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Identification and characterization of new impurity in Cefotaxime Sodium drug substance

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Abstract

Cefotaxime Sodium is a third generation cephalosporin drug substance. During the analysis of Cefotaxime Sodium in our lab, one unknown impurity was detected in gradient reverse phase High Performance Liquid Chromatography (HPLC) analysis at level ranging from 0.05 to 0.2% along with known impurities. This new impurity was detected by HPLC and have been identified by LCMS and was not reported in literature. The impurity was synthesized, isolated & characterized. Based on the spectral data, the impurity was named as, (6R,7R)-7-[(Z)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-[[(2-benzothiazolyl)thio]methyl]-3-cephem-4-carboxylic acid. The structure was established unambiguously by independent synthesis and co-injection in HPLC to confirm the retention time. Structural elucidation of this impurity by using spectral data (^{1}H NMR, 13C NMR, MS and IR), synthesis, isolation, formation, validation and stability elucidation of this impurity has been discussed in detailed in this paper.

Key words: Cefotaxime Sodium, Impurity, Isolation, Preparative HPLC, Characterization, Stability

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INTRODUCTION

Cefotaxime Sodium drug belongs to the third generation of cephalosporins .It has potent broad spectrum of activity against important pathogens [1, 2]. It is considered one of the first choice antibiotics in the therapy of spontaneous bacterial peritonitis in cirrhosis [3, 4]. Cefotaxime is a semi-synthetic cephalosporin consisting of an acetyl side-chain on aminothiazolyl ring and an

alpha-syn-methoxyimino group [5]. Cefotaxime is given by parenteral injection and it is marketed under various trade names including Claforan [6]. It is formulated as Cefotaxime Injection USP and Dextrose Injection and should be used only to treat or prevent infections that are proven or strongly suspected to be caused by bacteria [7]**.** The molecular formula is $C_{16}H_{16}N_5NaO_7S_2$ and molecular weight is 477. Its IUPAC name is sodium (6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-acetyl-oxymethyl-3-cephem-4-carboxylate.
During the HPLC analysis of Cefotaxime Sodium, one unknown impurity was detected along with six pharmacopoeial impurities [8] level ranging from 0.05 to 0.2%. The impurity profile study has to be carried out for any final drug substance as per the regulatory requirements to identify and characterize all the unknown impurities [9]**.** This paper describes the identification, isolation, characterization, formation, validation and stability evaluation of unknown impurity observed in Cefotaxime Sodium. Isolation and characterization of this new impurity was not reported till date to the best of our knowledge.

MATERIALS AND METHODS

Chemicals, reagents and samples

The investigated samples of Cefotaxime Sodium bulk drug and crude samples were synthesized in APL Research Centre Laboratories (a unit of Aurobindo Pharma Ltd., Hyderabad.). Disodium hydrogen orthophosphate anhydrous (AR grade), potassium dihydrogen orthophosphate (AR grade), orthophosphoric acid (~88%w/w, AR grade), acetonitrile (HPLC grade), methanol (HPLC grade), potassium hydroxide, ammonium acetate (GR grade), acetic acid (GR grade) were procured from E.Merck Limited, Mumbai, India. Distilled water was purified by using Milli-Q water purification system [Millipore, Bedford, MA].

High performance liquid chromatography (HPLC)

Chromatographic separations were performed on high performance liquid chromatograph system with Waters Alliance 2695 separation module equipped with 2996 photodiode array detector with Empower pro data handling system [Waters corporation, MILFORD, MA 01757, USA] . The analysis was carried out on YMC Pack ODS-A, 250 mm long, 4.6 mm i.d., 5µm particle diameter column. The mobile phase A was a mixture of phosphate buffer pH 6.2 and acetonitrile in the ratio of 98:2 v/v, (phosphate buffer was prepared by dissolving 3.6 g of disodium hydrogen orthophosphate anhydrous in 1000 ml water and adjusted to pH 6.2±0.05 with orthophosporic acid). The mobile phase B was a mixture of phosphate buffer pH 6.2 and acetonitrile in the ratio of 40:60 v/v and diluent was a mixture of 4.6 g of disodium hydrogen ortho phosphate anhydrous and 3.5 g of potassium dihydrogen orthophosphate in 1000 ml of water. UV detection was carried out at 235 nm, flow rate was kept at 1.0 ml/min, injection volume was 10µl and data acquired for 65 min. Pump mode was gradient and the program was as follows:
Time (min)/A(v/v):B(v/v);
$T_{0.01}$/100:0,T_{25}/80:20, T_{40}/60:40,T_{55}/0:100,T_{65}/0:100,T_{66}/100:0,T_{80}/100:0

