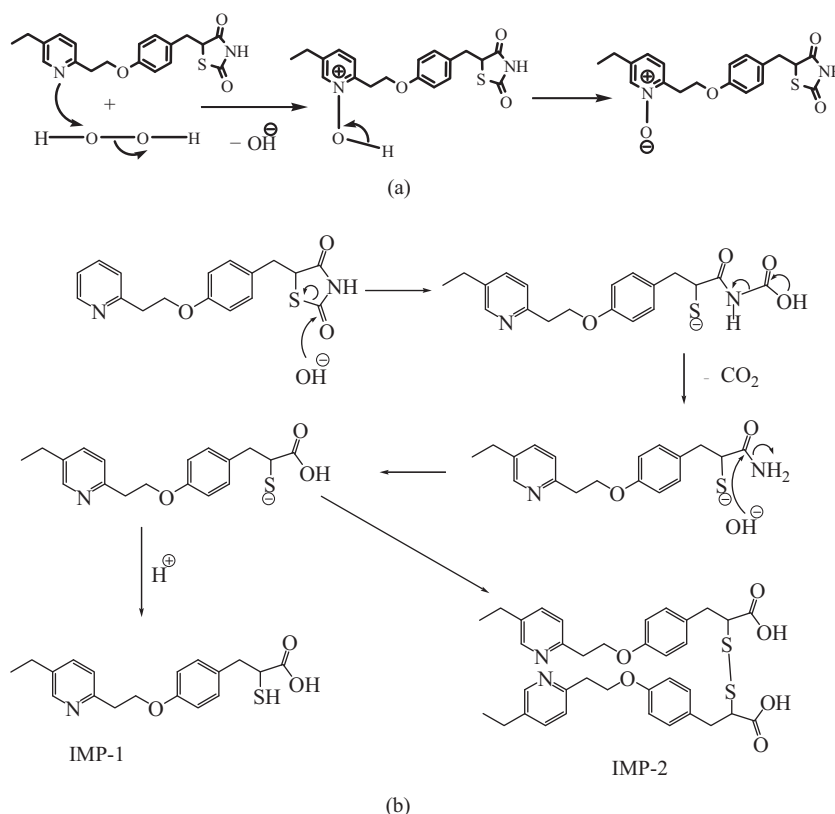
3.3.3. Isolation of degradation products by preparative HPLC

3.3.3.1. Isolation of base degradation products (impurity-1 and impurity-2). Pioglitazone hydrochloride (2.0 g) was treated with sodium hydroxide (0.5N, 25 mL) and kept at 80 °C for 24 h. Degraded solution was subjected

Table 1: NMR assignments of pioglitazone and N-oxide (oxidative degradation impurity)

Position ¹	Pioglitazone				N-oxide impurity			
	¹ H	δ (ppm)	² J	¹³ C	¹ H	δ (ppm)	² J	¹³ C
2	1H	8.36/d	2.0	148.40	1H	8.19/s	—	140.70
3	—	—	—	136.61	—	—	—	126.36
4	1H	7.57/m	—	135.66	1H	7.40/d	8.0	137.96
5	1H	7.27/d	8.1	122.98	1H	7.19/m	—	124.95
6	—	—	—	155.32	—	—	—	144.81
7	2H	3.13/t	6.6	36.27	2H	3.25/t	6.3	30.00
8	2H	4.29/t	6.6	66.66	2H	4.28/t	6.3	63.49
10	—	—	—	157.42	—	—	—	157.23
11, 15	2H	6.86/d	8.6	114.27	2H	6.89/d	8.7	114.29
12, 14	2H	7.13/d	8.6	130.26	2H	7.14/d	8.7	130.29
13	—	—	—	128.53	—	—	—	128.66
16	H _a	3.05/m	—	36.72	H _a	3.07/m	—	36.27
	H _b	3.02	—		H _b	3.03		
17	1H	4.84/m	—	52.98	1H	4.86/m	—	52.96
18	—	—	—	175.58	—	—	—	175.58
19	N-H	11.98/s	—	—	N-H	12.0/s	—	—
20	—	—	—	171.55	—	—	—	171.55
22	2H	2.59/q	7.6	24.94	2H	2.55/q	7.5	24.67
23	3H	1.20/t	7.6	15.36	3H	1.23/t	7.5	14.67

