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Short communication

Identification and characterization of degradation products of dicloxacillin in bulk drug and pharmaceutical dosage forms

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Abstract

Impurity profiling of dicloxacillin sodium bulk drug and pharmaceutical dosage forms subjected to stability studies is evaluated. Of many impurities detected in HPLC analysis, three were not reported in the literature. The impurities have been identified by LC-MS; isolated by preparative HPLC; and characterised by NMR, Mass spectroscopy and IR. Pure impurities obtained by isolation were co-injected with dicloxacillin sodium sample to confirm the retention times in HPLC. Structure elucidation of these degradation products by spectral data has been discussed in detail.
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Keywords: Dicloxacillin; Degradation products; Isolation; Preparative HPLC; Characterization

1. Introduction

Dicloxacillin (Fig. 1) is an isoxazolyl penicillin used in the treatment of infections due to staphylococci resistant to benzyl penicillin [1]. Isoxazolyl penicillins are likely to contain degradation products, viz., opened β-lactam ring products, rearrangement products derived from the nucleus as well as the side chain and the products of polymerization [2–4]. Reported literature was based mainly on HPLC methods for specific determination of isoxazolyl penicillins in the presence of their degradation products [2–8]. Present work involves identification, isolation and characterization via spectral analysis for the degradation products other than those specified in pharmacopoeia, formed in dicloxacillin bulk drug and pharmaceutical dosage forms subjected to stability studies. Three impurities of this kind were observed under accelerated stability conditions. Dicloxacillin bulk drug and pharmaceutical dosage forms do not show any of these three impurities initially but in 3 months accelerated stability conditions, they are formed in the range of 0.1–0.2% by area normalization. On further subjecting these samples to accelerated stability conditions, impurity-I forms up to 0.2%, impurity-II up to 0.5% and impurity-III up to 1.0%.

2. Experimental

2.1. Samples

Dicloxacillin bulk drug and available impurities were synthesized in CRD department of APL Research Centre (A Division of Aurobindo Pharma Ltd.) (Bachupally, Quthubullapur, Hyderabad 500072, India). Formulation department provided dicloxacillin 500 mg capsules. Dicloxacillin bulk drug samples, canister packing and dicloxacillin 500 mg capsules, in high density polyethylene container were kept at 40 °C/75% RH for 3 months in stability chamber.

2.2. Chemicals and reagents

Ammonium acetate, GR grade, methanol and acetonitrile of HPLC grade were obtained from E. Merck, India. Distilled water was prepared by using Milli-Q water purification system (Millipore, Bedford, MA). All other chemicals were of analytical

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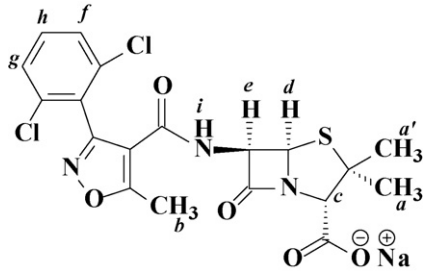


Fig. 1. Chemical structure of dicloxacillin.

grade obtained from E. Merck, India and were used without further purification.

2.3. Analytical LC conditions-bulk drug

Chromatographic separations were performed on High Performance Liquid Chromatograph system with Waters alliance 2695 separations module equipped with 2996 photodiode array detector with *Empower pro* data handling system [Waters Corporation, MILFORD, MA01757, USA]. A Hypersil ODS column with dimensions of 250 mm × 4.0 mm i.d., 5 μm particle size was employed for separations. The chromatographic conditions used were as per European Pharmacoepia with the mobile phase consisting of 75 volumes of 20 mM potassium dihydrogen orthophosphate, pH 5.0 ± 0.05 adjusted with dilute sodium hydroxide solution and 25 volumes of acetonitrile. Flow rate was kept at 1.5 ml/min and the column effluent was monitored at 225 nm [9].