LC-MS/MS analysis

LC-MS/MS analysis was carried out using a Perkin Elmer 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 5.5 kv and temperature was set at 375°C. The auxiliary gas and curtain gas used was high pure nitrogen. Zero air was used as nebulizer gas. LC-MS spectra were acquired from m/z 100-1000 in 0.1 amu steps with 2.0 s dwell time. The analysis was carried out by using Hypersil BDS C_{18} , 250 mm long, 4.6 mm i.d., 5µm particle diameter column. The mobile phase consisted of 0.01M ammonium acetate solution, pH adjusted to 6.0 with acetic acid (A) and acetonitrile: methanol (75:25 v/v) (B). UV detection at 235 nm , flow rate 1.0 ml/min , Injection volume 20µl and column was maintained at ambient conditions. Data acquisition time was 60 min. Water used as diluent. The gradient program was as follows, time (min)/A(v/v):B(v/v); $T_{0.01}$/98:2, T_5/98:2, T_{55}/40:60,T_{60}/40:60,T_{61}/98:2,T_{70}/98:2.

Preparative liquid chromatography

A Shimadzu LC-8A preparative liquid chromatograph equipped with SPD-10A VP, UV-Vis detector [Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan] was used. Hypersil BDS C18 (250 mm long, 21.2 mm i.d.) make: Thermo Scientific preparative column packed with 10 µm particle size was employed for isolation of impurity. The mobile phase A consists of 1.0% ammonium acetate solution and mobile phase B was acetonitrile. Flow rate was set as 25 ml/min and UV detection was carried out at 235nm. The gradient program was as follows, time (min)/A(v/v): B(v/v); $T_{0.01}$/95:5, T_{30}/90:10, T_{45}/80:20,T_{60}/75:25.

NMR spectroscopy

The ^{1}H NMR, ^{13}C NMR (proton decoupled) and DEPT spectra were recorded 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.

FT-IR Spectroscopy

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

RESULTS AND DISCUSSION

Detection and identification of impurity

The sample solutions equivalent to 1mg/ml of Cefotaxime Sodium prepared in diluent were injected into the analytical HPLC, using the analytical conditions mentioned in HPLC section. One unknown impurity (0.05 to 0.2%) was detected at relative retention time (RRT) of 1.72 respectively with Cefotaxime peak (retention time is about 27 min) along with phamacopoeial impurities. The same samples were subjected to LC-MS analysis using conditions as described in LCMS section to identify the mass of the impurity. The mass of the impurity recorded in positive ion mode was 562.8. Impurity was synthesized by chemical process and isolated by preparative HPLC; and co-injected with Cefotaxime Sodium sample into HPLC to confirm the retention time. The typical representative HPLC chromatograms of Cefotaxime sodium drug substance spiked with pharmacopoeial impurities including new impurity and Cefotaxime Sodium drug substance spiked with new impurity were shown in the

Fig.1a & 1b respectively and the chemical structures of Cefotaxime Sodium drug substance and impurity were given in Fig 2a & Fig 2b respectively.