¹ Refer Fig. 2 for numbering; ² This column gives ¹H - ¹H coupling constant; (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet



Scheme: Proposed degradation pathways for the (a) Oxidative degradation product, (b) base degradation products (impurity-1 and impurity-2)

to preparative LC as described in section 3.2.2. Fractions collected were analyzed by analytical HPLC as per the conditions mentioned in Section 3.2.1. Degradation impurity-1 and impurity-2 fractions were collected separately. Fractions of >95% were pooled together for impurity-1 and impurity-2 separately and concentrated on rotavapour to remove solvent mixture for both impurities. The concentrated fractions of impurity-1 and impurity-2 were again subjected to preparative HPLC using mobile phase consisting of a mixture of water and acetonitrile in the ratio of 50:50 (v/v) with UV detection at 225 nm and a flow rate of 10 mL/min. Again the eluate was concentrated using rotavapour for both impurities 1 and 2. To confirm the retention time of the isolated impurities the isolated fractions were analyzed by analytical

HPLC as mentioned in section 3.2.1. The impurity-1 is obtained as off-white solid and the chromatographic purity is 95.0%. The impurity-2 is obtained as off-white solid and the chromatographic purity is 96.0%.

3.3.3.2. Isolation of oxidative degradation product. Pioglitazone hydrochloride (2.0 g) was treated with hydrogen peroxide (5%, 25 mL) and kept at 80 °C for 24 h. Degraded solution was subjected to preparative LC as described in section 3.2.2. Fractions collected were analyzed by analytical HPLC as per the conditions mentioned in Section 3.2.1. Fractions of >95% were pooled, concentrated on rotavapour to remove solvent