2.4. Analytical LC conditions-formulation

The capsule samples were analyzed on a Lichrosphere RP 18e (250 mm× 4.0 mm, 5 μm) column with the mobile phase consisting of 20 mM potassium dihydrogen orthophosphate, pH 5.0 ± 0.05 adjusted with dilute sodium hydroxide solution (A) and acetonitrile (B). Flow rate was 1.5 ml/min and detection was carried out at 225 nm. The gradient program is given in Table 1.

2.5. Preparative LC conditions

A Shimadzu LC-8A preparative Liquid Chromatograph equipped with SPD-10A VP, UV–vis detector [Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan] was used. Inertsil ODS (250 mm × 20 mm i.d.; 10 μm) preparative column was employed for isolation of impurities. The mobile phase consisted of 0.2 M ammonium acetate solution, pH adjusted to 5.0 with glacial acetic acid (A) and acetonitrile (B). Flow rate was 40 ml/min and detection was carried out at 225 nm. The gradient program is given in Table 2.

Table 1
Formulation LC gradient program

Time (min)	% of Solvent A	% of Solvent B
0	75	25
18	75	25
23	65	35
30	65	35
31	75	25
40	75	25

Table 2
Preparative LC gradient program

Time (min)	% of Solvent A	% of Solvent B
0	100	00
10	95	05
20	90	10
30	85	15
60	80	20
80	75	25
100	75	25

2.6. LC-MS conditions

LC-MS analysis was carried out on Perkin-Elmer triple quadrupole mass spectrometer (API 2000, PE SCIEX) coupled with Shimadzu HPLC equipped with SPD 10 A VP UV–vis detector and LC 10 AT VP pumps. Analyst software was used for data acquisition and data processing. The turbo ion spray voltage was maintained at −4.5 kV and temperature was set at 375 °C. The auxiliary gas and sheath gas used was high pure nitrogen. Zero air was used as Nebuliser gas. LC-MS spectra were acquired from *m/z* 100–1000 in 0.1 amu steps with 2.0 s dwell time. LC-MS analysis of the stability samples was carried out using Hypersil-BDS C18 column with dimensions of 250 mm × 4.6 mm; 5.0 μm particle size. The mobile phase consisted of 10 mM ammonium acetate solution, pH adjusted to 5.0 with glacial acetic acid (A) and acetonitrile:methanol: [1:1, v/v] (B). Flow rate was 1.0 ml/min. The gradient program is given in Table 3.

2.7. NMR spectroscopy

The ^{1}H, ^{13}C experiments were performed on a Bruker Avance DPX-300 MHz [Faellanden, Switzerland] NMR spectrometer using DMSO-d_6 as solvent. ^{1}H chemical shift values were reported on the δ scale in ppm relative to TMS ($\delta = 0.00$ ppm) as internal standard and the ^{13}C chemical

Table 3
LC-MS gradient program

Time (min)	% of Solvent A	% of Solvent B
0	80	20
5	75	25
10	75	25
15	65	35
25	60	40
30	50	50
32	80	20
40	80	20

shift values were reported calibrating DMSO-d_6 signal to $\delta = 39.5$ ppm.

2.8. IR spectroscopy

The IR spectra were recorded in the solid state as KBr pellet using Perkin-Elmer instrument, model-spectrum one.

3. Results and discussions

3.1. Detection of impurities I, II and III

The stability samples were diluted to the required concentration and injected into the analytical LC using the conditions as described in Section 2.3. Major degradation products other than those specified in European Pharmacopoeia, eluted at 1.8, 3.0 and 11.8 min, respectively, while dicloxacillin eluted at 9.2 min. Typical chromatograms are shown in Fig. 2. The same samples were subjected to LC-MS using conditions as described in Section 2.6 to determine the molecular mass of the impurities. The masses of the impurities recorded in negative ion mode were 530 for impurity-I with dichloro pattern, 329 for impurity-II with dichloro pattern and 799 for impurity-III with tetrachloro pattern.

