Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 582–587

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Identification, isolation and characterization of a new degradation product in sultamicillin drug substance

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ARTICLE INFO

Article history:
Received 13 May 2010
Received in revised form 19 August 2010
Accepted 12 September 2010
Available online 19 September 2010

Keywords:
Sultamicillin
Stability
Impurities
2D-NMR
Characterization

ABSTRACT

A new degradant of sultamicillin drug substance was found during the gradient reverse phase HPLC analysis of stability storage samples. The level of this degradant impurity was observed up to 1.0%. The impurity (formaldehyde adduct with 5-oxo-4-phenylimidazolidin-1-yl moiety) was identified by LC/MS and was characterized by (^{1}H NMR, ^{13}C NMR, 2D-NMR (^{1}H–^{1}H COSY, NOESY, HSQC and HMBC), LC/MS/MS, MS/TOF, elemental analysis and IR. This impurity was prepared by isolation and co-injected into HPLC system to confirm the retention time.

1. Introduction

Sultamicillin, a mutual prodrug of ampicillin and sulbactam coupled together with ester linkage, is one of the antibiotics with adequate antimicrobial spectrum for the treatment of childhood pneumonia. It is available in both oral and parenteral preparations for pediatric use. Sultamicillin is also a valuable treatment option for a variety of pediatric infections, including those due to β-lactamase-producing organisms [1,2]. The use of β-lactam/β-lactamase inhibitor combinations, particularly ampicillin/sulbactam, as empiric therapy or prophylaxis for number of pediatric infections are well established, and have been extensively reviewed [1–5]. The antimicrobial activity of sultamicillin had been demonstrated *in vitro* against wide range of Gram-positive, Gram-negative organisms and anaerobes [1,6]. The oral suspension is highly palatable and can be administered without regard to meal times. This regimen is likely to be more convenient than others for the treatment of children, as it fits more easily into the normal daily routine, and may lead to greater compliance [7]. It was developed in 1987 by Pfizer Inc. and marketed under the trade name Unasyn [8]. The formation of degradants of sultamicillin during stability storage was studied in this research work. Sultamicillin was subjected to stress and formal stability storage conditions (accelerated and real time storage) as per International Conference on Harmonization (ICH) guidelines [9]. During the analysis of stability storage samples, an unknown degradant was observed other than the known impurities [10–13]. The present work deals with identification, isolation and structural elucidation of this degradant and with the mechanism of its formation.

2. Experimental

2.1. Chemicals, reagents and samples

The investigated samples of sultamicillin were taken from APL Research Centre Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). Sodium dihydrogen orthophosphate dihydrate, acetonitrile, methanol, ammonium acetate, orthophosphoric acid, formic acid, acetic acid and potassium bromide (IR spectroscopy grade) were procured from Merck (India) limited, formaldehyde (AR grade) was procured from Sigma–Aldrich (India) and pure Milli-Q water was prepared with the help of Millipore purification system.

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doi:10.1016/j.jpba.2010.09.016

2.2. High performance liquid chromatography (HPLC)

Chromatographic separations were performed on HPLC system with Waters alliance 2695 separation module equipped with 2996 photodiode array detector with Empower pro data handling system (Waters Corp., Milford, MA 01757, USA). The analysis was carried out on Kromasil C18, 100 mm × 4.6 mm, 3.5 μm particle size column. Mobile phase A was phosphate buffer pH 3.0 (prepared by dissolving 4.68 g of sodium dihydrogen orthophosphate dihydrate in 1000 ml water, and pH adjusted to 3.0 ± 0.05 using orthophosphoric acid). Mobile phase B was acetonitrile. Diluent was prepared by mixing solution A and solution B in the ratio of 70:30 (v/v) (solution A: methanol and acetonitrile in the ratio of 20:80, v/v and solution B: dissolved 1.56 g of sodium dihydrogen orthophosphate dihydrate in 900 ml of water and added 7.0 ml of orthophosphoric acid and diluted to 1000 ml with water). Injection volume was 10 μl, flow rate 1.0 ml/min and column oven temperature 25 °C. UV detection was carried out at 215 nm and data acquisition time was 20 min. The gradient program was as follows:

Time (min)/A (v/v):B(v/v); $T_{0.01}$/95:05, T_{15}/30:70, T_{20}/30:70, T_{22}/95:05, T_{30}/95:05.