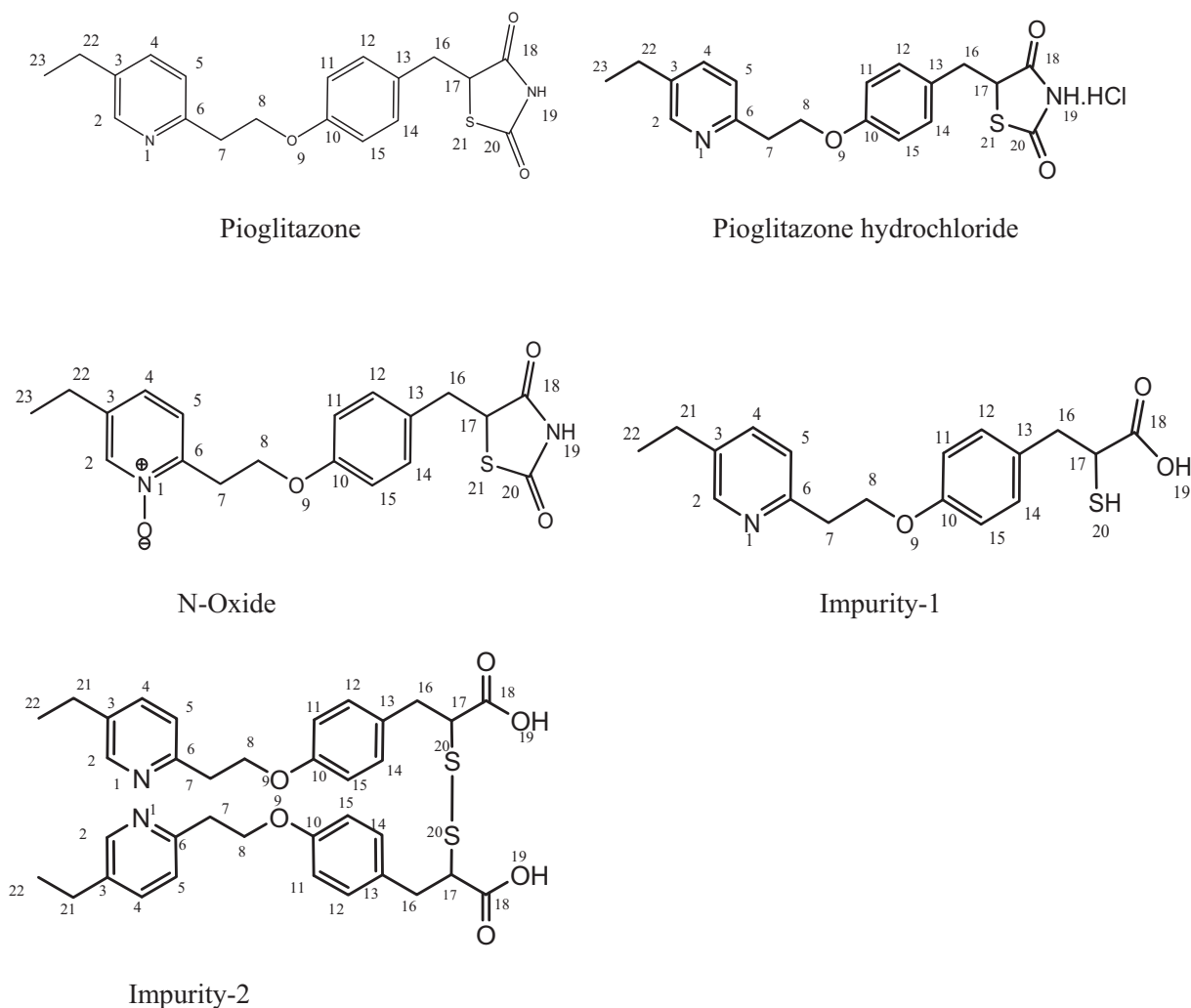


Fig. 2: Structures of Pioglitazone and degradation products

mixture. The concentrated fractions were again subjected to preparative HPLC using mobile phase consisting of a mixture of water and acetonitrile in the ratio of 50:50 (v/v) with UV detection at 225 nm and a flow rate of 10 mL/min. Again the eluate was concentrated on rotavapour. To confirm the retention time of the isolated impurity the isolated fraction was analyzed by analytical HPLC as mentioned in section 3.2.1. The impurity was obtained as off-white solid and the chromatographic purity is 97.0% by area normalization.

3.4. Structure elucidation of degradation products

3.4.1. Oxidative degradation impurity

The HR MS data of this oxidative degradation impurity showed exact mass of the protonated molecular ion at m/z 373.1204 (Calcd. 373.1222 for $C_{19}H_{21}N_2O_4S$) which corresponds to the molecular formula $C_{19}H_{20}N_2O_4S$.

The 1H and ^{13}C NMR spectral data of oxidative degradation impurity were compared with those of pioglitazone in Table 1. The numbering Scheme for the NMR assignments is shown in Fig. 2. The number of protons and carbon signals obtained in NMR spectra of degradation impurity are same as that of signals present in pioglitazone. However, the 1H and the ^{13}C chemical shifts of the CH groups attached to the nitrogen atom in the pyridine ring are shifted to down-field when compared to those of pioglitazone. The NMR experiments were done using $DMSO-d_6$ solvent. This observation lends support to the hypothesis of the formation of N-Oxide in the pyridine ring. The electrospray ionization (ESI) mass spectrum of this impurity exhibited a molecular ion peak at m/z , 373 amu (MH^+) in positive ion mode which is more than 16 amu to the molecular ion of pioglitazone. The IR (KBr) spectral data of impurity was compared with those of pioglitazone in Table 2. In IR absorption spectrum of degradation impurity, the absorption bands corresponding to major functional groups are similar to pioglitazone. IR absorption bands for this impurity (cm^{-1})