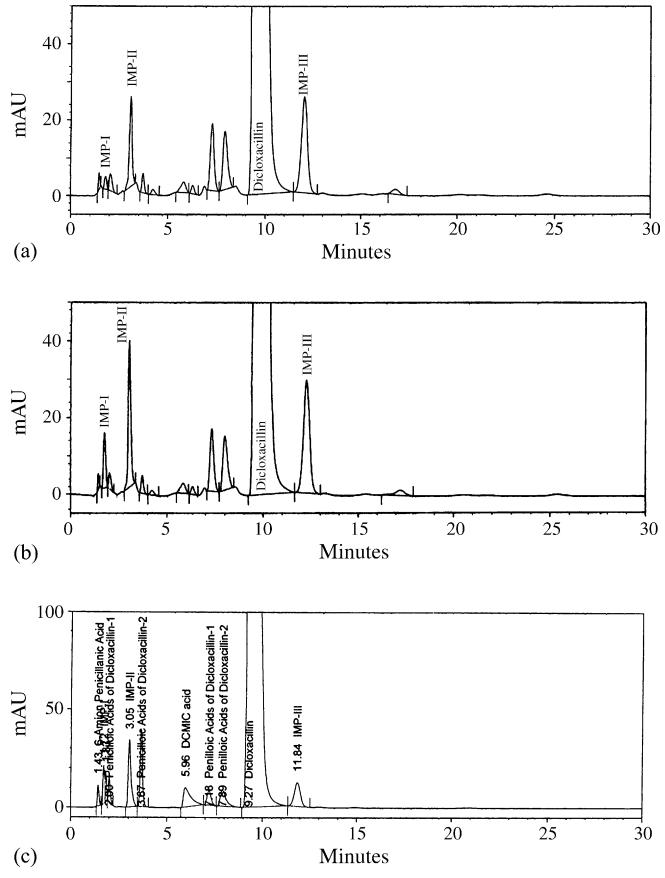


Fig. 2. Typical representative chromatograms: (a) HPLC chromatogram of dicloxacillin sodium bulk drug, (b) HPLC chromatogram of dicloxacillin sodium bulk drug spiked with impurities I, II and III and (c) HPLC chromatogram of dicloxacillin sodium spiked with pharmacopoeial impurities along with impurities I, II and III.

3.2. Enrichment of degradation products in dosage forms

Approximately 10 gm of capsule blend was subjected to autoclave for 20 min at temperature 120–24 °C and pressure 15–17 psi. The degradation products, which were 0.2–1.0% initially, were enriched up to 0.5–5.0% levels. The purity of the blend before and after autoclave was determined using LC conditions as described in Section 2.4.

3.3. Isolation of degradation product(s) by preparative HPLC

Two grams of sample enriched with impurities were subjected to preparative HPLC under the conditions described in Section 2.5. Fractions collected were analyzed by analytical LC conditions described in Section 2.4. Fractions of >95% purity were pooled together; concentrated on rotavapor to remove acetonitrile. Concentrated fractions were passed through the preparative column by using water:acetonitrile (50:50) as mobile phase to remove ammonium acetate. Again the eluate was concentrated using rotavapor to remove acetonitrile and the aqueous solution was lyophilised using a freeze dryer (Virtis advantage 2XL, Gardiner, NY 12525, USA). Impurity-I was obtained as a white powder with chromatographic purity of 99.0%, Impurity-II was obtained as a white powder with chromatographic purity 99.6% and Impurity-III was obtained as a white powder with chromatographic purity 97.0%.