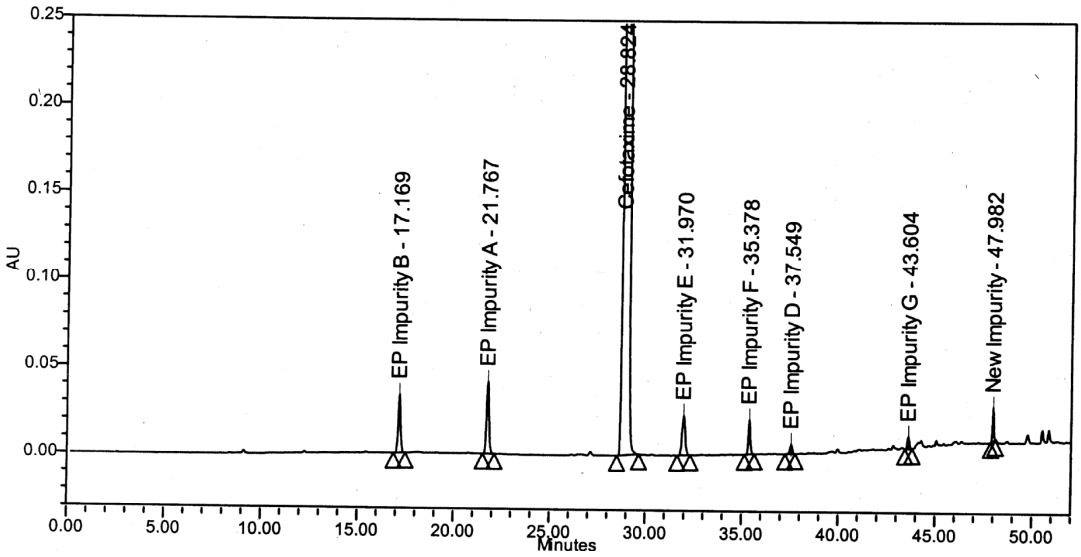

Figure 1a. A typical representative HPLC chromatogram of Cefotaxime Sodium drug substance spiked with pharmacopoeial impurities including new impurity.

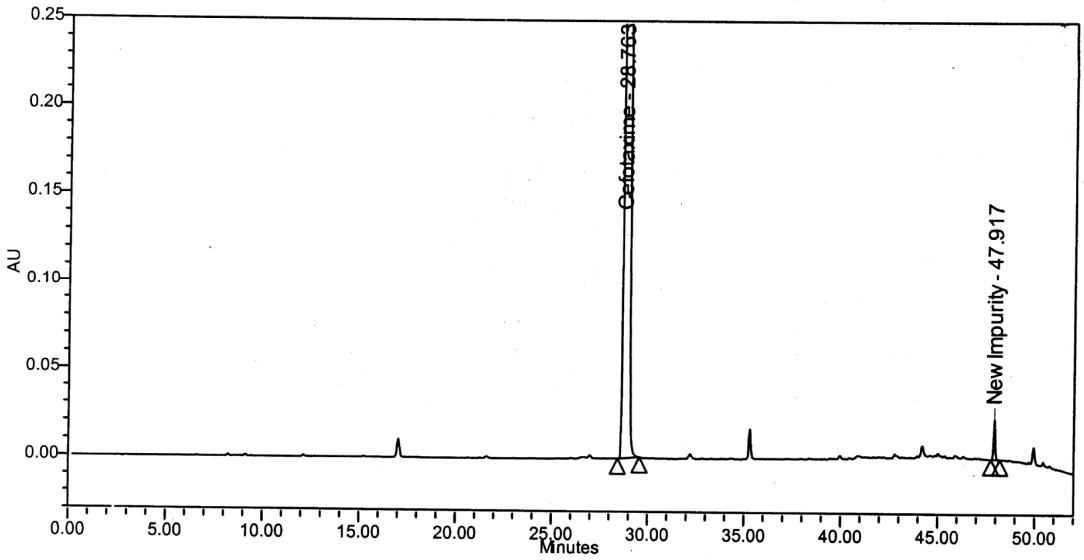

Figure 1b. A typical representative HPLC chromatogram of Cefotaxime Sodium drug substance spiked with new impurity.