Table 2: FT-IR spectral data of pioglitazone and impurities

Compound	IR ^a
Pioglitazone	3416 (N-H stretching), 3084 (aromatic C-H stretching), 1692 (C=O stretching), 1509 (aromatic C=C stretching), 1461 (C-H bending), 1243 (C-O stretching).
N-Oxide	3422 (N-H stretching), 3090 (aromatic C-H stretching), 1701 (C=O stretching), 1513 (aromatic C=C stretching), 1421 (C-H bending), 1246 (C-O stretching).
Impurity-I	3446 (O-H stretching, broad), 3030 (aromatic C-H stretching), 2567 (S-H weak, sharp), 1685 (C=O stretching), 1512 (aromatic C=C stretching), 1404 (C-H bending), 1211 (C-O stretching), 1136 (C-O stretching).
Impurity-II	3440 (O-H stretching, broad), 3070 (aromatic C-H stretching), 1692 (C=O stretching), 1512 (aromatic C=C stretching), 1404 (C-H bending), 1200 (C-O stretching), 1028 (C-O stretching).

^a KBr

Table 3: NMR assignments of impurity-1 and impurity-2 (base degradation impurities)

Position ¹	Impurity-1				Impurity-2			
	¹ H	δ (ppm)	² J	¹³ C	¹ H	δ (ppm)	² J	¹³ C
2	1H	8.36/d	1.7	148.47	1H	8.71/s	—	144.93
3	—	—	—	136.58	—	—	—	141.08
4	1H	7.56/m	—	135.61	1H	8.36/d	7.3	140.58
5	1H	7.26/d	7.8	122.97	1H	7.93/d	8.0	126.88
6	—	—	—	155.48	—	—	—	151.51
7	H _a	3.19/m	—	36.85	2H	3.43/t	5.9	32.66
	H _b	3.15	—	—	—	—	—	—
8	2H	4.39/t	6.3	66.68	2H	4.36/t	6.1	65.49
10	—	—	—	156.65	—	—	—	156.69
11, 15	2H	6.79/d	8.8	113.95	2H	6.84/d	8.3	114.35
12, 14	2H	7.09/d	8.8	129.98	2H	7.11/d	8.3	130.12
13	—	—	—	132.51	—	—	—	130.03
16	H _a	3.13/m	—	42.00	H _a	2.99/m	—	35.86
	H _b	3.11	—	—	H _b	2.95/m	—	—
17	1H	3.33/m	—	47.21	1H	3.75/m	—	53.76
18	—	—	—	174.98	—	—	—	171.86
21	2H	2.58/q	7.3	24.97	2H	2.77/q	7.5	24.65
22	3H	1.18/t	7.5	15.38	3H	1.23/t	7.5	14.61

¹ Refer Fig. 2 for numbering; ²: This column gives ¹H - ¹H coupling constant; (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet

are 3422 (N-H stretching), 3090 (aromatic C-H stretching), 1701 (C=O stretching), 1513 (aromatic C=C stretching), 1421 (C-H stretching), 1246 (C-O stretching). From the spectral data, the structure of this degradation impurity is characterized as pioglitazone N-Oxide with molecular formula C₁₉H₂₀N₂O₄S and molecular weight 372.1.