3.4. Structural elucidation of degradation products

3.4.1. Dicloxacillin sodium

^{1}H NMR values (Fig. 1) in DMSO-d_6: δ(ppm) 1.45 and 1.47 (2s, 6H, a and a^1), 2.73 (s, 3H, b), 3.93 (s, 1H, c), 5.41–5.47 (m, 2H, d and e), 7.54–7.65 (m, 3H, f, g and h), 8.10 (d ($J = 7.41$ Hz), 1H, i); ^{13}C NMR values in DMSO-d_6: δ(ppm) 13.8, 28.1, 33.2, 58.8, 65.4, 67.6, 74.5, 113.0, 127.6, 129.5(2C), 133.5, 135.3, 135.7, 158.2, 160.4, 171.3, 173.1(2C). ESI mass spectrum of dicloxacillin sodium in negative ion mode showed molecular ion peaks at *m/z*-468 [(M−H)$^-$], 470 [(M−H+2)$^-$], 472 [(M−H+4)$^-$] with intensities in the ratio of (100:64:10) indicating dichloro pattern. FT-IR absorption frequencies (cm^{-1}): 3364(m) NH stretch; 2969, 2931, 2868(w) CH_3 stretch; 1786(s) β-lactam C=O stretch; 1657(s) C=O stretch (amide); 1604(s) C=O stretch (carboxyl); 1621, 1505(s) C=C and C=N stretch; 1445, 1370(m) CH_3 bending; 793, 787, 777 m C–Cl stretch and =C–H out of plane bend. Molecular formula is $C_{19}H_{17}Cl_2N_3O_5S$ with molecular mass of 470.

3.4.2. Impurity-I

ESI mass spectrum of this impurity in negative ion mode showed molecular ion peaks at *m/z*-528 [(M−H)$^-$], 530 [(M−H+2)$^-$], 532 [(M−H+4)$^-$] with intensities in the ratio of (100:64:10) indicating dichloro pattern. This impurity is having molecular mass 60 amu more than dicloxacillin. ^{1}H NMR values (Fig. 3a) in DMSO-d_6: δ(ppm) 1.27 and 1.49 (2s, 6H, a and a^1), 1.81 and 2.12 (2s, 3H, b), 2.74 (s, 3H, c), 4.05 (s, 1H, d), 4.91 (m, 1H, e), 5.54 (m, 1H, f), 7.03 (d ($J = 7.14$ Hz), 1H, g), 7.51–7.61 (m, 3H, ArH). From ^{1}H NMR

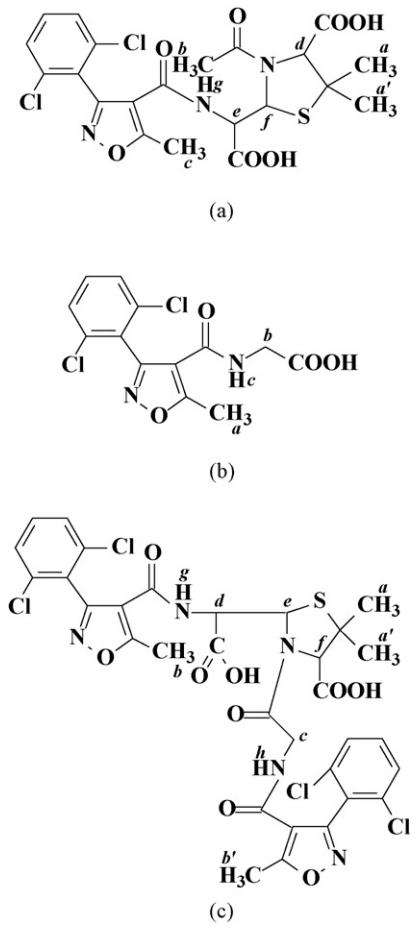


Fig. 3. Chemical structures of dicloxacillin impurities. (a) Impurity-I, (b) Impurity-II and (c) Impurity-III.