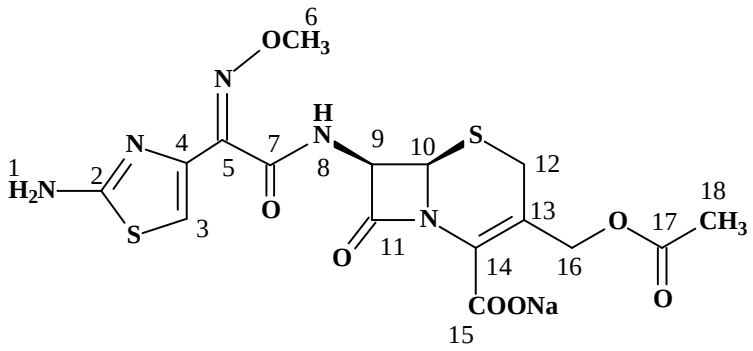

Fig. 2a Chemical structure of Cefotaxime Sodium

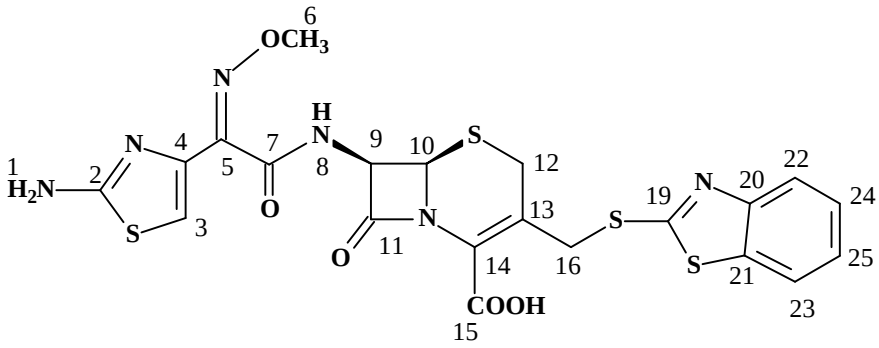

Fig. 2b Chemical structure of impurity

Synthesis of Impurity

7-aminocephalosporanic acid (7-ACA) was reacted with 2-mercaptobenzothiazole (MBT) in presence of BF_3 in acetonitrile solution at 0-5°C for 2 hours. Thereafter, the pH of the reaction mass was adjusted to 3.0 with aqueous ammonia and the resulting precipitate was filtered, washed with water followed by methylene chloride. This compound was dried and suspended in a mixture of water and THF at 0-5°C.

2-mercaptobenzothiazolyl[(*Z*)-2-(2-amino-4-thiazolyl)-2-methoxyimino]acetate (MAEM) and dimethyl acetamide (DMAC) were added at 0-5°C. Thereafter, the reaction mixture was stirred for 4 hours at 0-5°C while maintaining the pH between 7.8 and 8.2 by the addition of triethylamine. Methylene chloride added to the reaction mixture and the aqueous layer was separated and its pH was adjusted to 3.0 using 15% w/w aqueous Hydrochloric acid. The precipitate solid was filtered, wash with water and dried at 30-35°C under reduced pressure to afford the impurity (6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-[[(2-benzothiazolyl)thio]methyl]-3-cephem-4-carboxylicacid, having the HPLC chromatographic purity of 86%. Further it was purified by using preparative HPLC.

Isolation of impurity by preparative HPLC

Crude sample was subjected to preparative HPLC as per the conditions described in preparative HPLC section. Fractions collected were analyzed by analytical HPLC. Fractions of > 95% were pooled together; concentrated on rotavapor to remove acetonitrile. The concentrated fractions were passed through the preparative column using water: acetonitrile (50:50) as mobile phase to remove the buffers used for isolation. Again the eluate was concentrated in a Rotavapor to remove acetontrile. The aqueous solutions were lyophilized using freeze dryer (Virtis advantage 2XL). The impurity was obtained as an off-white powder with 95.0% chromatographic purity.