3.4.2. Structure elucidation of base degradation impurity-1

The HR MS data of impurity-1 showed exact mass of the protonated molecular ion at *m/z* 332.1332 (Calcd. 332.1320 for C₁₈H₂₂NO₃S) which corresponds to the molecular formula C₁₈H₂₁NO₃S. The ¹H and ¹³C NMR spectral data of impurity-1 (Table 3) was compared with those of pioglitazone (Table 1). The numbering scheme for the NMR assignments is shown in Fig. 2. The ¹H and the ¹³C chemical shifts of the CH group at the 17th position of impurity-1 are shifted to down-field when compared with the pioglitazone. In ¹³C NMR, the presence of carbonyl group at 20th position in pioglitazone, was missing in impurity-1. Thus the impurity-1 structure can be rationalized in terms of opening of thiazolidine ring. The electrospray ionization (ESI) mass spectrum of this impurity exhibited a molecular ion peak at *m/z*, 332 amu (MH⁺) in positive ion mode which is less than 25 amu to the molecular ion of pioglitazone, indicating that loss of one carbon, one nitrogen and addition of one hydrogen atom in the molecule. IR absorption spectrum also supporting that loss of one carbonyl functional group and formation of the thiol functional group in the impurity-1. The IR (KBr) spectral data of impurity-1 was compared with those of pioglitazone in Table 2. IR absorption bands for this impurity (cm⁻¹) are 3446 (O-H stretching, broad), 3030 (aromatic C-H stretching), 2567 (S-H weak, sharp) 1685 (C=O stretching), 1512 (aromatic C=C stretching), 1404 (C-H bending), 1211 (C-O stretching) and 1136 (C-O stretching). From the spectral data, the structure of this impurity-1 is characterized as 3-(4-(2-(5-ethylpyridine-2yl) ethoxy) phenyl)-2-mercaptopropanoic acid with molecular formula C₁₈H₂₁NO₃S and molecular weight 331.1.

3.4.3. Structure elucidation of base degradation impurity-2

The HR MS data of impurity-2 showed exact mass of the protonated molecular ion at *m/z* 661.2383 (Calcd. 661.2406 for C₃₆H₄₀N₂O₆S₂) which corresponds to the molecular formula C₃₆H₄₀N₂O₆S₂. The ¹H and ¹³C NMR spectral data of impurity-2 (Table 3) was compared with those of pioglitazone (Table 1). The numbering scheme for the NMR assignments is shown in Fig. 2. The proton chemical shifts of the CH group at the 17th position of impurity-2 are shifted to down-field when compared with the pioglitazone. In ¹³C NMR, the presence of carbonyl group at 20th position in pioglitazone, was missing in impurity-2. Thus the impurity-2 structure can be rationalized in terms of opening of thiazolidine ring. The electrospray ionization (ESI) mass spectrum of this impurity exhibited a molecular ion peak at *m/z*, 661 amu (MH⁺) in positive ion mode which is more than 304 amu to the molecular ion of pioglitazone and the 329 amu to the molecular ion of impurity-1. Thus spectral data indi-

cating that it can be the dimmer impurity of impurity-1. To confirm that ¹H and ¹³C NMR spectral data of impurity-2 was compared with those of impurity-1 (Table 3). The ¹H and the ¹³C chemical shifts of the CH group at the 17th position of impurity-2 are shifted to up-field when compared with the impurity-1. This is in well agreement with the IR pattern of impurity-2. The IR (KBr) spectral data of impurity-2 was compared with those of impurity-1 in Table 2. IR (KBr) absorption bands for this impurity (cm⁻¹) are 3440 (O-H stretching, broad), 3070 (aromatic C-H stretching), 1692 (C=O stretching), 1512 (aromatic C=C stretching), 1404 (C-H bending), 1200 (C-O stretching), 1028 (C-O stretching). From the spectral data, the structure of this impurity is characterized as 2-(1-carboxy-2-{4-[2-(5-ethylpyridine-2yl)-ethoxy] phenyl}-ethyl disulfanyl)-3-{4-[2-(5-ethylpyridine-2yl)-ethoxy] phenyl} propanoic acid with molecular formula C₃₆H₄₀N₂O₆S₂ and molecular weight 660.2.

3.5. Formation of impurities

Oxidative degradation impurity formed in the presence of peroxide stress degradation, to the corresponding to pyridine ring. Both impurity-1 and impurity-2 were formed in the presence of base stress degradation. Impurity-1 is formed due to cleavage of thiazolidine ring and the impurity-2 is obtained as the dimmer impurity of impurity-1 by forming the disulfide bond formation. The probable degradation pathway is shown in the Scheme 1.

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