values an upfield shift of proton 'e' was observed compared to dicloxacillin, showing the breakage of β-lactam ring. An extra CH_3 signal appeared at 2.12 ppm, showing the presence of acetyl group. ^{13}C NMR values in DMSO-d_6: δ(ppm) 13.4, 24.7, 27.5, 34.2, 51.4, 57.9, 65.7, 78.2, 113.6, 127.9, 129.2, 129.3(2C), 133.1, 135.4(2C), 158.4, 159.5, 171.8, 172.4, 172.8. In IR spectrum, band at 1786 cm^{-1} corresponding to β-lactam C=O stretch was absent. FT-IR absorption frequencies (cm^{-1}): 3401 and 3196 (br and s), 2935(m) CH_3 stretch; 1715(m) C=O stretch (carboxyl); 1652(s) C=O stretch (amide); 1602, 1510(s) C=C and C=N stretch; 780(m) C–Cl stretch, 732(m) =C–H out of plane bend. The above spectral data support the assigned structure as (2*RS*,4*S*)-3-acetyl–2-[carboxy[[[3- (2, 6-dichlorophenyl)-5-methylisoxazole-4-yl] -carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxy - lic acid [acetylated penicilloic acids of dicloxacillin] with molecular formula $C_{21}H_{21}Cl_2N_3O_7S$ and molecular mass of 530. This impurity exists as a mixture of two stereoisomers.

3.4.3. Impurity-II

ESI mass spectrum of this impurity in negative ion mode showed molecular ion peaks at *m*/*z*-327 [(M−H)$^-$], 329 [(M−H+2)$^-$], 331 [(M−H+4)$^-$] with intensities in the ratio of (100:64:10) indicating dichloro pattern. ^1H NMR values (Fig. 3b) in DMSO-d_6: δ(ppm) 2.72 (s, 3H, a), 3.74 (d (J = 5.76 Hz), 2H, b), 7.51–7.62 (m, 3H, ArH), 8.04 (t, 1H, c). Signal at 8.04 ppm got exchanged with D_2O. ^1H NMR spectrum showed the absence of 6-aminopenicillanic acid moiety. Doublet at 3.74 ppm and triplet at 8.04 ppm indicated the O=C–NH–CH_2– linkage. ^{13}C NMR values in DMSO-d_6: δ(ppm) 13.4, 42.1, 114.0, 128.1, 129.2(2C), 133.0, 135.4(2C), 158.8, 160.7, 171.3, 171.7. FT-IR absorption frequencies (cm^{-1}): 3269 (br) NH stretch; 3072 Aryl CH stretch; 2982, 2936 CH_3 and CH_2 stretch; 1732 C=O stretch of COOH; 1669 C=O stretch (amide); 1607; 1532 C=C and C=N; 784(m) C–Cl stretch. The above spectral data are in conformity with the proposed structure as 2-[[[3-(2,6-dichlorophenyl)-5-methylisoxazole-4-yl] carbonyl] amino] acetic acid with molecular formula $C_{13}H_{10}Cl_2N_2O_4$ and molecular mass of 329.