2.3. Preparative liquid chromatography

A Shimadzu LC-8A preparative liquid chromatograph equipped with SPD-10A VP, UV–vis detector (Shimadzu Corp., Analytical Instruments Division, Kyoto, Japan) was used. Hyperprep HS C_{18} 500 mm × 30 mm (Thermo Scientific) 10 μm particle size column was employed. Mobile phase A was 0.2% ammonium acetate solution, pH adjusted to 5.0 ± 0.1 with glacial acetic acid and mobile phase B was acetonitrile. Flow rate was 30 ml/min and UV detection was carried out at 215 nm. The gradient program was as follows:

Time (min)/A (v/v):B (v/v); $T_{0.01}$/100:0, T_{10}/90:10, T_{25}/80:20, T_{40}/70:30, T_{55}/60:40, T_{65}/55:45, T_{80}/50:50, T_{100}/40:60, T_{120}/20:80.

2.4. LC/MS/MS

LC/MS/MS analysis was carried out on Applied biosystems triple quadrupole mass spectrometer (API 2000, PE SCIEX) coupled with a Shimadzu HPLC equipped with SPD 10 AT 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 5500 mV and temperature was set at 375 °C. High purity nitrogen gas was used as auxiliary gas and curtain gas. Zero air was used as nebulizer gas. LC/MS spectra were acquired from m/z 100 to 1000 in 0.1 amu steps with 2.0 s dwell time. The analysis of the samples was carried out by using YMC PACK ODS-AQ, 250 mm × 4.6 mm, 5 μm particle size column. Mobile phase A was 0.1% formic acid and mobile phase B was acetonitrile. UV detection was carried out at 215 nm, flow rate was 1.0 ml/min and data acquisition time was 30 min. The gradient program was as follows:

Time (min)/A (v/v):B (v/v); $T_{0.01}$/95:05, T_{20}/30:70, T_{30}/20:80, T_{32}/95:05, T_{40}/95:05.

2.5. MS/TOF

The MS part consisted of Micro Q-TOF spectrometer (Waters micromass) operated with MassLynks (version 4.1) software. The fragmentation profile of the samples was established by carrying out MS/TOF studies in positive electrospray ionization (+ve ESI) mode. The mass parameters for MS/TOF analysis were, hexapole Rf, capillary voltage 3000 V, cone voltage 25 V, source temperature 120 °C, desolvation temperature 300 °C, ion energy 1.0 V and collision energy 8 V. The samples were directly infused using a syringe at a concentration of 2 mg/ml in methanol.

2.6. NMR

1D (^{1}H NMR, ^{13}C NMR) and 2D (DEPT, ^{1}H–^{1}H COSY, NOESY, HSQC and HMBC) NMR experiments were performed on Bruker 300 MHz NMR spectrometer (Bruker AG Industries, Faellanden, Switzerland) using deuterated dimethylsulfoxide (DMSO-d_6) as solvent and tetramethylsilane (TMS) as internal standard at 25 °C. For ^{1}H NMR and ^{13}C NMR spectrometer, the operating frequencies were 300.1315 and 75.4748 MHz; number of scans was 32 and 1062, respectively. LB 0.3 Hz and SF 300 MHz parameters were used for data processing.