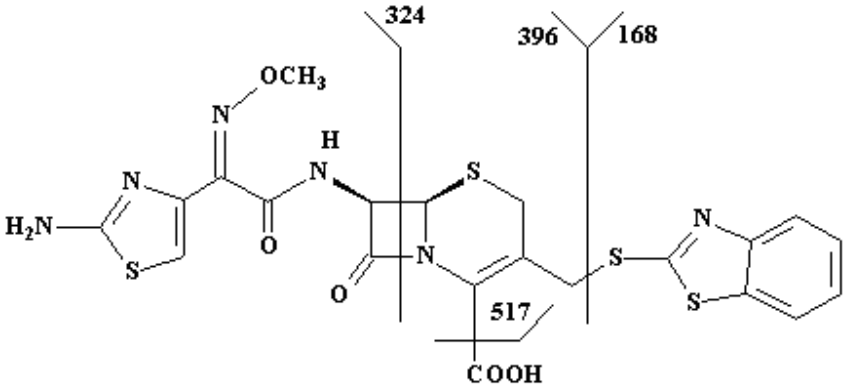

Fig.3 Fragmentation pattern of the ion at m/z 563 corresponding to impurity

Structural elucidation

The electrospray ionization mass spectrum of the impurity showed a protonated molecular ion peak at m/z 563, which indicating the molecular weight of the impurity could be 562. The even number of molecular weight indicating the presence of even number nitrogen atoms. In the ^{1}H NMR spectrum in DMSO-d6, the signal assigned to the acetyl function attached to the cephem ring of the cephem moiety was disappeared in the impurity. The specific ABq of $-CH_2$ group in cefotaxime will appear at 4.77 and 4.97 ppm, which was shifted to 4.30 and 4.71 ppm in the impurity. This was suggesting that acetyl group in cefotaxime was replaced by a relatively less electronegative function. Also, the ABq at 3.45 and 3.70 ppm in the impurity indicating the presence of $S-CH_2$. The multiplet at 7.31-7.49 ppm corresponding two protons and two doublets at 7.87 and 8.01 ppm corresponding to two protons suggesting the presence of a substituted benzene ring. In ^{13}C NMR spectrum of the impurity, the signals at 21.6 ppm and 163.9 ppm corresponds to acetyl function of cefotaxime was found absent. The signal corresponds to $–CH_2$ carbon of cefotaxime at 58.2 ppm was shifted to 36.5. The signals at 122.1, 122.6, 125.2 and 127.1 ppm are observed in the impurity. Also, the signals corresponding to quaternary carbon atoms at 144.1,145.0 and 153.4 ppm suggesting the presence of a substituted benzene ring. Comparative ^{1}H, ^{13}C NMR and DEPT assignments for Cefotaxime Sodium and its impurity was shown in Table 1. The major protonated fragmentation ions m/z 517, 396, 324 and 168 in mass spectrum supporting the structure. The fragementation pattern is shown in Fig 3. In the IR spectrum of the impurity, the absorption band at 3425 cm^{-1} indicating the presence of N-H stretch, the absorption band at 3061 cm^{-1} indicating the presence of aromatic C-H stretch, the

absorption at 1770 cm^{-1} indicating the presence of C=O of β-lactam, the absorption band at 1621 cm^{-1} indicating the presence of C=O of amide linkage. The FT-IR spectral assignments are given in Table 2. From ^{1}H NMR and ^{13}C NMR, Mass and IR spectral studies the impurity name was proposed as (6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-[[(2-benzothiazolyl)thio]methyl]-3-cephem-4-carboxylic acid . Finally a pure sample of this impurity was synthesized and co-injected with cefotaxime sample in HPLC and retention time was found same.

Table1 Comparative ^{1}H, ^{13}C NMR and DEPT assignments for Cefotaxime Sodium and its new impurity

Position[a]	Cefotaxime Sodium			Impurity		
	^{1}H δ (ppm), multiplicity	^{13}C δ (ppm)	DEPT	^{1}H δ (ppm), multiplicity	^{13}C δ (ppm)	DEPT
1	7.25 (brs, 2H)	-	-	7.21 (brs, 2H)	-	-
2	-	163.0	C	-	164.1	C
3	6.73 (s,1H)	109.9	CH	6.72 (s,1H)	109.7	CH
4	-	143.5	C	-	143.4	C
5	-	149.5	C	-	149.8	C
6	3.84 (s,3H)	62.7	CH_3	3.81 (s,3H)	62.7	CH_3
7	-	169.3	C	-	169.1	C
8	9.53 (d,1H)	-	-	9.55 (d,1H)	-	-
9	5.59 (dd,1H)	65.4	CH	5.66 (dd,1H)	59.1	CH
10	5.01 (d,1H)	58.9	CH	5.05 (d,1H)	58.3	CH
11	-	171.4	C	-	169.1	C
12	3.22,3.47 (ABq,2H)	26.2	CH_2	3.45,3.70 (ABq,2H)	27.6	CH_2
13	-	112.9	C	-	144.1	C
14	-	135.9	C	-	135.6	C
15	-	165.0	C	-	163.8	C
16	4.77,4.97 (ABq,2H)	58.2	CH_2	4.30,4.71 (ABq,2H)	36.5	CH_2
17	-	163.9	C	-	-	-
18	8.00(s,3H)	21.6	CH_3	-	-	-
19	-	-	-	-	164.1	C
20,21	-	-	-	-	153.4,145.0	C
22,23	-	-	-	7.87,8.01 (2d,2H)	122.1,122.6	CH
24,25	-	-	-	7.31-7.49 (m,2H)	125.2,127.1	CH

s, singlet; d, doublet; dd, doublet of doublet; m, multiplet; brs, broad singlet; q,quartet; t, triplet, ABq, AB quartet. a, refer chemical structures (Fig. 2a & 2b) for numbering.