3.4.4. Impurity-III

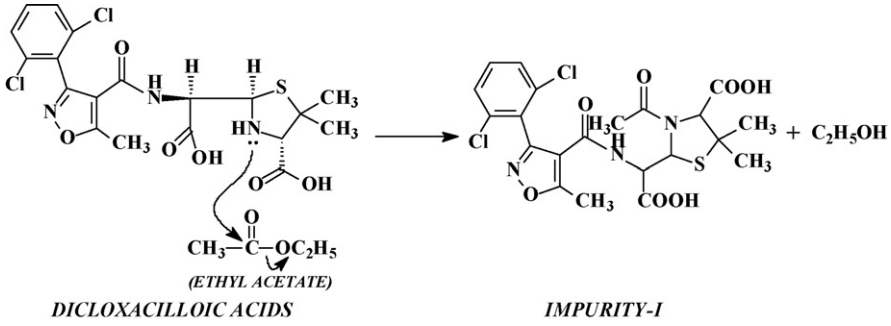
ESI mass spectrum of this impurity in negative ion mode showed molecular ion peaks at *m*/*z*-796 [(M−H)$^-$], 798 [(M−H+2)$^-$], 800 [(M−H+4)$^-$], 802 [(M−H+6)$^-$] with intensities in the ratio of (78:100:48:10) indicating tetrachloro pattern. ^1H NMR values (Fig. 3c) in DMSO-d_6: δ(ppm) 1.47 and 1.54 (2s, 6H, a and a^1), 2.67 and 2.74 (2s, 6H, b and b^1), 3.67 and 4.11 (2d (J = 16.47 Hz), 2H, c), 4.63 (brs, 1H, d), 5.27 (brs, 1H, e), 5.56 (s, 1H, f), 7.07(brs,1H, g), 7.55(m, 6H, ArH), 8.07(brs, 1H, h), 13.3(brs). In ^1H NMR spectrum, signals at 5.41–5.47 ppm corresponding to d and e protons in dicloxacillin were absent and new signals appeared with upfield shift at 4.63 and 5.27 ppm. This is an evidence to confirm that the β-lactam ring is absent. In addition to this, two methyl signals appeared at 2.67 and 2.74 ppm instead of one methyl signal at 2.73 ppm in dicloxacillin. Signals at 7.55 ppm appeared with six protons indicating presence of two dichlorophenyl-methyl isoxazole groups. These spectral data indicate that this impurity is an adduct of 2-[[[3-(2,6-dichlorophenyl)-5-methylisoxazole-4-yl] carbonyl] amino] acetic acid and dicloxacillin. ^{13}C NMR values in DMSO-d_6: δ(ppm) 13.4, 13.5, 26.5, 33.8, 43.0, 51.6, 56.0, 66.3, 73.5, 113.1, 114.0, 127.6, 128.0, 129.1(2C), 129.3, 129.4, 133.0, 133.3, 135.3(3C), 135.5, 158.3, 158.7, 160.5, 160.6(2C), 170.9, 171.4(2C), 172.6. IR absorption spectrum showed the absence of β-lactam C=O stretch. FT-IR absorption frequencies (cm^{-1}): 3397(m) NH stretch; 3086 Aryl CH stretch; 2972, 2933 CH_3 stretch; 1735 C=O stretch of COOH; 1659 CONH; 1603; 1518, 1432 C=C and C=N; 780(m) C–l stretch. The above spectral data supports the assigned structure as 2-[carboxy[[[3-(2,6-dichlorophenyl)-5-methylisoxazole-4-yl]carbonyl]amino]methyl]-3-[2-[[3-(2,6-dichlorophenyl)-5-methylisoxazole-4-yl]carbonyl]amino]acetyl]5,5-dimethylthiazolidine-4-carboxylic acid with molecular formula $C_{32}H_{27}Cl_4N_5O_9S$ and molecular mass of 799.

3.5. Formation of impurities

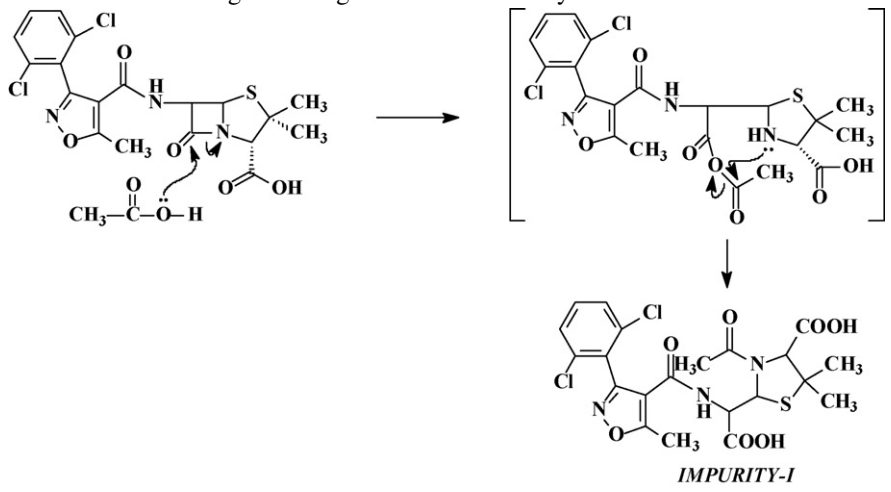
3.5.1. Impurity-I

β-lactam ring of dicloxacillin sodium undergoes hydrolysis under the forced degradation conditions resulting in the formation of penicilloic acids of dicloxacillin (dicloxacilloic acids).