2.7. FT-IR

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

2.8. Stability studies

As per ICH guidelines [9] sultamicillin drug substance was subjected to thermal exposure 60 ± 2 °C and analyzed at different time intervals: 12 h, 1 day, 2, 5, 10 and 15 days, respectively. The degradation at elevated temperature and humidity, i.e. 40 ± 2 °C/75 ± 5%RH, 25 ± 2 °C/60 ± 5%RH was also investigated at various time intervals from 1 to 3 months. The samples were also kept at refrigerator condition (5 ± 3 °C) for 2, 3 and 6 months. All these samples were packed in a low-density polyethylene bag, which was closed with a twist tie with a plastic fastener. This bag was inserted into a second low-density polyethylene bag, which was heat sealed and further placed in a triple laminate of 20 μm thickness (polyethyleneterephthalate/aluminium foil/linear low-density polyethylene) bag, which was also heat sealed. Finally, this pack was placed in a well closed high-density polyethylene (HDPE) container. The samples were diluted to the required concentration and were injected into HPLC using the analytical conditions described in Section 2.2. From the chromatograms, an unknown degradant was found along with known impurities listed in Ph. Eur. [10]. This impurity was eluted after the elution of sultamicillin EP impurity G at a relative retention time around 1.44 with respect to sultamicillin. The level of this degradant during the stability storage studies at different conditions is given in Table 1. The impurity was prepared in small quantities by preparative isolation and co-injected with sultamicillin into the HPLC for confirmation of relative retention time and also analyzed by FT-IR in order to acquire additional identification data. Typical HPLC chromatograms of sultamicillin spiked with pharmacopoeial impurities along with new impurity and sultamicillin spiked with new impurity are shown in Fig. 1.

3. Results and discussion

3.1. Structural elucidation of impurity

In the course of the LC/MS/MS studies the ESI mass spectrum of this impurity displayed a protonated molecular ion at m/z 607 amu [$(M+H)^+$] in positive ion mode, which is 12 units more than that of sultamicillin protonated molecular ion. The MS/TOF spectrum shows a molecular ion peak at m/z 629.1368 amu [$(M+Na)^+$] as sodium adduct against the theoretical mass of m/z 629.1352 amu [$(M+Na)^+$]. The mass error between calculated and observed masses is 2.5 ppm. The ^{1}H NMR and ^{13}C NMR spectra of this impurity show chemical shift (δ) values which are comparable with sultamicillin

Table 1
Formation of impurity at different stability storage conditions.

Time period	Sulbactam, %	Ampicillin, %	Impurity at RRT ~ 1.44, %	Total related substances, %
Formal stability storage conditions[a]				
40 ± 2 °C/75 ± 5%RH				
Initial	0.12–0.12	0.16–0.22	ND–0.11	1.32–1.62
1 month	0.36–0.55	0.31–0.36	0.58–0.77	2.56–3.30
2 months	0.37–0.50	0.31–0.36	0.66–0.92	3.06–3.80
3 months	0.36–0.45	0.28–0.32	0.64–0.87	2.92–3.73
25 ± 2 °C/60 ± 5%RH				
1 month	0.27–0.38	0.21–0.33	0.27–0.37	1.77–2.36
2 months	0.28–0.40	0.30–0.36	0.36–0.52	2.19–2.82
3 months	0.31–0.41	0.29–0.42	0.38–0.48	2.23–2.99
5 ± 3 °C				
2 months	0.13–0.18	0.19–0.26	0.11–0.19	1.57–1.89
3 months	0.13–0.19	0.18–0.26	0.11–0.15	1.39–1.76
6 months	0.19–0.26	0.24–0.31	0.12–0.20	1.64–1.93
Stress stability storage conditions[b]				
60 ± 2 °C				
Initial	ND	0.19	0.11	1.79
12 h	0.20	0.41	0.41	3.27
1 day	0.46	0.54	1.26	6.27
2 days	0.40	0.56	1.16	5.87
5 days	0.36	0.54	1.02	4.96
10 days	0.34	0.59	1.12	6.29
15 days	0.34	0.70	1.11	7.46

[a] These results are based on three batches analysis and reported in %w/w (by HPLC).
[b] These results are based on the single batch analysis and reported in %w/w (by HPLC).