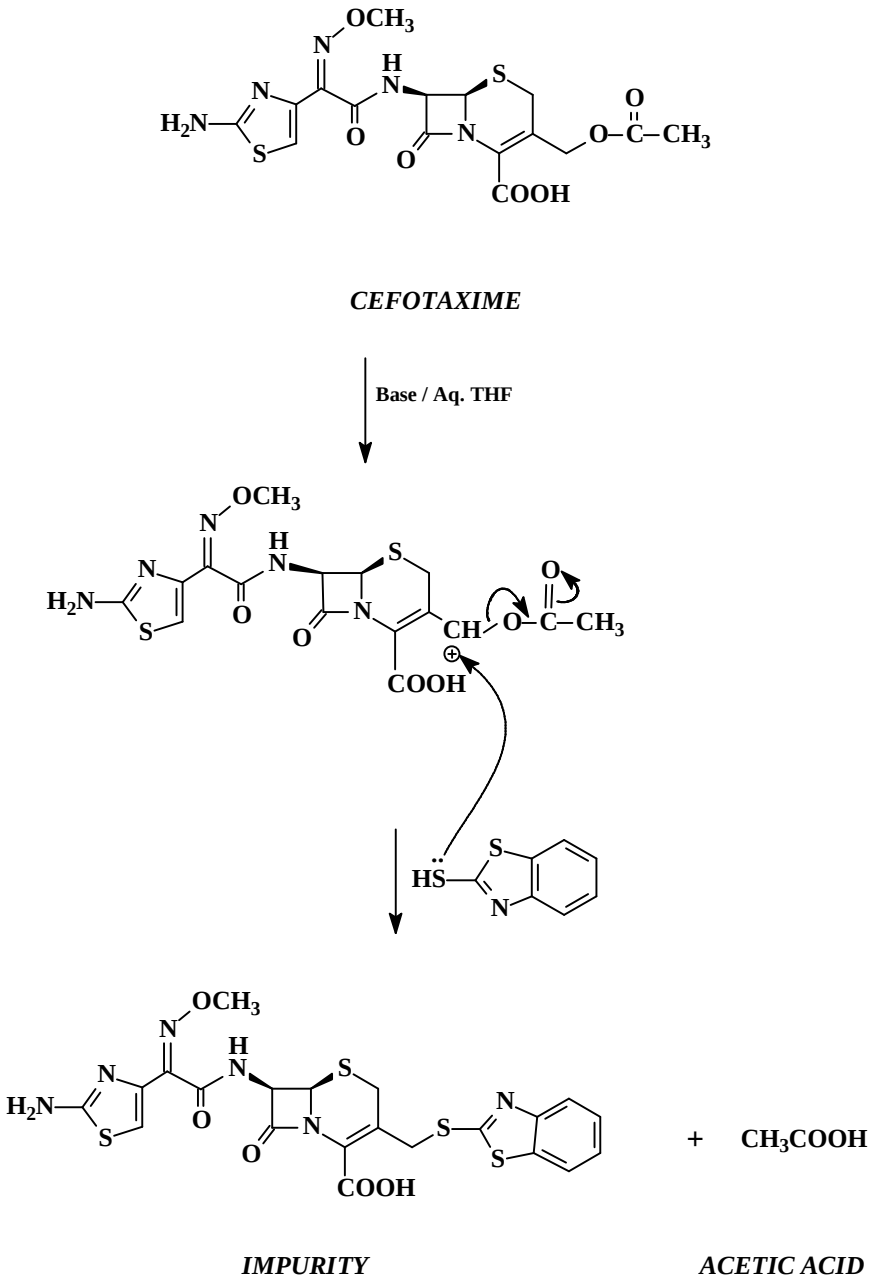

Fig. 4 Mechanism for the formation of impurity

Table 2 FT-IR spectral data for Cefotaxime Sodium and its new impurity.