The basic nitrogen of the thiazolidine ring of dicloxacilloic acids probably undergoes acetylation under degradation conditions (temperature and/or pressure) in presence of ethyl acetate present during crystallization from ethyl acetate medium to produce detectable quantities of *N*-acetyl dicloxacilloic acid.

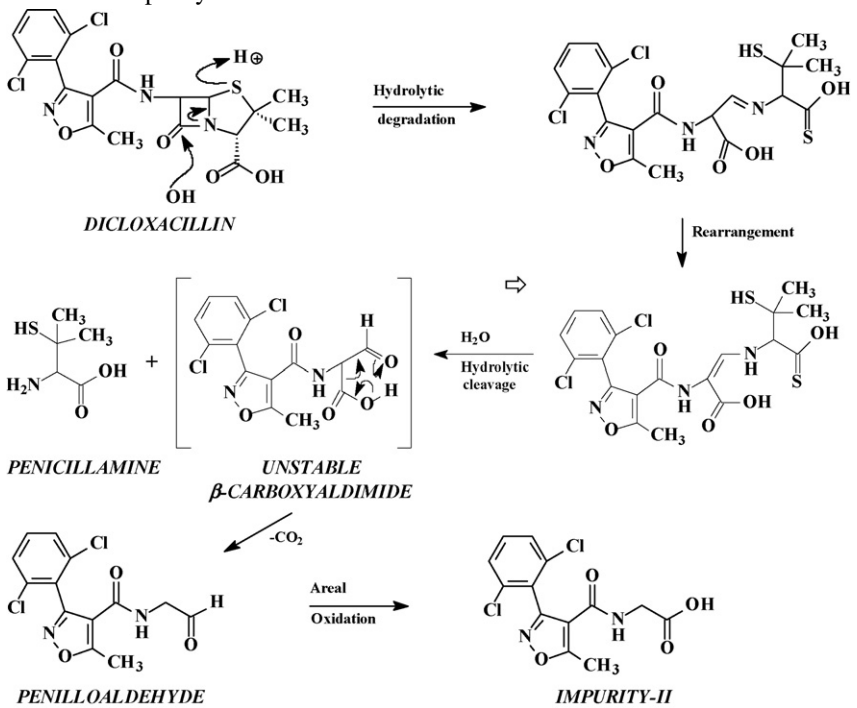


An alternate mechanism is that acetic acid, byproduct of ethyl acetate hydrolysis could open the β-lactam ring to a transient acetic-penicilloic anhydride that could undergo rearrangement to the *N*-acetyl dicloxacilloic acid.



3.5.2. Impurity-II

Penilloaldehyde [10] a known impurity of the penicillin on atmospheric oxidation under the degradation conditions is the most likely pathway for the formation of Impurity-II.



3.5.3. Impurity-III

Impurity-II forms adduct with dicloxacillin sodium to give Impurity-III.

4. Conclusion

Dicloxacillin sodium bulk drug and pharmaceutical dosage forms subjected to stability studies were evaluated for degradation products. Apart from pharmacopoeial impurities, three unknown impurities not reported in the literature were observed. These degradation products were isolated and characterised by spectroscopic techniques IR, NMR and MS. The most probable structures are proposed for these impurities based on the spectral data. Of the three degradation products isolated, impurity-III was the major degradation product in accelerated stability study.

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