except at phenyl glycine and lactam moieties of ampicillin linkage. In the NMR data of sultamicillin and the impurity in Table 2 appreciable ^{13}C chemical shifts have been observed at 13, 14 and 18 positions. At signal position 14, downfield shift from δ 58.8 to δ 66.25, at signal position 13, an upfield shift from δ 68.2 to δ 60.17 and at signal position 18, an upfield shift from δ 62.8 to δ 60.90 have been observed, indicating the possibility of cyclization in the phenyl glycine moiety. At position 24, additional signals of δ 4.52 and δ 4.79 in ^1H NMR and δ 82.1 in DEPT and ^{13}C NMR represent the methylene signal of imidazolone moiety of this impurity. In comparison with sultamicillin, amide NH signal is found to be absent at position 16 and an additional chemical shift value δ 4.00 corresponds to NH signal of imidazolone moiety at position 19, which is an exchangeable proton with D_2O. This phenomenon has fur-

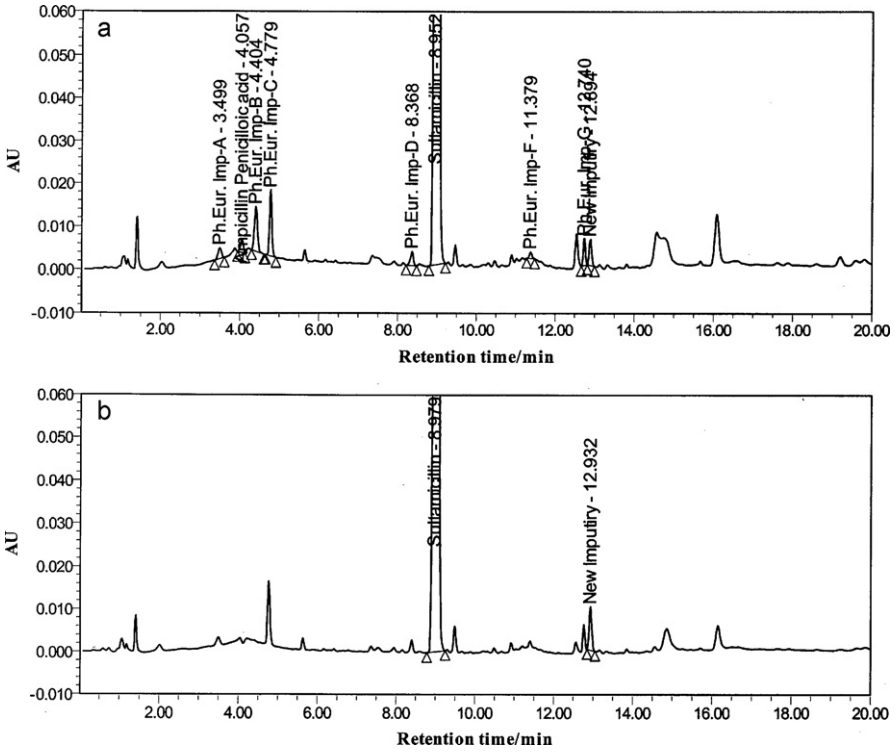

Fig. 1. HPLC chromatograms of sultamicillin drug substance spiked with Ph. Eur. impurities including new impurity (a); and sultamicillin drug substance spiked with new impurity (b).

Table 2
Comparative ^{1}H, ^{13}C NMR, DEPT ,HSQC and HMBC assignments for sultamicillin and its impurity.