IR (KBr) absorption bands (cm^{-1})			
Cefotaxime Sodium		**Impurity**	
3430, 3347(s,br)	NH stretch	3425(s)	NH stretch
2938(m)	Aliphatic C-H stretch	3061(s)	Aromatic C-H stretch
1760(s)	C=O (lactam) stretch	2972(s), 2934(s)	Aliphatic C-H stretch
1729(s)	C=O (carboxylic ester)	1770(s)	C=O (lactam) stretch
1647(s)	C=O (amide) stretch	1621(s)	C=O (amide) stretch
1610,1536(s)	C=C & C=N stretch	1615,1532(s)	C=C , C=N stretch
1386,1355(s)	Aliphatic C-H bend	1424,1355(s)	Aliphatic C-H bend
1062(s)	C-O stretching	1036(s)	C-O stretching

s: strong; m: medium; br: broad.

Formation of impurity

Cefotaxime was synthesized by the condensation of 7-ACA and MAEM, wherein, MBT is formed as the byproduct. The reaction of MBT with cefotaxime results in the Impurity. The possible mechanism for formation of this impurity is due to nucleophilic substitution of acetyl group in cefotaxime with byproduct MBT. The mechanism for the formation of impurity is given in Fig 4.

Analytical validation for this impurity

The analytical Reverse Phase HPLC method was validated as per ICH guidelines [10]. Method validation was performed in terms of specificity, precision, linearity, LOD & LOQ determination and accuracy and validation data of this impurity is tabulated in Table 3.

Specificity

The interference from related impurities of Cefotaxime, which were already listed in pharmacopoeia monographs and known substances, was investigated.

Precision

Precision of the method was evaluated in terms of method precision and inter day precision (ruggedness). Six preparations individually using Cefotaxime Sodium drug substance spiked with impurity at known concentration level and injected each solution as per methodology.

Table 3 Analytical method validation data for identified impurity

Validation Parameter	Results
Repeatability (n=6, %R.S.D)	
Method Precision	0.9
Intermediate Precision	0.8
LOD & LOQ	
Limit of Detection (%w/w)	0.008
Limit of Quantification (%w/w)	0.012
Precision at LOD (%R.S.D)	18.9
Precision at LOQ (%R.S.D)	6.0
Linearity	
Range (µg/ml)	0.10-15.2
Number of Points	7
Slope	27040
Intercept	757
Residual sum of squares	1903
Correlation coefficient	0.9999

Accuracy

	LOQ level	50% level	100% level	150% level
Added (%w/w)	0.012	0.097	0.194	0.294
Recovered (%w/w)	0.012	0.100	0.201	0.275
%Recovery	100.0	103.1	103.6	93.5
%R.S.D	5.1	2.5	0.5	0.2

Linearity

Impurity solutions were prepared between 0.10µg/ml to 15.2µg/ml range and injected into HPLC.

Limit of Detection (LOD) and Limit of Quantification(LOQ)

The LOQ refers to the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. There are different approaches to determine the LOQ and LOD. Based on residual standard deviation of regression line and slope from set of concentrated solutions, LOQ and LOD values were predicted and précised from six preparations of solutions.

Accuracy
It is measure of the closeness of test results obtained by a method to the true value. It is determined by the applying the method to samples to which known amount of analyte have been added. Cefotaxime Sodium sample solutions were prepared in triplicate by spiking Impurity at four different levels and injected each solution into HPLC as per methodology and recovery values were calculated.

Evaluation of stability studies
Stability studies were carried out under ICH prescribed storage conditions [11], *viz.*, stress, accelerated and real time. The drug substance was subjected to various storage conditions to evaluate the stability studies of impurity under the influence of a variety of environmental factors such as temperature and humidity i.e 40°C/75%RH, 30°C/65%RH & 25°C/60%RH at various time points from 1 month to 24 months and also stress studies were conducted at 105°C for 120 hours. All the samples were withdrawn from respective conditions in respective time periods and analyzed as per HPLC methodology. Based on these studies, no significant change was observed in impurity level when compared with initial or control sample.

CONCLUSION

This impurity was observed during monitoring by HPLC in Cefotaxime Sodium bulk drug substance was identified by LC-MS and characterized by spectroscopic techniques. The structure for this impurity was confirmed based on the spectral data. And also stability of this impurity and validation studies for this impurity was discussed.

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