Position[a]	Sultamicillin			Impurity		
	^{1}H, δ (ppm), multiplicity	^{13}C, δ (ppm)	DEPT, HMQC & HMBC	^{1}H, δ (ppm), multiplicity	^{13}C, δ (ppm)	DEPT, HMQC & HMBC
1 & 1′	1.36 & 1.42 (2s, 6H)	18.4, 20.3	2× CH_3[c]	1.36 & 1.45 (2s, 6H)	17.5, 19.4	2× CH_3[c]
2	–	63.0	C[d]	–	70.1	C[d]
3	5.20 (dd, 1H, J=5.21, 1.65 Hz)	61.3	CH[b,c]	5.20 (dd, 1H, J=5.21, 1.65 Hz)	60.43	CH[b,c]
4	3.26 & 3.70 (ABq, 2H)	38.2	CH_2[b,c]	3.26 & 3.70 (ABq, 2H)	38.2	CH_2[b,c]
5	–	167.2	C[d]	–	167.1	C[d]
6	4.57 (s, 1H)	70.5	CH[c]	4.64 (s, 1H)	69.50	CH[c]
7	–	174.8	C[d]	–	174.7	C[d]
8	5.92 & 5.95 (ABq, 2H)	81.9	CH_2[c]	5.92 & 5.95 (ABq, 2H)	81.9	CH_2[c]
9	–	173.9	C[d]	–	172.9	C[d]
10 & 10′	1.47 & 1.60 (2s, 6H)	27.2, 30.8	2× CH_3[c]	1.47 & 1.65 (2s, 6H)	26.4, 29.6	2× CH_3[c]
11	–	64.9	C[d]	–	70.4	C[d]
12	4.50 (s, 1H)	58.8	CH[c]	4.56(s, 1H)	61.92	CH[c]
13	5.50 (d, 1H)	68.2	CH[c]	5.61 (m, 1H)	60.17	CH[c]
14	5.59 (d, 1H)	58.8	CH[c]	5.61 (m, 1H)	66.25	CH[c]
15	–	166.7	C[d]	–	166.7	C[d]
16	8.75 (brs, 1H)	–	–	–	–	–
17	–	172.9	C[d]	–	172.2	C[d]
18	4.51 (s, 1H)	62.8	CH[c]	4.54 (s, 1H)	60.90	CH[c]
19	–	–	–	4.00 (m, 1H)	–	–
20	–	140.0	C[d]	–	138.0	C[d]
21 & 21′	7.38 (d, 2H)	128.9	2× CH[c]	7.38 (d, 2H)	129.1	2× CH[c]
22 & 22′	7.25 (dd, 2H)	127.6	2× CH[c]	7.26 (dd, 2H)	128.0	2× CH[c]
23	7.28 (t, 1H)	128.0	1× CH[c]	7.28 (t, 1H)	128.7	1× CH[c]
24	–	–	–	4.52 & 4.79 (2m, 2H)	82.1	CH_2[b,c]

s, singlet; d, doublet; dd, doublet of doublet; m, multiplet; brs, broad singlet; q, quartet; t, triplet; ABq, AB quartet; J, spin coupling constant.
[a] Refers chemical structures for numbering.
[b] Deduced from ^{1}H–^{1}H-COSY.
[c] Deduced from HSQC.
[d] Deduced from HMBC.

ther been confirmed from the correlation observed between cross peaks of key protons at positions 19 and 24 in ^{1}H–^{1}H COSY NMR spectrum. All the signal assignments and correlations have further been confirmed by using 2D-NMR experiments like ^{1}H–^{1}H COSY, NOESY, HSQC and HMBC. During the interpretation of HMBC spectrum of the impurity, long range correlation between δ 4.52 and δ 4.79 at position 24 and δ 172.2 at position 17 together with their chemical shifts reveals that 14, 17 and 24 position carbons are connected to nitrogen atom. Further all quaternary carbons (positions indicated in Table 2) were interpreted based on the long range correlations observed in the HMBC spectral cross peaks. The structure of the impurity proposed for the ion at m/z 607 is supported by the presence of major fragments (see Fig. 2b) at m/z 577, 405 and 361 obtained from the mass spectra. In addition to that, the structure of this impurity was further confirmed by FT-IR spectral data. The absence of a characteristic strong absorption band at 1641 cm^{-1} in comparison with sultamicillin corresponding to amide C=O stretch supports the presence of imidazolone moiety. The elemental anal-

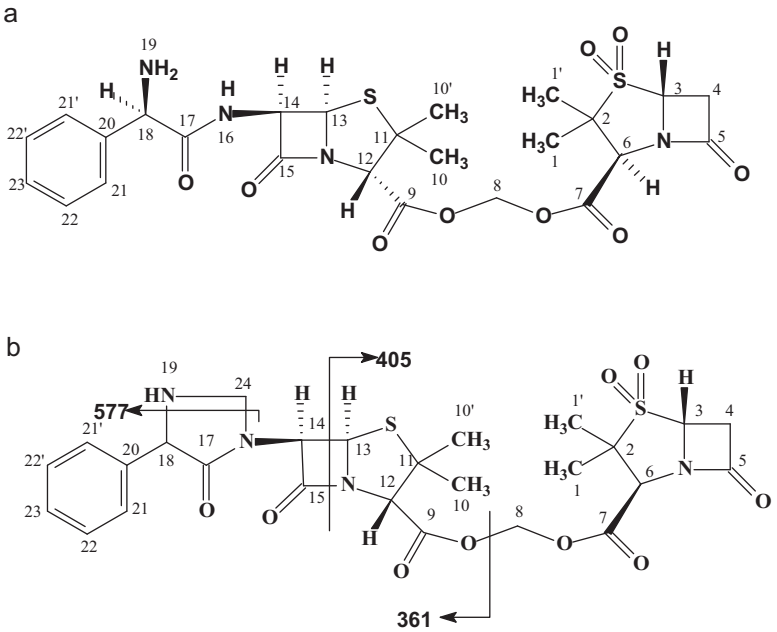

Fig. 2. Chemical structures of sultamicillin (a) and the new impurity (b).

Table 3
Formation of the impurity at 60 ± 2 °C.

Time period	Sulbactam, %	Ampicillin, %	Impurity at RRT ~ 1.44, %	Total related substances, %
Sultamicillin drug substance				
Initial	0.06	0.12	ND	1.01
12 h	0.08	0.18	0.89	1.74
24 h	0.34	0.67	0.82	4.47
Sultamicillin mixed with paraformaldehyde (10%, w/w)				
1 h	0.11	0.36	1.10	2.69
12 h	0.11	0.25	11.56	13.73
24 h	0.44	0.47	21.21	29.67

ysis (theoretical values C, 51.47; H, 4.98; N, 9.24; S, 10.57 and observed values C, 51.38; H, 4.91; N, 9.18; S, 10.50) suggests that the elemental composition of this impurity is $C_{26}H_{30}N_4O_9S_2$ with m/z 606 and LC/MS/TOF analysis is also justifying the obtained chemical formula and mass. The proposed structure of the impurity based on the obtained spectral data is shown in Fig. 2b together with that of sultamicillin in Fig. 2a. To the best of our knowledge, this impurity has not been reported in the literature.

3.2. Isolation of impurity by preparative HPLC

To enrich the degradant in sultamicillin, about 1 g of drug substance was taken in a petridish, small amount of water was spilled on the sample, kept at temperature 80 °C for about 16 h in a vacuum oven. The initial and enriched samples were subjected to HPLC analysis. The required degradant was increased to a level of around 2.5% when compared with initial sample. The enriched sample was taken in a beaker, added about 10 ml mixture of water–acetonitrile 40:60 (v/v), and was sonicated to dissolve. This solution was loaded into the preparartive column using the conditions described in Section 2.3. Fractions of ≥95% were pooled together, concentrated on rotavapor to remove acetonitrile. Concentrated fractions were loaded into a preparative column and eluent was treated with water for exclusion of ammonium acetate used for isolation. Finally, the column was washed with water–acetonitrile 20:80 (v/v). Again eluent was concentrated using rotavapor to remove acetonitrile. The aqueous solution was lyophilized using freeze dryer (Virtis advantage 2XL). The impurity was obtained as an off-white powder, with a chromatographic purity >95.0%.

3.3. Formation of impurity

Stability studies data indicate that the formation of this degradant impurity depends on the temperature. As shown in Table 1, the level of the impurity formed in samples stored at 60 ± 2 °C for 15 days was more than that of samples stored at 40 ± 2 °C/75 ± 5%RH, 25 ± 2 °C/60 ± 5%RH and 5 ± 3 °C. Under thermal stress conditions sultamicillin degrades to ampicillin, sulbactam and formaldehyde is released as a byproduct. Formaldehyde further reacts with amino group in sultamicillin leading to formation of this impurity. The formation of impurity was further verified by carrying out the experiments in the laboratory. Sultamicillin was mixed with paraformaldehyde (10%, w/w) and packed in the same packaging conditions as mentioned in Section 2.8 and stored at 60 ± 2 °C up to 24 h, the exposed samples were subjected to HPLC analysis. The results showed that, this impurity was found at about 10% level in 12 h sample, and further it increased with time. The experimental results are reported in Table 3. The probable mechanism for the formation of this impurity is given in Fig. 3.

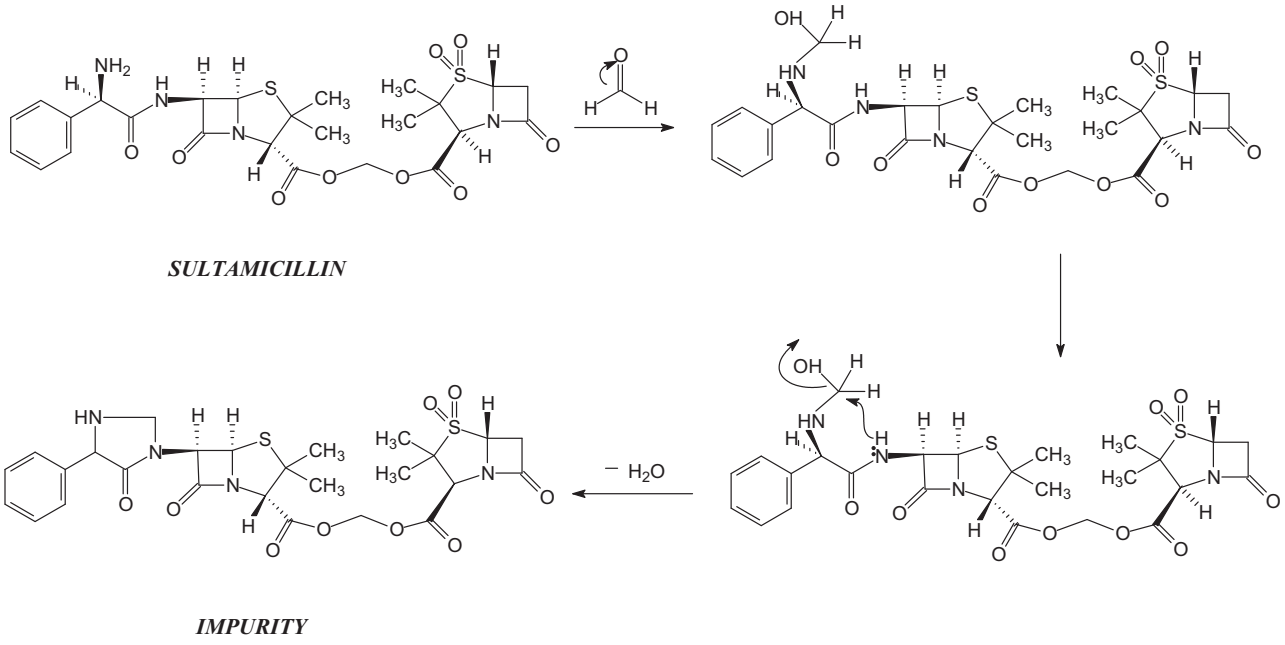

Fig. 3. Mechanism of the formation of the impurity.

Acknowledgements

The authors gratefully acknowledge the management of APL Research Centre (A Division of Aurobindo Pharma Ltd.), for allowing us to carry out the present work. The authors are also thankful to the colleagues of Analytical Research Department and Chemical Research Department for their co-operation.

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